Modulation of calcium responses by altered peptide ligands in a human T cell clone

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To determine whether altered peptide ligands (APL) affect calcium signaling events, we investigated changes in intracellular calcium concentration ($[Ca^{2+}]_i$) in human T cell clone stimulated with either the fully agonistic peptide M12p54–68, the partially agonistic analogue E63V or the simple antagonistic analogue E58M. Both E63V and E58M stimulated a Ca^{2+} response in ~40 % of T cells, whereas M12p54–68 did so in ~70 % of T cells. The most predominant pattern of a Ca^{2+} increase induced by M12p54–68 was a small sinusoidal peak followed by a sustained high response. The most frequent pattern of calcium response induced by E63V was a continuous high response without a preceding sinusoidal peak, whereas that induced by E58M was large with frequent oscillations. Genistein, an inhibitor of the protein tyrosine kinases (PTK), markedly inhibited the wild-type peptide-induced increase in $[Ca^{2+}]_i$, whereas it marginally inhibited the response induced by E63V or E58M. In contrast, GF109203X, a protein kinase C (PKC)-specific inhibitor, markedly inhibited the E63V- or E58M-induced Ca^{2+} response, whereas it marginally affected the wild peptide-induced Ca^{2+} response. Furthermore, in nominal Ca^{2+}-free medium, the E58M-induced Ca^{2+} response was almost completely blocked, while the M12p54–68- or E63V-induced responses were only partially inhibited. Our results suggest that the Ca^{2+} response induced by the fully agonistic peptide depends on activation of the genistein-sensitive signaling pathway, including PTK, whereas the Ca^{2+} response to a simple antagonistic APL completely depends on extracellular Ca^{2+} and activation of the GF109203X-sensitive signaling pathway, including PKC. These differences in the $CA^{2+}{}_i$ response in recognition of different APL may parallel the unique T cell activation patterns induced by APL in human T cells.

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1 Introduction

An increase in concentration of intracellular free calcium ions ($[Ca^{2+}]_i$) regulates and controls many cellular processes and functions, including adhesion, motility, gene expression and proliferation in various cell types [1–6]. With direct observation of changes in $[Ca^{2+}]_i$ at the single cell level, using ratiometric fluorescence dyes, calcium signaling proved to be an important step and a specific consequence of T cell activation [7–13]. The elevated calcium level activates the calcium/calmodulin-dependent phosphatase calcineurin and leads to translocation of the cytoplasmic component of the T cell-specific transcription factor, nuclear factor of activated T cell (NF-AT), to the nucleus and to the initiation of transcription in several genes, including the IL-2 gene [14].

Studies of calcium signaling activity in mouse T cells stimulated with altered peptide ligands (APL) indicated that the Ca^{2+} response induced by antagonistic ligands was smaller in amplitude and shorter in duration than that induced by fully agonistic ligands [15–17]. A recent study on a mouse T cell clone also showed that the APL-stimulated Ca^{2+} response is initiated and sustained at lower levels than that stimulated by a strong agonistic signal, but it resembles that stimulated by a weaker agonistic stimulus [15]. Increases in $[Ca^{2+}]_i$ can occur as a single transient, as repetitive oscillations or as a sustained plateau; amplitude and duration would reflect

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Abbreviations: $[Ca^{2+}]_i$: Intracellular free calcium concentrations **APL:** Altered peptide ligand **Fura-2/AM:** Fura-2/acetoxy-methyl-ester **IP_3:** Inositol 1,4,5-triphosphate **PTK:** Protein tyrosine kinase

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specificity of the transcription factors activated [6, 16]. In B lymphocytes of mice, for example, different Ca^{2+} responses induce activation of different transcription factors: NF-AT c/p is significantly more Ca^{2+} sensitive than NF-κB and JNK1/ATF-2, and this enhanced Ca^{2+} sensitivity enables NF-AT to be selectively activated by the low Ca^{2+} increase [6]. However, it is not clear whether TCR antagonism, and TCR partial agonism (in this report, partial agonism means TCR antagonism with partial activation, not weak agonism; see Sect. 4.1) can stimulate the intracellular Ca^{2+} response as does a fully agonistic peptide and whether different types of APL induce different calcium signaling in human T cells.

In previous work, we characterized peptide-binding motifs for HLA-DR4 molecules [18] and analyzed the effects of APL on IL-12 production by APC [19] and T cell survival [20]. We also analyzed responses of a human T cell clone to a large panel of analogue peptides derived from an antigenic peptide presented by DR4 (DRB1*0406), the objective being to investigate frequencies and characteristics of TCR agonism, TCR partial agonism and TCR antagonism [21]. Our results showed that these APL frequently stimulated a human T cell clone to exhibit either agonism or antagonism. The minor proportion of antagonistic APL exhibited partial activation which induced increases in cell size and expression levels of several CD markers, without cell proliferation. Utilizing the well characterized T cell clone and APL, we measured $[Ca^{2+}]_i$, using fura-2-acetoxy-methyl-ester (fura-2/AM) video imaging methods and we compared Ca^{2+} responses of a T cell clone to different APL exhibiting full agonism, partial agonism or antagonism. The different APL induced different patterns of Ca^{2+} response in T cells together with differences in sensitivities to the protein tyrosine kinase (PTK) inhibitor genistein, the PKC inhibitor GF109203X or an extracellular calcium ion-free condition.

2 Results

2.1 Non-agonistic APL induced an increase in $[Ca^{2+}]_i$ in the T cell clone

A direct measurement of $[Ca^{2+}]_i$ was made using a ratio-imaging system with fura-2/AM in individual T cells. The different Ca^{2+} responses to various stimuli, including the immunogenic ligand and APL, were examined. To observe differences among various peptide-induced Ca^{2+} responses, we compared the distribution of the Ca^{2+} imaging ratio in 100 T cells stimulated with each TCR ligand. One hundred T cells contacting APC, as determined by microscopic images, were selected for analysis. Almost all cells stimulated with an irrelevant peptide, A60D (95/100 cells, A60D stands for an analogue peptide of M12p54–68 carrying a replacement of Ala 60 to Asp) or with medium only (97/100 cells) showed no detectable increase in $[Ca^{2+}]_i$ throughout the 30-min observation period. In contrast, 68 % (68/100), 43 % (43/100), or 42 % (42/100) of the T cells stimulated with fully agonistic M12p54–68, partially agonistic E63V or simple antagonistic E58M, respectively, showed an increase in $[Ca^{2+}]_i$ (Δ ratio ≥ 0.2, Fig. 1). The percentage of cells strongly responding with Δ ratio ≥ 1.0 (half of the maximum Δ ratio value) among the responding cells (Δ ratio ≥ 0.2) was 23 % (15/64 cells) for stimulation with the wild-type peptide at 7 min after start of measurements. However, the percentage of strongly responding cells was 65 % (26/40 cells) for E63V and 55 % (21/38 cells) for E58M. These results indicate that more cells stimulated by E63V or E58M showed strong Ca^{2+} responses during the early period of measurement, as compared with stimulation by the wild-type peptide,

