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Early Signal Transduction by the Antigen Receptor Without Commitment to T Cell Activation

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The T lymphocyte antigen–receptor complex mediates antigen-specific cell activation, at least in part, through the production of inositolphospholipid-derived second messengers. Little is known about how second messenger events, typically measured within minutes of ligand binding, eventually lead to distal biologic responses such as expression of lymphokine genes. Several monoclonal antibodies directed against the receptor complex were tested for their ability to elicit transmembrane signaling in the parental Jurkat line and in a somatic mutant (J.CaM1) with a deficient receptor function. One antibody elicited substantial early Ca^{2+} mobilization responses in both cells but was unable to promote expression of the interleukin-2 gene in J.CaM1. In J.CaM1 there was a diminished production of phosphatidylinositol second messengers, and the elevation in intracellular free Ca^{2+} was transient. Thus, short-term Ca^{2+} mobilization does not always indicate complete signal transmission and lead to a full cellular response.

THE T LYMPHOCYTE ANTIGEN RECEPTOR recognizes foreign antigen and transduces this recognition into intracellular biochemical events that result in biologic responses. The receptor complex is a multimeric structure consisting of the antigen-binding disulfide-linked heterodimer (Ti) noncovalently associated with three to seven invariant proteins (CD3) (*1, 2*). This complex is functionally coupled to the phosphatidylinositol (PI) second messenger pathway (*3*) responsible for mobilization of intracellular free Ca^{2+} and activation of protein kinase C (PKC) (*4, 5*). These second messenger events contribute to later cellular responses, such as lymphokine gene expression (*2*).

Recently we described a system for isolating and characterizing T cell somatic mutants with deficient signal transduction by the antigen-receptor complex (*6*). The first such mutant, J.CaM1 (derived from the leukemic cell line Jurkat), was selected primarily for its inability to increase the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in response to monoclonal antibodies (mAbs) directed against the antigen receptor complex, which function as agonists for the wild-type Jurkat cells. In J.CaM1, lectins and mAbs against the idiotype of the receptor (Ti) or certain mAbs against the invariant CD3 molecules associated with the receptor (Fig. 1B) are ineffective in eliciting the rapid increase in $[Ca^{2+}]_i$ that is characteristic of Jurkat (Fig. 1A) and other cell lines (*7–11*). However, some mAbs that alone are nonagonists for J.CaM1 (for example, OKT3 and C305) when used in combination are potent activators of Ca^{2+} mobilization in J.CaM1 (*6*); in addition, at least one mAb directed against CD3 (mAb 235) (*12*) appears to act alone as an effective agonist for both Jurkat (Fig. 1C) and J.CaM1 (Fig. 1D). Thus, the CD3-Ti complex expressed on J.CaM1 appears to be inefficiently coupled to the PI pathway, but certain potent mAbs to CD3 can initiate PI metabolism in this cell.

In Jurkat, expression of the interleukin-2 (IL-2) gene can be induced by phorbol myristate acetate (PMA) combined with a Ca^{2+}-mobilizing agonist, such as a lectin, an ionophore, or a mAb against CD3-Ti (*7, 13*). Since the short-term Ca^{2+} responses to mAb 235 are comparable in the parental and mutant cell lines, we were surprised to find a discrepancy in the ability of mAb 235 to synergize with PMA to produce IL-2 in J.CaM1 and Jurkat. The parental Jurkat cells secreted substantial amounts of IL-2 during a culture overnight in the presence of PMA and mAbs directed against Ti (C305) or against CD3 (OKT3 or mAb 235), as expected (Fig. 2). However, neither the agonist combination of OKT3 and C305 nor mAb 235 alone synergized with PMA to elicit substantial production of IL-2 by J.CaM1 (Fig. 2) even though they elicited large immediate Ca^{2+} responses in this cell (Fig. 1) (*6*). Both cell lines produced substantial IL-2 in response to the ionophore ionomycin in the presence of PMA (Fig. 2), indicating that the failure to respond was not due to a defect in the distal gene expression apparatus.