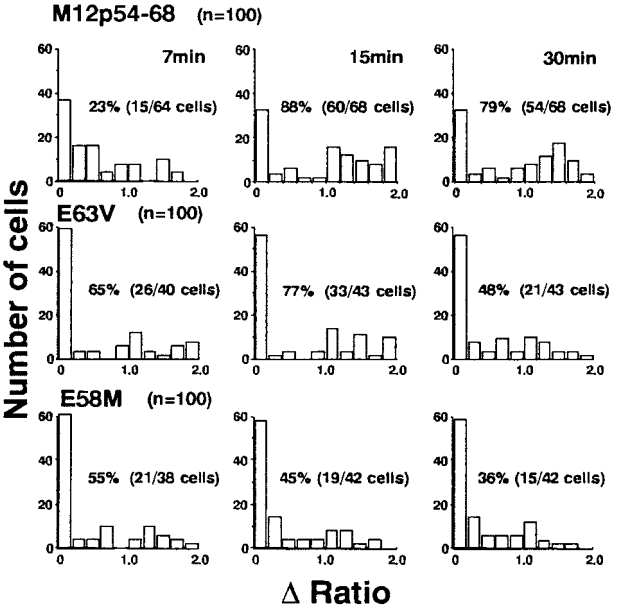

Figure 1. Distribution at three different time points of the Ca^{2+} response in the YN5–32 T cell clone after stimulation with three different peptide ligands. Fura-2/AM-loaded T cells were immobilized on poly-L-lysine-coated surfaces and stimulated with L-cell transfectants expressing DR4 (DRA + DRB1*0406) pulsed with 100 μM peptide. The images were obtained 15 min after start of measurements, using an image processor system (ARGUS-50/CA). Distribution of 340-nm/380-mm ratio levels in 100 T cells contacting APC for each group was analyzed 7, 15 and 30 min after start of measurements. Note the numbers in the figure indicating the percentage and the ratio of cells showing a strong Ca^{2+} response (Δ ratio ≥ 1.0) to cells showing a positive response (Δ ratio ≥ 0.2).

even though the total numbers of responding cells were fewer for E63V or E58M stimuli than for the wild-type peptide stimulus. The wild-type peptide continued stimulating a strong Ca^{2+} response in 88 % (60/68) and 79 % (54/68) of the cells, respectively at 15 and 30 min after starting the measurements. E63V still elicited strong responses in 77 % (33/43) and 48 % (21/43) of the cells after 15 and 30 min, respectively. The corresponding measurements for E58M showed 45 % (19/42) and 36 % (15/42). These data indicate differences in kinetics and frequencies of Ca^{2+} response in T cells for stimulation with M12p54–68, E63V or E58M peptides. Unexpectedly, a small fraction of cells stimulated with E63V or E58M reached a high $[Ca^{2+}]_i$ level earlier than did cells stimulated with the wild-type peptide.

2.2 Patterns of Ca^{2+} response in T cells stimulated with various TCR ligands

As shown in Fig. 2 and Table 1, various tracing patterns of Ca^{2+} responses were observed in T cells stimulated with M12p54–68, E63V or E58M. We divided the patterns of Ca^{2+} responses of YN 5–32 into the following five types: 1) a transient and small sinusoidal peak (Δ ratio ≥ 0.3, duration 2–3 min) followed by a high sustained Ca^{2+} response (Δ ratio ≥ 1.0, duration > 10 min) as shown in the top panel of Fig. 2A; 2) a continuous high response (Δ ratio ≥ 1.0 and duration > 10 min) without a preceding sinusoidal peak as shown in the top panel of Fig. 2B; 3) large and frequent oscillations (Δ ratio ≥ 1.0; frequency, three to five times/min, total number of oscillations ≥ 60), as shown in the top panel of Fig. 2C; 4) a small and sustained response ($0.2 < \Delta$ ratio < 1.0, duration > 10 min) as shown in the left top panel of Fig. 4A; 5) no response (Δ ratio ≤ 0.2). As shown in Table 1, the wild-type peptide predominantly induced the type 1 response in 56 % (38/68) of responding cells; the partially agonistic E63V mainly induced the type 2 response in 51 % (22/43) of responding cells. The simple antagonistic E58M, mainly induced the type 3 response in 55 % (23/42) of responding cells. To determine whether the E58M-induced change in $[Ca^{2+}]_i$ is a specific effect of this peptide, we also investigated effects of other simple antagonistic peptides (E63R and E58I) on the Ca^{2+} response. Both APL stimulated a strong and frequent oscillatory Ca^{2+} response like that of E58M (data not shown).

2.3 Onset time and peak response of an increase in $[Ca^{2+}]_i$

Fig. 3A shows differences in the onset time of the Ca^{2+} response, that is from start of measurements to initiation

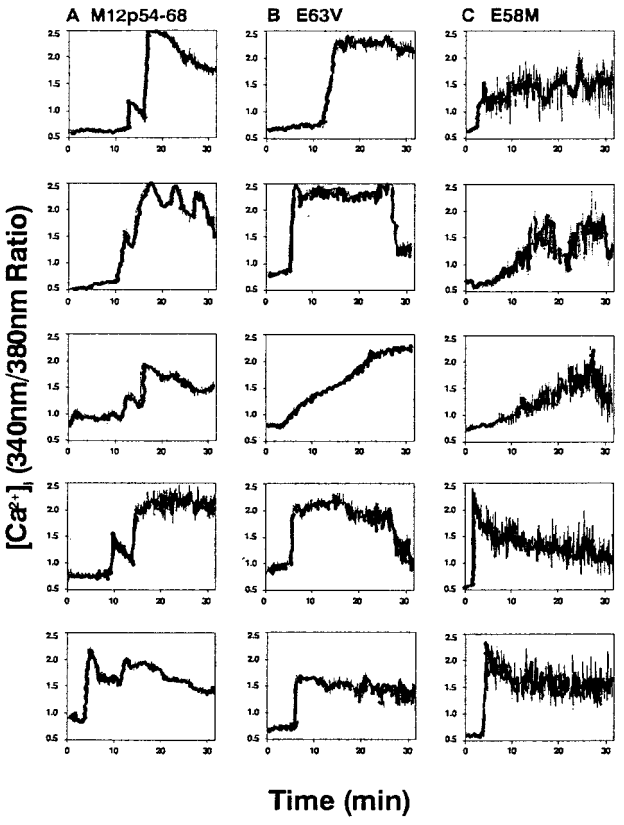

Figure 2. Predominant patterns of changes in $[Ca^{2+}]_i$ in the YN5–32 T cell clone after stimulation with three different peptide ligands. The five representative records of ratio image analysis for each stimulus are shown. (A) A fully agonistic wild-type peptide, M12p54–68, (B) a partially agonistic peptide, E63V, (C) a simple TCR antagonistic peptide, E58M.

of $[Ca^{2+}]_i$ increase, as stimulated by the three peptides. In 68 cells stimulated with the fully agonistic peptide, there was a positive increase in $[Ca^{2+}]_i$, within 7.7 ± 0.8 min (mean ± S.E.). However, the onset time of Ca^{2+} response in those cells stimulated with the partial agonist (5.7 ± 0.6 min, $n = 43$) was earlier than that stimulated with the wild-type peptide. This shortening of the onset time was marked in cells stimulated with the simple antagonistic peptide (4.0 ± 0.3 min, $n = 42$, $p < 0.05$, as compared with the wild-type peptide). Fig. 3B shows mean values of peak amplitudes in increase in $[Ca^{2+}]_i$ observed during the 30-min observation period in response to the wild-type peptide and its analogues in T cells showing a positive Ca^{2+} response. Unexpectedly, we found no significant differences in these values between the three different peptide ligands.

Table 1. Patterns of Ca^{2+} response in a T cell clone stimulated with various peptide ligands[a)]

Peptide	Number of cells showing different patterns of $[Ca^{2+}]_i$ increase					Number of responding cells
M12p54-68 (Full agonist/WT)	**56%** (38/68)	19% (13/68)	12% (8/68)	13% (9/68)	32% (32/100)	68% (68/100)
E63V (Partial agonist)	16% (7/43)	**51%** (22/43)	12% (5/43)	21% (9/43)	57% (57/100)	43% (43/100)
E58M (Simple antagonist)	12% (5/42)	12% (5/42)	**55%** (23/42)	21% (9/42)	58% (58/100)	42% (42/100)
A60D (irrelevant)	0	0	2	3	**95%** (95/100)	5% (5/100)
Medium	0	0	0	3	**97%** (97/100)	3% (3/100)

a) Ca^{2+} response patterns of 100 cells for each peptide ligand were divided into five types and are summarized as indicated. Percentages of cells and in parentheses the real number of cells per number of cells investigated are given for each pattern of response. Percentages indicated in the left four columns indicate the number of T cells showing each pattern of response in proportion to that of total responding T cells. For detailed descriptions see Sects. 2.1 and 2.2.

2.4 The dose dependence of the wild-type peptide-induced $[Ca^{2+}]_i$ elevation

We asked whether lower doses of the wild-type peptide would stimulate similar patterns of Ca^{2+} response as induced by E63V or E58M. APC were prepared by pulsing an L cell transfectant with the wild-type peptide, in three concentrations (0.2, 2, 20 μM) lower than the 100 μM used in the former experiment. As shown in Fig. 4A, although the amplitude of the Ca^{2+} response showed dependence on the peptide concentration, the pattern of Ca^{2+} response induced by the wild-type peptide did not change to match those observed for E63V and E58M. Furthermore, even by stimulation with a low concentration of peptide (0.2 μM), the small sinusoidal peak in the Ca^{2+} response was observed. As shown in Fig. 4B, the patterns of $[Ca^{2+}]_i$ increases observed in T cells were divided into the following three groups, according to the definition described in Sect. 2.2: (1) a transient and small sinusoidal peak followed by a high sustained Ca^{2+} response; (2) a transient and small sinusoidal peak alone and (3) a small and sustained Ca^{2+} response. Percentages of total responding T cells showing one of these three Ca^{2+} response patterns were 53.3 % (32/60) for 0.2 μM peptide, 56.7 % (34/60) for 2 μM peptide and 63.3 % (38/60) for 20 μM peptide. Along with the decrease in peptide concentration, the number of T cells showing pattern (1) decreased, whereas those showing pattern (3) increased. The continuous high response without a preceding sinusoidal peak, typical for a partially agonistic APL, and the oscillatory Ca^{2+} response, typical for simple antagonistic APL, were observed only in a small number (< 5 %) of T cells stimulated with 20 μM peptide-pulsed APC but not 2 μM or 0.2 μM peptide-pulsed APC. Thus, Ca^{2+} responses induced by a lower concentration of the wild-type peptide did not mimic those induced by E63V or E58M.

2.5 Effects of extracellular calcium on the Ca^{2+} response

To determine whether $[Ca^{2+}]_i$ oscillations are related to Ca^{2+} influx through the plasma membrane, a calcium-free solution was applied to test the Ca^{2+} response of T cells. When stimulated by the simple antagonistic peptide E58M, T cells exhibited almost no Ca^{2+} response, whereas in cells exposed to the wild-type peptide, a small and fluctuating response was evident though it had a lower plateau level or no plateau. Under these conditions, the response of T cells to E63V was also markedly suppressed (Fig. 5A). As shown in Fig. 5B, the distribution of 340 nm/380 nm fluorescence in 60 independent T cells stimulated with each peptide was analyzed at three different time points. In response to the wild-type peptide, 23 % (14/60 cells), 42 % (25/60 cells) or 38 % (23/60 cells) were positive (Δ ratio ≥ 0.2) at 7, 15 and 30 min, respectively. E63V induced a similar response but with less fluctuation. These data indicate that the response to E58M strongly depends on extracellular Ca^{2+}, whereas responses induced by the wild-type peptide or by E63V are only partially dependent.

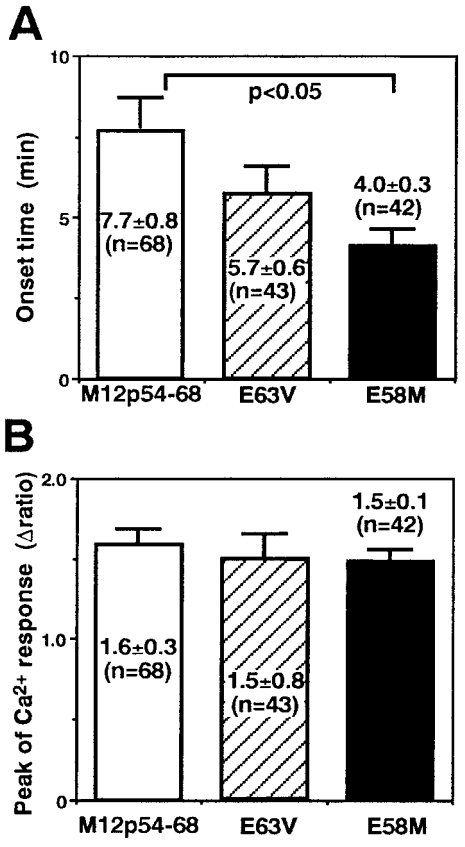

Figure 3. (A) Differences in onset time of calcium response to M12p54–68 and its analogues. The mean values ± S.E. of time required for onset of respone are indicated. (B) No differences in amplitude of peak Ca^{2+} response stimulated by the three peptide ligands. The peak amplitude of Ca^{2+} response observed during the recording period from 0 to 30 min was estimated for each individual T cell and the mean value ± S.E. of peak amplitudes for each peptide is indicated.