Since production of IL-2 is regulated primarily pretranslationally (*2, 14*), we investigated the possibility that the failure of mAb 235 to stimulate production of IL-2 by J.CaM1 was also observable at the level of mRNA for IL-2. Analysis of IL-2 mRNA generation by these cells confirmed that mAb 235 was unable to synergize with PMA to promote transcription of the IL-2

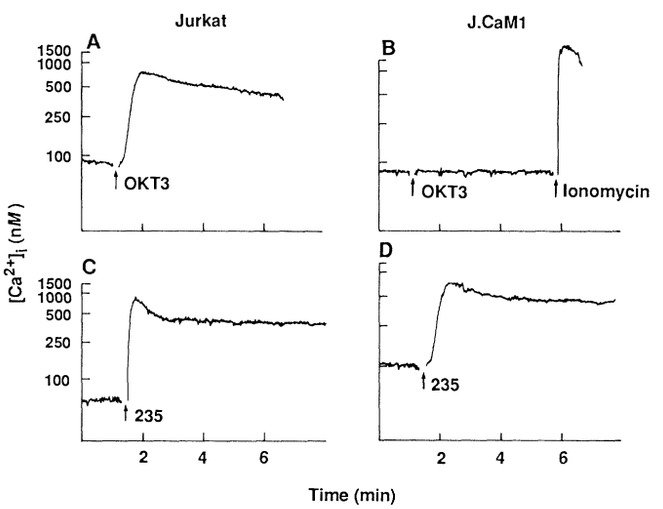

Fig. 1. Mobilization of Ca^{2+} by Jurkat and a signaling mutant derived from Jurkat. Cells were loaded with the fluorescent Ca^{2+}-sensitive indicator Indo-1 (*18*) at 3 μM, and fluorescence profiles were obtained with a Spex Fluorolog II spectrofluorometer as described (*6, 7*). Jurkat cells showed large increases in $[Ca^{2+}]_i$ in response to (**A**) OKT3 or (**C**) mAb 235 (ascites, 1:1000 dilution). J.CaM1 cells failed to respond to (**B**) OKT3 but showed a substantial response to (**D**) mAb 235, which was comparable to that of Jurkat cells. Ionomycin was added where indicated (1 μM) to demonstrate intracellular Indo-1 and releasability of Ca^{2+} stores.

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gene by J.CaM1, despite its effectiveness in Jurkat. Therefore, despite the comparable rapid Ca^{2+} mobilization elicited in the two cell lines by mAb 235 (Fig. 1) and the combination of OKT3 and C305 (*6*), these ligands are relatively ineffective in promoting at least one of the usual biologic consequences—IL-2 production—in J.CaM1.

This discrepancy between acute Ca^{2+} mobilization and IL-2 production appears to be at odds with current models of T cell activation in which the receptor-mediated increase in $[Ca^{2+}]_i$ synergizes with PMA-mediated events to promote more distal cellular responses. Because the receptor-induced Ca^{2+} increase is a consequence of the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) (*3*), we examined this second messenger system for a biochemical explanation of this dissociation between Ca^{2+} mobilization and IL-2 production. During a long-term kinetic study in wild-type Jurkat, mAb 235 elicited an immediate and dramatic increase in the PIP_2-derived second messenger inositol 1,4,5-trisphosphate (IP_3) (Fig. 3A), which is thought to be responsible for mobilizing Ca^{2+} from intracellular pools (*3, 4*), and IP_3 or its metabolites, for regulating Ca^{2+} channels (*15, 16*). This increase was marked at 3 min, peaked at 20 min, and declined gradually thereafter. A twofold increase was still measurable 80 min after stimulation. The IP_3 metabolites inositol 1,4-bisphosphate (IP_2) and inositol 1-monophosphate (IP_1) lagged slightly behind IP_3 (Fig. 3B), as expected. PI second messenger production in J.CaM1 in response to mAb 235 was markedly diminished (Fig. 3, A and B), however, when compared to that in Jurkat. Although the timing of second messenger generation appeared similar to that of Jurkat, IP_3 in J.CaM1 reached a maximum only 3-fold above basal levels (in contrast to the 12-fold increase in Jurkat) and declined more rapidly. The metabolites IP_2 and IP_1 were concomitantly diminished in J.CaM1. Thus, second messengers in J.CaM1 in response to the Ca^{2+}-agonist mAb 235, although detectable, were produced at levels substantially lower than those in Jurkat.

These findings suggest that short-term Ca^{2+} responses do not always indicate substantial PI second messenger production. Furthermore, since the PI second messengers may have a role in intracellular communication beyond the initial transient mobilization of Ca^{2+}, the detection of a short-term change in $[Ca^{2+}]_i$ does not always imply effective signal transduction. For example, IP_3 or a metabolite may play a role in sustaining the elevation of $[Ca^{2+}]_i$ beyond the first few minutes (*15, 16*). Because previous studies with Jurkat cells demonstrated that induction of the IL-2 gene requires receptor occupancy for 2 to 4 hours (*17*), it seems likely that there is a requirement for sustained second messenger activities (Ca^{2+} mobilization or PKC activation) to complete the transmission of induction signals. Since the generation of IP_3 and metabolites was substantially reduced in J.CaM1, these lower levels of second messengers might be unable to sustain Ca^{2+} mobilization during the several hours required for commitment to IL-2 expression.

Therefore, we sought to determine whether the Ca^{2+} responses to mAb 235 during the first 10 min in Jurkat and J.CaM1 were maintained beyond the initial phase. Standard fluorimetry could not be used beyond the first 20 min, since the fluorescent Ca^{2+}-indicator Indo-1 (*18*) leaked from the cells after that time. Consequently, we turned to the fluorescence-activated cell sorter (FACS) for analysis. Since the fluorescence emission spectrum of Indo-1 shifts from a peak near 486 nm in the

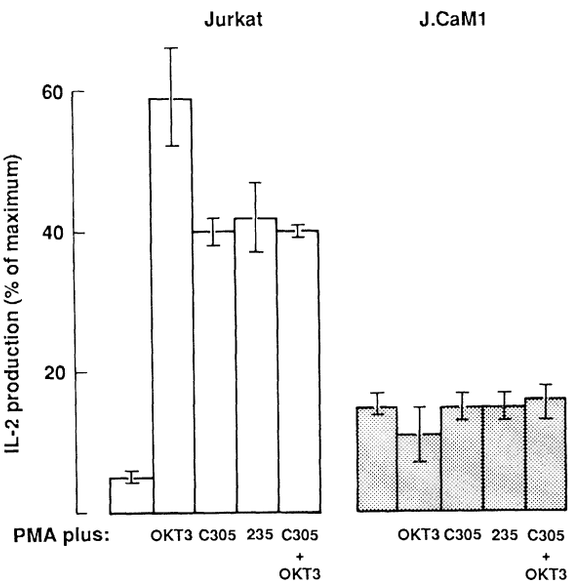

Fig. 2. Production of IL-2 by Jurkat and J.CaM1 in response to various stimuli. Jurkat and J.CaM1 cells were cultured overnight with PMA (50 ng/ml) in combination with saturating concentrations of the individual mAb indicated (ascites, 1:1000 dilution), supernatants were harvested, and IL-2 secretion was determined in a bioassay with CTLL-20 cells (*13, 24*). IL-2 production is reported as the mean of triplicate cultures with standard errors, expressed as a percentage of maximal production by each cell type (determined with the ionophore ionomycin at 1 μM in combination with PMA) to normalize for clonal variability. In this assay, maximum IL-2 production by Jurkat was 209 U/ml and by J.CaM1 was 55 U/ml. The quantitative difference in maximum production of IL-2 between Jurkat and J.CaM1 is not a consistent finding and does not explain the results with receptor mAb. The small apparent IL-2 response to PMA alone is due to the direct effect of PMA on the CTLL-20 cells in the bioassay.