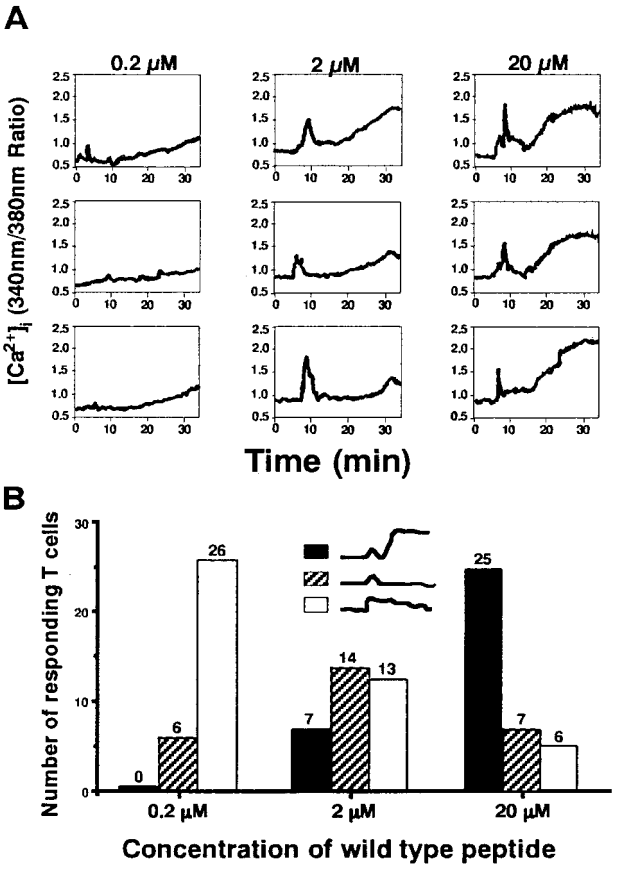

Figure 4. Dose dependence of the wild-type peptide-induced Ca^{2+} response. (A) Sample recordings of change in $[Ca^{2+}]_i$ in the three representative T cells are shown. L-cell transfectants were prepulsed with the three concentrations of wild-type peptide for 5 h. (B) Summary of distribution of T cells showing three different patterns of Ca^{2+} response stimulated by L-cell transfectants prepulsed with three different concentrations of M12p54–68 peptide. The closed bar indicates the number of T cells showing a transient and small sinusoidal peak followed by a high sustained Ca^{2+} response. Hatched and open bars indicate those showing a transient and small sinusoidal Ca^{2+} response alone and a small sustained Ca^{2+} response, respectively. The definition of three patterns of Ca^{2+} response is according to that described in Sect. 2.2. Few T cells (< 5 %) stimulated with 20 μM peptide-pulsed APC showed a continuous high response without a preceding sinusoidal peak or an oscillatory response.

2.6 Effects of genistein on the Ca^{2+} response

Genistein, a inhibitor of PTK inhibits activation of PLCγ mediated by Fyn and Lck [22, 23]. In this assay, T cells were pretreated with 3 μM genistein for 3 h, as described before [22] and the Ca^{2+} response was measured in the presence of 3 μM genistein throughout the experimental period. As shown in Fig. 6A, the Ca^{2+} response induced by APC prepulsed with the wild-type peptide (100 μM) was completely blocked by treatment with genistein. Furthermore, genistein also inhibited the Ca^{2+} response induced by APC prepulsed with a lower concentration (10 μM) of the wild-type peptide (data not shown). However, genistein showed only marginal inhibition of the Ca^{2+} response induced by the partially agonistic peptide E63V or the simple antagonistic peptide E58M. As shown in Table 2, the Ca^{2+} response was divided into high and low response; Δ ratio ≥ 1 was taken as a high response whereas 0.2 < Δ ratio < 1 was taken as a low response. There were no cells showing high response to the wild-type peptide by treatment with genistein, whereas 26 % (26/100) and 28 % (28/100) of the cells responding to the partially agaonistic peptide and the

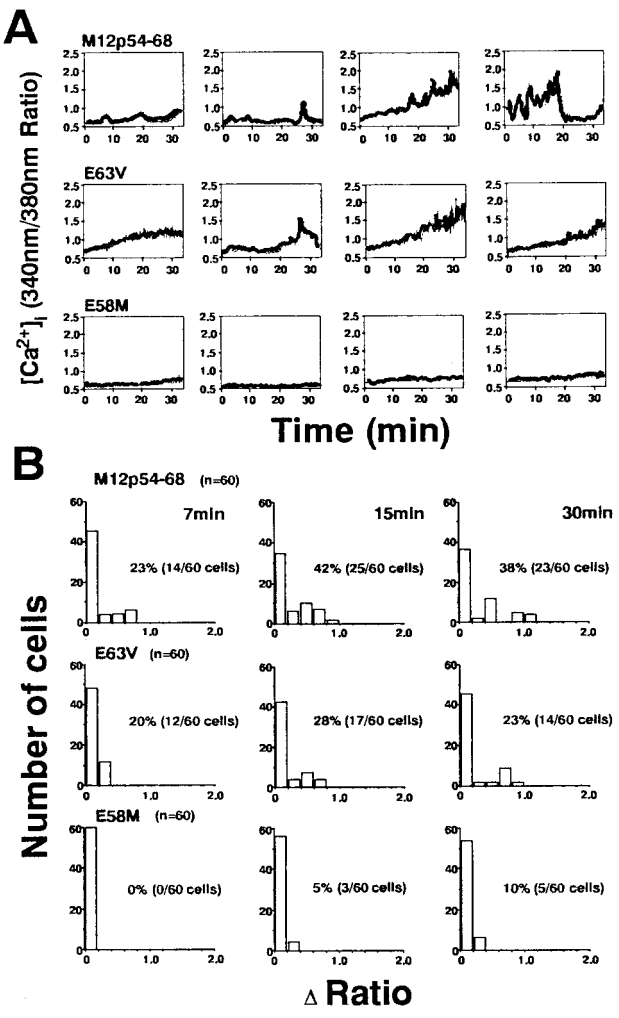

Figure 5. Effects of extracellular Ca^{2+}-free solution on $[Ca^{2+}]_i$ increases induced by M12p54–68 and its analogues in the YN5–32 T cell clone. (A) Sample recordings of changes in $[Ca^{2+}]_i$ in four representative T cells after stimulation with APC prepulsed with 100 μM different peptide ligands for 5 h. (B) Distribution of $[Ca^{2+}]_i$ increase in 60 cells stimulated with each peptide at 7, 15 and 30 min after start of measurements. All cells (T cells and APC) were washed twice with the nominal Ca^{2+}-free solution before measurement, and measurement of $[Ca^{2+}]_i$ was also done in the absence of extracellular Ca^{2+} ions. Numerals in the figure indicate percentages and numbers of cells showing a positive response (Δ ratio ≥ 0.2) in proportion to 60 cells investigated.

simple antagonistic peptide, respectively, showed a high response. This means that the wild-type peptide-induced Ca^{2+} response is absolutely dependent on activation of the genistein-sensitive signaling pathway, including PTK. In contrast, the Ca^{2+} response induced by antagonistic peptide or partially agonistic peptide involves signaling pathways resistant to genistein.