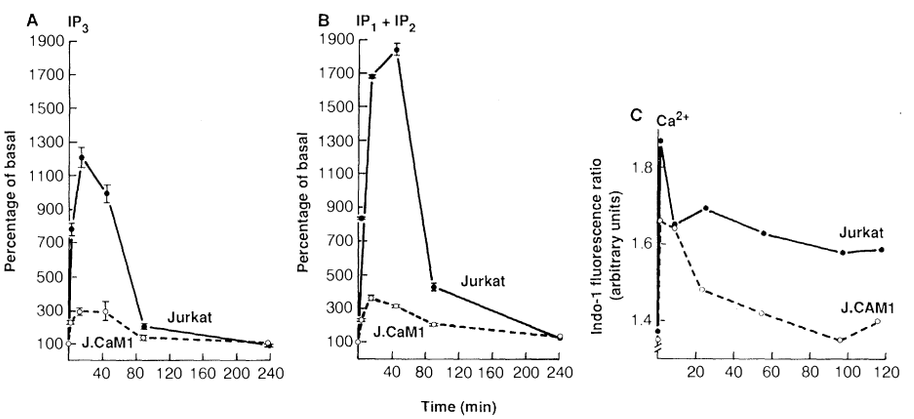

Fig. 3. Second messenger generation in Jurkat and J.CaM1 in response to mAb 235. (**A** and **B**) Cells were labeled with [^{3}H]myoinositol and then exposed to mAb 235 (ascites, 1:1000) for the indicated time interval. Cells were then lysed and levels of (A) IP_3 and (B) IP_1 + IP_2 were determined essentially as described earlier (*3*). Results are shown as the mean and standard error of triplicate cultures, expressed as a percentage of the levels measured in the absence of stimulus. (**C**) Cells were loaded with Indo-1 and cultured in the presence of mAb 235. The culture tube was intermittently sampled on a Becton-Dickinson FACS IV with excitation at 364 nm and emission detection at 404 and 486 nm. Indo-1 fluorescence is plotted as the mean ratio (7000 cells) of fluorescence at 404 nm (Ca^{2+}-bound peak) to 486 nm (Ca^{2+}-free peak). For each data point, a control tube was assessed to demonstrate that the baseline fluorescence ratio had not moved. The difference in peak values for the two cells at the earliest time point in this experiment has not been consistently observed, but the marked difference between Jurkat and J.CaM1 in the later phase has been. Furthermore, the difference at the early time points was judged not to be substantial on the basis of the calibrated fluorimetry tracings in Fig. 1.

absence of Ca^{2+} to a peak of 404 nm when bound to Ca^{2+} (*18*), we can use a ratio of the fluorescence intensities at these two wavelengths as an indicator of $[Ca^{2+}]_i$ that is independent of the concentration of Indo-1 in a given cell; fluorescence from extracellular dye does not interfere because of the large dilutional effects in the FACS.

As expected, Jurkat cells responded promptly to mAb 235 with a marked rise in their mean Indo-1 ratio to a peak that then declined slightly to a plateau that was well above the baseline (Fig. 3C); this elevated plateau persisted for at least 2 hours. In contrast, the Indo-1 ratio of J.CaM1 rose markedly immediately after stimulation, but there was no subsequent plateau phase (Fig. 3C); the Indo-1 ratio in these cells began to decay within 10 min after stimulation and was at or below the baseline within 80 to 90 min. Therefore, despite the comparable early changes in $[Ca^{2+}]_i$ in Jurkat and J.CaM1 in response to mAb 235, the rise in J.CaM1 was only transient, while it was sustained for at least 2 hours in Jurkat. These results are consistent with the view that an elevation of $[Ca^{2+}]_i$ must be sustained for more than 2 hours to promote IL-2 gene expression and that a short-term rise is not sufficient to indicate effective signal transduction by the antigen receptor complex. Moreover, the inability of J.CaM1 to sustain an elevation of $[Ca^{2+}]_i$ may be related to the weak receptor-mediated PI second messenger generation.

These findings suggest several properties of signal transduction by the antigen-receptor complex that may have implications for other PI-coupled receptors. First, early changes in $[Ca^{2+}]_i$ that result from receptor-ligand binding do not alone imply effective and full signal transmission; communication between the receptor and certain activation genes requires more than just a transient elevation in $[Ca^{2+}]_i$. However, certain biologic responses in other systems, such as exocytosis of prepackaged hormones, are indeed responsive to transient Ca^{2+} responses. For example, in pituitary GH_3 cells there are at least two phases of prolactin secretion in response to thyrotropin-releasing hormone, with the immediate phase corresponding to the immediate Ca^{2+} increase (*19*) and the later plateau phase related either to a plateau of elevated $[Ca^{2+}]_i$ (*19*) or to sustained diacylglycerol generation (*20*). In T lymphocytes, a similar immediate response might be stimulated in cytotoxic T cells containing prepackaged cytolytic granules (*21*).

A second conclusion is that the peak Ca^{2+} mobilization responses measured within minutes of receptor engagement do not always correlate with the levels of IP_3 generated. Therefore, substantial changes in $[Ca^{2+}]_i$ can be detected with only minimal production of Ca^{2+}-mobilizing second messengers. A similar observation was made in a study of the induction of nonresponsiveness in T cell clones, in which antigen presented on chemically modified antigen-presenting cells stimulated an increase in $[Ca^{2+}]_i$ without any measurable increase in IP_3 (*8*). Although it remains possible that a signal pathway distinct from PI is involved in that situation, our results demonstrate that detection of Ca^{2+} mobilization can be a much more sensitive indicator of receptor perturbation than is the measurement of inositol phosphates, even when the Ca^{2+} response derives from activation of the PI second messenger pathway. Thus, the use of short-term Ca^{2+} mobilization as a marker of PI pathway activation may at times be deceptive.

Finally, there is a requirement for sustained second messenger generation in the receptor-mediated induction of IL-2 gene expression. It is unclear from the studies described thus far whether it is the sustained elevation of $[Ca^{2+}]_i$ itself that is required for commitment to IL-2 expression, as suggested by Fig. 3C, or whether this prolonged Ca^{2+} response is an associated marker of other second messenger activities required during this period. To investigate these possibilities directly, we examined the ability of Jurkat cells to produce IL-2 in response to mAb 235 plus PMA in the presence and absence of extracellular Ca^{2+}, since extracellular Ca^{2+} is required for sustaining the elevation of $[Ca^{2+}]_i$ caused by CD3-Ti ligands (*3*). Assays of both secreted IL-2 bioactivity and mRNA for IL-2 revealed that addition of EGTA (1 m*M*, to chelate Ca^{2+}) as late as 150 min after exposure of the cells to mAb 235 plus PMA completely abrogated production of IL-2 by these cells. Addition of EGTA even 4 to 6 hours after stimulation substantially reduced but did not abrogate expression of IL-2 mRNA and production of IL-2. These results are consistent with the reported kinetics of IL-2 mRNA production in Jurkat, in which mRNA for IL-2 is detectable beginning 2 to 4 hours after exposure to receptor ligands (*14*). That the findings with EGTA are not the result of general toxicity to the cells is suggested by three lines of evidence: (i) the cells were trypan blue–excluding even after 16 hours of culture with EGTA; (ii) the production of mRNA for the Ti-β chain was unaffected by the presence of EGTA; and (iii) EGTA has been shown to have no effect on the ability of human T cell clones to proliferate in response to exogenous IL-2 (*8*). These findings are consistent with the interpretation that a sustained elevation of $[Ca^{2+}]_i$ is the relevant missing event in J.CaM1 that prevents stimulation of IL-2 production.

Reported studies of T cell nonresponsiveness (*22*) and of Thy-1 signal transduction in Jurkat and mutant transfectants (*23*) have also shown that Ca^{2+} mobilization can be observed in the absence of gene induction. The kinetic analysis described in our studies suggests that the paradox may lie in the ability or inability of a stimulus to promote sustained signal transduction activities. These results support the earlier finding that engagement of the antigen receptor does not trigger an irreversible cascade of events, but rather that the receptor must be continuously occupied and engaged in signal transduction for several hours for resultant IL-2 gene induction (*17*). Further studies of proximal signal transduction and of distal biologic responses may lead to a better understanding of how these events are connected.

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