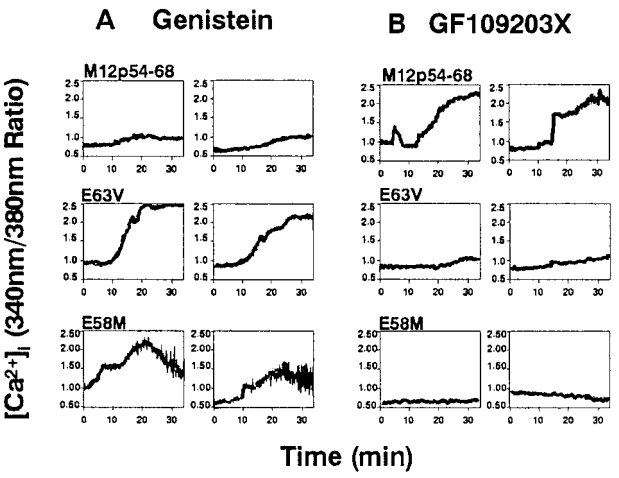

Figure 6. Effects of genistein (A) and GF109203X (B) on the Ca^{2+} response induced by M12p54–68 and APL. Sample records of changes in $[Ca^{2+}]_i$ in two representative T cells after stimulation with APC prepulsed with 100 μM different peptide ligands for 5 h. T cells were pretreated with 3 μM genistein or 100 nM GF109203X for 3 h and measurement of $[Ca^{2+}]_i$ was done under the same conditions throughout the experimental period.

2.7 Effects of GF109203X on the Ca^{2+} response

GF109203X, a PKC inhibitor with a high selectivity for PKC, as compared to five other protein kinases [24], was used. T cells were pretreated with 100 nM GF109203X as reported [24] for 3 h before measurement. Treatment of T cells with GF109203X abrogated the Ca^{2+} response induced by either the partially agonistic E63V or the simple antagonistic E58M, but it did not inhibit the wild-type peptide-induced Ca^{2+} response even when APC prepulsed with a lower concentration (10 μM) of the peptide were applied (data not shown). Forty-six percent (46/100) cells still showed a high Ca^{2+} response by stimulation with the wild-type peptide, whereas no cell showed a high response to partially agonistic or antagonistic peptides in the presence of GF109203X. We conclude that the Ca^{2+} response triggered by simple antagonistic or by partially agonistic peptides depends on activation of GF109203X-sensitive signaling pathways, including PKC.

3 Discussion

In the present study, we characterized the response of a human T cell clone to the M12p54–68 peptide or its analogues which exhibited characteristics of full agonism, partial agonism or antagonism for TCR. The results clearly demonstrated that although these peptides

Table 2. Differences in effects of genistein and GF109203X on calcium responses induced by three peptide ligands

Inhibitor	Number of responding cells in 100 T cells					
	M12p54–68 (Full agonist/WT)		E63V (Partial agonist)		E58M (Simple antagonist)	
	High response[a)]	Low response[b)]	High response	Low response	High response	Low response
Genistein (PTK inhibitor)	0	4	26	10	28	9
GF109203X (PKC inhibitor)	46	15	0	3	0	4

a) High response means Ca^{2+} response $\triangle$ ratio $\geqq 1$.
b) Low response means Ca^{2+} response, $0.2 < \triangle$ ratio < 1.

exhibited different effects on activation of T cells, all induced an increase in $[Ca^{2+}]_i$. The amplitudes of peak responses of the $[Ca^{2+}]_i$ to E63V or E58M were much the same as those induced by M12p54–68 (Fig. 3B). However, the proportion of responding cells and the patterns of Ca^{2+} responses to these different stimuli showed considerable variation. These observations suggested that the different Ca^{2+} responses induced by the immunogenic ligand and its analogues may directly couple with different types of T cell activation.

Stimulation with a strong antagonistic peptide, E58M, resulted in the more rapid onset of the Ca^{2+} response in a small fraction of T cells than seen with the wild-type peptide. Furthermore, during the early period of measurements, the number of T cells strongly responding to an antagonistic peptide exceeded that responding to the wild type. These phenomena differ from findings in mouse T cells [16]. In these studies, the onset delay increased when using a weaker agonist and this increase was marked in response to an antagonistic peptide. Studies on physicochemical interactions between purified TCR and its ligand demonstrated that, in most cases, partially agonistic and antagonistic ligands dissociate more rapidly from a given TCR than do agonistic ligands [25–27]. Because oligomerization of the MHC-peptide-TCR trimolecular complexes and induction of accumulation of intracellular signaling molecules are thought to be delayed in recognition of partial agonists or antagonists, we expected that both may cause in an increase in onset time of intracellular signaling by antagonistic APL [28]; it is possible, that the duration of TCR ligation is shorter in recognition of partially agonistic or antagonistic APL than is that of the full agonistic peptide [29]. This rapid but incomplete engagement of TCR with APL may cause the rapid but altered Ca^{2+} response we observed. According to this model, the kinetic advantage of antagonists would interfere with the rate of productive TCR engagements by agonists and result in suppression of signaling processes needed for cell proliferation.

The release of Ca^{2+} from IP_3-sensitive intracellular stores is followed by a sustained influx of Ca^{2+} into the cytoplasm from the extracellular space via a mechanism designated as IP_3-induced calcium release [1, 30, 31]. This mechanism induces a biphasic increase in $[Ca^{2+}]_i$, a single small peak followed by a sustained high level, as observed at the single cell level in mouse T cells stimulated with peptide-MHC complexes [16] and in Chinese hamster ovary cells stimulated by platelet-derived growth factor [32]. However, as shown in Fig. 2, the sinusoidal peak only appeared in recognition of the wild-type peptide and not in recognition of the antagonistic APL. As these small peaks observed in response to the wild-type peptide remained in the absence of extracellular Ca^{2+}, the sinusoidal peak may correspond to the IP_3-induced calcium release from intracellular stores.

Oscillatory signals in numerous biological systems have long been recognized. Some of the most striking examples are oscillations in membrane potentials are seen in neuronal and cardiac cells [33]. To our knowledge, Ca^{2+} oscillation is a response of a cell to various continuous stimulations; it plays an important role in mitogenic activation of T cell and natural killer cells [34]. The elevated levels of IP_3 perhaps act with inositol-(1,3,4,5)-tetrakisphosphate ($InsP_4$) to maintain a constant influx of calcium or constant oscillation [1, 35–37]. However, in the present study, the E58M-induced Ca^{2+} oscillation was blocked by elimination of extracellular Ca^{2+} ions in almost all cells used. This finding suggests that the E58M-induced oscillation would mainly be dependent on the influx of extracellular Ca^{2+}. Oscillatory Ca^{2+} responses

responses induced by anti-CD3 mAb in human T cells were seen to be dependent on extracellular Ca^{2+} and these responses were reduced by membrane depolarization [38].

On the other hand, E63V induced the pattern of a high sustained Ca^{2+} response without a preceding sinusoidal peak. It may be that a single peak appeared at the same time as the high response. However, this peptide cannot induce proliferation of T cells. It was reported that artificial cytosolic Ca^{2+} elevation, for example by addition of ionomycin together with the partially agonistic APL, did not stimulate proliferation or IL-2 production by T cells [15]. Thus, not all high sustained Ca^{2+} responses can induce proliferation of T cells. The results also indicate that the peptide-induced activation of T cells does not merely depend on amplitude and duration of increase in $[Ca^{2+}]_i$, but rather upon the pattern of $[Ca^{2+}]_i$ increase and signals mediated by substances other than Ca^{2+}.

It is possible that differences in density of TCR ligands on the surface of APC may induce different patterns of Ca^{2+} response in T cells. Thus, the Ca^{2+} response to fully agonistic peptide expressed in low density may mimic that induced by partially agonistic or simple antagonistic APL. But this was not the case, and a difference in quality but not in quantity of MHC-peptide ligand seems to determine different patterns of Ca^{2+} response induced by each peptide ligand in T cells. This was also confirmed by the observation that Ca^{2+} responses stimulated by a lower concentration of wild-type peptide were still sensitive to genistein but not to GF109203X, a characteristic of Ca^{2+} response to fully agonistic peptide.

The effect of genistein on the APL-induced change in $[Ca^{2+}]_i$ suggests that the fully agonistic peptide-induced calcium mobilization involves the phospholipase Cγ1-IP_3 pathway; previous reports showed that genistein prevented TCR-CD3-mediated activation of phospholipase C through inhibition of activities of PTK, Fyn and Lck [22, 23, 39]. However, the partially agonistic and antagonistic peptide-induced increase in $[Ca^{2+}]_i$ may be bypassed by an alternative second messenger which may be dependent on the activation of PKC since the elevation of $[Ca^{2+}]_i$ induced by these APL was completely inhibited by treatment of T cells with GF109203X. As noted in other studies, there are two domains in PKC molecules, one is the catalytic domain contacting ATP and the other is a regulatory domain which interacts with calcium, diacylglycerol (DAG) and phosphatidylserine [24]. GF109203X specifically interacts with the catalytic domain to inhibit the activation of PKC. It may be that PKC-dependent Ca^{2+} responses induced by E58M or E63V are stimulated by substances other than DAG, such as phosphatidylserine, because genistein, a potent inhibitor of PTK and the subsequent production of DAG, showed marginal inhibitory effects on Ca^{2+} responses induced by the two APL.

Our observations correspond to findings in previous reports in which the antagonistic peptide did not induce full phosphorylation of the CD3 ζ-chain and Zap70 [40, 41] and another report in which partially agonistic APL did not stimulate any detectable inositol phosphate turnover in mouse T cells [42]. One possible explanation for the non-agonistic APL-induced increase in $[Ca^{2+}]_i$ in mouse T cells [15, 16] or in our human T cell clone is that APL may stimulate IP_3-independent signaling pathway(s) to induce a Ca^{2+} response in T cells. It is known that Ca^{2+} mobilization occurs independently of the PLC-IP_3 pathway in many cellular systems. Examples include sphingosine-stimulated Ca^{2+} increase in Jurkat human T cells [43], α_2-adrenoceptor-induced Ca^{2+} signaling in human erythroleukemia cells [44], thrombin-activated Ca^{2+} increase in neonatal rat ventricular myocytes [45] and cyclopiazonic acid-mediated Ca^{2+} response in cultured bovine pulmonary arterial endothelial cells [46]. Furthermore, it was recently reported that the PKC activator TPA stimulated calcium influx in ciliary cells and that the calcium response was sensitive to PKC inhibitors including GF109203X but resistant to the PLC inhibitor [47]. However, we cannot be conclusive on our observations obtained from only one T cell clone and peptide combination. Further investigations utilizing other human T cell clones and peptides are needed. To better understand mechanisms underlying the increase in $[Ca^{2+}]_i$ triggered by partially agonistic and antagonistic peptide, signaling molecules activated by APL and membrane-associated channel(s) involved in transport of Ca^{2+} need to be identified, as was done for mouse T cells [40–42].

In conclusion, stimulation of a human T cell clone with various APL induced a Ca^{2+} response with different patterns of increases in $[Ca^{2+}]_i$. Our findings suggest that the full agonist-induced calcium mobilization fully depends on genistein-sensitive signaling pathways, including PTK, phospholipase Cγ1 and IP_3 pathways, whereas that induced by a simple antagonist depends on extracellular Ca^{2+} ions and GF109203X-sensitive pathways, including PKC. The partially agonistic peptide-induced increase in $[Ca^{2+}]_i$ may involve several more complex mechanisms. Our characterization of the Ca^{2+} response in individual human T cells provides important information which will contribute to studies on mechanisms of activation and proliferation of human T cells.

4 Materials and methods

4.1 Peptides and T cell clone

Peptides and the T cell clone (YN5–32) were prepared as described elsewhere [21, 48, 49]. In brief, the $CD4^+$ $\alpha\beta$ T cell clone YN5–32 restricted by HLA-DRB1*0406 was established by stimulating PBMC with soluble M12p54–68 peptide, as described elsewhere [21]. The peptide M12p54–68 (NRDLEQAYNELSGEA), which corresponds to amino acid residues 54 to 68 of the streptococcal M protein derived from group A β hemolytic streptococcal strain 12 [50], was used as a fully agonistic wild-type peptide. M12p54–68 and its analogues carrying single residue substitutions, E63V, E63R, E58M, E58I and A60D, used in this study were synthesized using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu), based on the Fmoc strategy. All peptides were purified by reversed-phase high performance liquid chromatography (Millipore). Among APL derived from M12p54–68, E63V was found to be the strongest TCR partial agonist; it induced increases in cell size and expression levels of several cell surface molecules without cell proliferation, while E58M is the strongest simple TCR antagonist. TCR antagonism is an Ag-specific phenomenon in which T cells are inhibited by interactions involving TCR residues required for the recognition of conventional Ag and altered residues in peptide analogues (see review [28]). In the presence of 100 μM soluble E58M peptide, the proliferative response of the T cell clone YN5–32 to irradiated PBMC prepulsed for 2 h with 3 μM wild-type peptide was almost completely inhibited. This inhibition was not associated with induction of T cell anergy or increase in cell size and expression levels of several CD markers, which were observed for the partially agonistic APL, E63V. The evidence that single residue substituted APL-mediated inhibition of the proliferative response of YN5–32 to wild-type peptide is not due to competitive inhibition of peptide binding to HLA-DR molecules, but rather to TCR antagonism was reported in our previous publication [21]. A60D is an irrelevant analogue which exhibits neither agonism nor antagonism.

4.2 Measurements of $[Ca^{2+}]_i$

For video imaging experiments [51], T cells (5×10^5 cells/ml) were incubated with 3.5 μM fura-2/AM (Dojindo Laboratories, Kumamoto) for 45 min at 37 °C, then the cells were washed four times with the medium. To prevent movement of T cells during the assay, the fura-2/AM-loaded T cells (1×10^5/100 μl) were placed on a poly-L-lysine-coated mounted coverslip dish (35 mm diameter, MatTek Corp. Ashland, MA) and incubated for 30 min followed by addition of peptide-pulsed APC. The APC was prepared by incubating L-cells expressing DRA and DRB1*0406 genes [52, 53] with 100 μM peptide in a water bath at 37 °C for 5 h followed by three washings with medium. Then, the cells (2×10^5/100 μl) were added to the mount dish for another 30 min to obtain optimum contact between APC and T cells. Microfluorometric measurements were made in saline solution containing: 140 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, and 1 mg/ml BSA at pH 7.4 (adjusted with NaOH) at room temperatures (24–26 °C). The cells were exposed to UV light produced by a 100 W xenon arc lamp at alternating excitation wavelengths of 340 and 380 nm on an inverted microscope (Nikon, TMD-EFQ, Tokyo) equipped with 20x and 40x objectives. The emitted light was collected through a 510 nm emission filter with an intensifying CCD video camera linked to an image processor system (ARGUS-50/CA, Hamamatsu Photonics Corp, Hamamatsu). The background at each wavelength was acquired from a region free of cells on the glass coverslip and was subtracted, on line, from the 340 and the 380 nm images. The ratio of 340 nm/380 nm images was collected and stored on an MO disk every 4 s until analysis. In some experiments, Ca^{2+} response was investigated in the presence of genistein or GF109203X, or in the absence of extracellular Ca^{2+} ions. Details of the treatments are described in legends for Figs. 5 and 6.

4.3 Reagents and statistics

All salts for preparing solutions and poly-L-lysine, genistein, GF109203X and other pharmacological agents were purchased from Sigma (St. Louis, MO). Data were analyzed by basic statistical methods, including analysis of the two-tailed Student's *t*-test (unpaired), and expressed as the arithmetic means ± S.E. A $p < 0.05$ value was considered to be statistically significant.

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5 References

1 **Berridge, M. J.,** Inositol trisphosphate and calcium signalling. *Nature* 1993. **361:** 315–325.

2 **Crabtree, G. R. and Clipstone, N. A.,** Signal transmission between the plasma membrane and nucleus of T lymphocytes. *Annu. Rev. Biochem.* 1994. **63:** 1045–1083.

3 **Gold, M. R. and DeFranco, A. L.,** Biochemistry of B lymphocyte activation. *Adv. Immunol.* 1994. **55:** 221–295.

4 **Fanger, C. M., Hoth, M., Crabtree, G. R. and Lewis, R. S.,** Characterization of T cell mutants with defects in capacitative calcium entry: genetic evidence for the physiological roles of CRAC channels. *J. Cell Biol.* 1995. **131:** 655–667.

5 **Ghosh, A. and Greenberg, M. E.,** Calcium signaling in neurons: molecular mechanisms and cellular consequences. *Science* 1995. **268:** 239–247.

6 **Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C. and Healy, J. I.,** Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. *Nature* 1997. **386:** 855–858.

7 **Berridge, M. J. and Irvine, R. F.,** Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* 1984. **312:** 315–321.

8 **Damjanovich, S., Szollosi, J. and Tron, L.,** Transmembrane signalling in T cells. *Immunol. Today* 1992. **13:** A12–15.

9 **Gardner, P.,** Calcium and T lymphocyte activation. *Cell* 1989. **59:** 15–20.

10 **Crabtree, G. R.,** Contingent genetic regulatory events in T lymphocyte activation. *Science* 1989. **243:** 355–361.

11 **Chakrabarti, R., Chang, J. Y. and Erickson, K. L.,** T cell receptor-mediated Ca^{2+} signaling: release and influx are independent events linked to different Ca^{2+} entry pathways in the plasma membrane. *J. Cell. Biochem.* 1995. **58:** 344–359.

12 **Donnadieu, E., Cefai, D., Tan, Y. P., Paresys, G., Bismuth, G. and Trautmann, A.,** Imaging early steps of human T cell activation by antigen-presenting cells. *J. Immunol.* 1992. **148:** 2643–2653.

13 **Rotnes, J. S. and Bogen, B.,** Ca^{2+} mobilization in physiologically stimulated single T cells gradually increases with peptide concentration (analog signaling). *Eur. J. Immunol.* 1994. **24:** 851–858.

14 **Northrop, J. P., Ho, S. N., Chen, L., Thomas, D. J., Timmerman, L. A., Nolan, G. P., Admon, A. and Crabtree, G. R.,** NF-AT components define a family of transcription factors targeted in T-cell activation. *Nature* 1994. **369:** 497–502.

15 **Sloan-Lancaster, J., Steinberg, T. H. and Allen, P. M.,** Selective activation of the calcium signaling pathway by altered peptide ligands. *J. Exp. Med.* 1996. **184:** 1525–1530.

16 **Wülfing, C., Rabinowitz, J. D., Beeson, C., Sjaastad, M. D., McConnell, H. M. and Davis, M. M.,** Kinetics and extent of T cell activation as measured with the calcium signal. *J. Exp. Med.* 1997. **185:** 1815–1825.

17 **Rabinowitz, J. D., Beeson, C., Wülfing, C., Tate, K., Allen, P. M., Davis, M. M. and McConnell, H. M.,** Altered T cell receptor ligands trigger a subset of early T cell signals. *Immunity* 1996. **5:** 125–135.

18 **Matsushita, S., Takahashi, K., Motoki, M., Komoriya, K., Ikagawa, S. and Nishimura, Y.,** Allele specificity of structural requirement for peptides bound to HLA-DRB1*0405 and -DRB1*0406 complexes: implication for the HLA-associated susceptibility to methimazole-induced insulin autoimmune syndrome. *J. Exp. Med.* 1994. **180:** 873–883.

19 **Matsuoka, T., Kohrogi, H., Ando, M., Nishimura, Y. and Matsushita, S.,** Altered TCR ligands affect antigen-presenting cell responses: up-regulation of IL-12 by an analogue peptide. *J. Immunol.* 1996. **157:** 4837–4843.

20 **Matsushita, S., Kohsaka, H. and Nishimura, Y.,** Evidence for self and nonself peptide partial agonists that prolong clonal survival of mature T cells in vitro. *J. Immunol.* 1997. **158:** 5685–5691.

21 **Chen, Y. Z., Matsushita, S. and Nishimura, Y.,** Response of a human T cell clone to a large panel of altered peptide ligands carrying single residue substitutions in an antigenic peptide: characterization and frequencies of TCR agonism and TCR antagonism with or without partial activation. *J. Immunol.* 1996. **157:** 3783–3790.

22 **Mustelin, T., Coggeshall, K. M., Isakov, N. and Altman, A.,** T cell antigen receptor-mediated activation of phospholipase C requires tyrosine phosphorylation. *Science* 1990. **247:** 1584–1587.

23 **June, C. H., Fletcher, M. C., Ledbetter, J. A., Schieven, G. L., Siegel, J. N., Phillips, A. F. and Samelson, L. E.,** Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. *Proc. Natl. Acad. Sci. USA* 1990. **87:** 7722–7726.

24 **Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F. et al.,** The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* 1991. **266:** 15771–15781.

25 **al-Ramadi, B. K., Jelonek, M. T., Boyd, L. F., Margulies, D. H. and Bothwell, A. L.,** Lack of strict correlation of functional sensitization with the apparent affinity of MHC/peptide complexes for the TCR. *J. Immunol.* 1995. **155:** 662–673.

26 **Alam, S. M., Travers, P. J., Wung, J. L., Nasholds, W., Redpath, S., Jameson, S. C. and Gascoigne, N. R.,** T-cell-receptor affinity and thymocyte positive selection. *Nature* 1996. **381:** 616–620.

27 **Lyons, D. S., Liebermann, S. A., Hampl, J., Boniface, J. J., Chien, Y., Berg, L. J. and Davis, M. M.,** A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. *Immunity* 1996. **5:** 53–61.

28 **Sloan-Lancaster, J. and Allen, P. M.,** Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology. *Annu. Rev. Immunol.* 1996. **14:** 1–27.

29 **Valitutti, S. and Lanzavecchia, A.,** Serial triggering of TCRs: a basis for the sensitivity and specificity of antigen recognition. *Immunol. Today* 1997. **6:** 299–303.

30 **Furuichi, T., Kohda, K., Miyawaki, A. and Mikoshiba, K.,** Intracellular channels. *Curr. Opin. Neurobiol.* 1994. **4:** 294–303.

31 **Furuichi, T. and Mikoshiba, K.,** Inositol-1, 4, 5-trisphosphate receptor-mediated Ca^{2+} signaling in the brain. *J. Neurochem.* 1995. **64:** 953–960.

32 **Mathias, R. S., Mikoshiba, K., Michikawa, T., Miyawaki, A. and Ives, H. E.,** IP_3 receptor blockade fails to prevent intracellular Ca^{2+} release by ET-1 and α-thrombin. *Am. J. Physiol.* 1998. **274:** C1456–C1465.

33 **Fewtrell, C.,** Ca^{2+} oscillations in non-excitable cells. *Annu. Rev. Physiol.* 1993. **55:** 427–454.

34 **Hess, S. D., Oortgiesen, M. and Cahalan, M. D.,** Calcium oscillations in human T and natural killer cells depend upon membrane potential and calcium influx. *J. Immunol.* 1993. **150:** 2620–2633.

35 **Irvine, R. F.,** 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates – a possible mechanism. *FEBS Lett.* 1990. **263:** 5–9.

36 **Bird, G. S., Rossier, M. F., Hughes, A. R., Shears, S. B., Armstrong, D. L. and Putney, J. W. Jr.,** Activation of Ca^{2+} entry into acinar cells by a non-phosphorylatable inositol trisphosphate. *Nature* 1991. **352:** 162–165.

37 **Irvine, R. F.,** Is inositol tetrakisphosphate the second messenger that controls Ca^{2+} entry into cells? *Adv. Second Messenger Phosphoprotein Res.* 1992. **26:** 161–185.

38 **Donnadieu, E., Bismuth, G. and Trautmann, A.,** Calcium fluxes in T lymphocytes. *J. Biol. Chem.* 1992. **267:** 25864–25872.

39 **Nakayama, K. and Loh, D. Y.,** No requirement for $p56^{lck}$ in the antigen-stimulated clonal deletion of thymocytes. *Science* 1992. **257:** 94–96.

40 **Sloan-Lancaster, J., Shaw, A. S., Rothbard, J. B. and Allen, P. M.,** Partial T cell signaling: altered phospho-zeta and lack of Zap70 recruitment in APL-induced T cell anergy. *Cell* 1994. **79:** 913–922.

41 **Madrenas, J., Wange, R. L., Wang, J. L., Isakov, N., Samelson, L. E. and Germain, R. N.,** ζ phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. *Science* 1995. **267:** 515–518.

42 **Sloan-Lancaster, J., Evavold, B. D. and Allen, P. M.,** Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature* 1993. **363:** 156–159.

43 **Sakano, S., Takemura, H., Yamada, K., Imoto, K., Kaneko, M. and Ohshika, H.,** Ca^{2+} mobilizing action of sphingosine in Jurkat human leukemia T cells. Evidence that sphingosine releases Ca^{2+} from inositol trisphosphate- and phosphatidic acid-sensitive intracellular stores through a mechanism independent of inositol trisphosphate. *J. Biol. Chem.* 1996. **271:** 11148–11155.

44 **Michel, M. C., Brass, L. F., Williams, A., Bokoch, G. M., LaMorte, V. J. and Motulsky, H. J.,** α_2**-**adrenergic receptor stimulation mobilizes intracellular Ca^{2+} in human erythroleukemia cells. *J. Biol. Chem.* 1989. **264:** 4986–4991.

45 **Jiang, T., Kuznetsov, V., Pak, E., Zhang, H., Robinson, R. B. and Steinberg, S. F.,** Thrombin receptor actions in neonatal rat ventricular myocytes. *Circ. Res.* 1996. **78:** 553–563.

46 **Pasyk, E., Inazu, M. and Daniel, E. F.,** CPA enhances Ca^{2+} entry in cultured bovine pulmonary arterial endothelial cells in an IP_3-independent manner. *Am. J. Physiol.* 1995. **268:** H138–146.

47 **Levin, R., Braiman, A. and Priel, Z.,** Protein kinase C induced calcium influx and sustained enhancement of ciliary beating by extracellular ATP. *Cell Calcium* 1997. **21:** 103–113.

48 **Ikagawa, S., Matsushita, S., Chen, Y. Z., Ishikawa, T. and Nishimura, Y.,** Single amino acid substitutions on a Japanese cedar pollen allergen (Cry j 1)-derived peptide induced alterations in human T cell responses and T cell receptor antagonism. *J. Allergy Clin. Immunol.* 1996. **97:** 53–64.

49 **Chen, Y. Z., Matsushita, S. and Nishimura, Y.,** A single residue polymorphism at DR β^{37} affects recognition of peptides by T cells. *Hum. Immunol.* 1997. **54:** 30–39.

50 **Robbins, J. C., Spanier, J. G., Jones, S. J., Simpson, W. J. and Cleary, P. P.,** *Streptococcus pyogenes* type 12 M protein gene regulation by upstream sequences. *J. Bacteriol.* 1987. **169:** 5633–5640.

51 **Fujii, T., Tokutomi, N., Hirata, A., Negi, A. and Nishi, K.,** Cytoplasmic Ca^{2+} mobilization and Ca^{2+}-dependent membrane currents in dispersed bovine ciliary muscle cells. *Curr. Eye Res.* 1997. **16:** 436–444.

52 **Gregersen, P. K., Goyert, S. M., Song, Q. L. and Silver, J.,** Microheterogeneity of HLA-DR4 haplotypes: DNA seqence analysis of LD"KT2" and LD"TAS" haplotypes. *Hum. Immunol.* 1987. **19:** 287–292.

53 **Inoko, H., Bodmer, J. G., Heyes, J. M., Drover, S., Trowsdale, J. and Marshall, W. H.,** Joint report on the transfectant/monoclonal antibody component. In **Tsuji, K., Aizawa, M. and Sasazuki T.** (Eds.) *HLA 1991.* Oxford Science Publications, Oxford 1992, p 919.

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