Regulation of Inositol 1,4,5-Trisphosphate Kinase Activity after Stimulation of Human T Cell Antigen Receptor

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Abstract

Inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3), a Ca^{2+}-mobilizing messenger, can be phosphorylated by a cytoplasmic kinase, yielding inositol 1,3,4,5-tetrakisphosphate (Ins-1,3,4,5-P_3). We observed that stimulation of the antigen receptor on a malignant human T cell line, Jurkat, led to substantial, sustained increases in Ins-1,4,5-P_3 and $InsP_4$. The Ins-1,4,5-P_3 kinase partially purified from resting Jurkat cells had a maximum velocity (V_{max}) of 0.09 nmol/min/mg protein and an apparent Michaelis constant (K_m) of 0.2 μM. When the kinase was partially purified 10 min after stimulation of the antigen receptor or after the addition of phorbol myristate acetate, the V_{max} was increased twofold. The activity of the Ins-1,4,5-P_3 kinase obtained from either resting or stimulated Jurkat cells was enhanced in vitro by increasing the concentration of free Ca^{2+} from 0.1 to 0.5 μM. These results indicate that the activity of the Ins-1,4,5-P_3 kinase is regulated as a consequence of stimulating the T cell antigen receptor.

Introduction

Transmembrane signaling by a variety of cell-surface receptors involves the release of Ca^{2+} from intracellular stores (reviewed in 1, 2). Stimulation of these Ca^{2+}-mobilizing receptors results in the hydrolysis of a membrane phospholipid, phosphatidylinositol-4,5-bisphosphate, generating diacylglycerol and inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3)[1] (1, 2). Diacylglycerol is an activator of protein kinase C (2). Ins-1,4,5-P_3, on the other hand, releases Ca^{2+} from the endoplasmic reticulum of broken cells and is thought to mediate receptor-induced intracellular Ca^{2+} mobilization within intact cells (1, 2).

The metabolism of Ins-1,4,5-P_3 is complex. Specific phosphatases can sequentially remove phosphate groups from the inositol ring, eventually converting Ins-1,4,5-P_3 to free inositol (3). Alternatively, a cytoplasmic kinase that appears to have a wide tissue distribution can phosphorylate Ins-1,4,5-P_3, yielding inositol 1,3,4,5-tetrakisphosphate (Ins-1,3,4,5-P_4) (4–6). Although increases in Ins-1,3,4,5-P_4 and its immediate breakdown product, inositol 1,3,4-trisphosphate (Ins-1,3,4-P_3), have been observed after receptor stimulation, it is not known what role, if any, the Ins-1,3,4,5-P_4 pathway plays in receptor-mediated regulation of cellular activities (4–7). At a minimum, however, the existence of two pathways for the metabolism of Ins-1,4,5-P_3 suggests a mechanism for differential control of the levels of this Ca^{2+}-mobilizing messenger. In view of the investment of adenosine triphosphate in the formation of Ins-1,3,4,5-P_4, it is also possible that Ins-1,3,4,5-P_4 has a second messenger function of its own.

An important issue in defining the physiological role of the Ins-1,3,4,5-P_4 pathway is whether the activity of the Ins-1,4,5-P_3 kinase is regulated as a consequence of receptor stimulation. To address this question, we examined the effects of stimulating the T cell antigen receptor (T3/Ti) and used a malignant human T cell line, Jurkat, which has served as a model for studies of T cell activation (8–11). T3/Ti is composed of a disulfide-linked polymorphic heterodimer, Ti, that is noncovalently associated with at least three invariant T3 polypeptides (12). Because T lymphocytes recognize specific antigen on the surface of specialized accessory cells, physiological stimulation of T3/Ti requires cell–cell contact (12). Under appropriate conditions, however, monoclonal antibodies (MAbs) with specificity for T3/Ti can mimic the effects of antigen and activate T lymphocytes (13). The addition of T3/Ti MAbs to Jurkat cells induces substantial increases in $InsP_3$ and concentration of cytoplasmic free calcium ($[Ca^{2+}]i$) and stimulates the translocation of protein kinase C activity from the cytosol to a membrane fraction (8–11). The validity of the use of these MAb as receptor agonists is supported by the demonstrations that antigen-primed accessory cells stimulate antigen-specific T cell clones to generate $InsP_3$ and increase $[Ca^{2+}]i$ (14–16). Stewart et al. recently reported that permeabilized Jurkat cells have Ins-1,4,5-P_3 kinase activity and observed an increase in the level of $InsP_4$ 10 min after the addition of a T3/Ti MAb (6). Herein we demonstrate that stimulation of T3/Ti increases the activity of Ins-1,4,5-P_3 kinase in Jurkat cells.

Methods

Cells and reagents. Jurkat E6-IL2 was passaged as described (17). C305, a mouse IgM kappa MAb, recognizes Ti on Jurkat (17). All chemicals were from Sigma Chemical Co., St. Louis, MO. [^{3}H]Inositol (17.1 Ci/mmol) was obtained from New England Nuclear, Boston, MA. [^{3}H]Ins-1,4,5-P_3 (1 Ci/mmol; Amersham Corp., Arlington Heights, IL) was dried under nitrogen and resuspended in water before use. Protein measurements were by the colorimetric method of Bio-Rad Laboratories, Richmond, CA.

Separation of inositol polyphosphates by high-performance liquid chromatography (HPLC). Jurkat cells, labeled with [^{3}H]inositol as de-

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1. *Abbreviations used in this paper:* $[Ca^{2+}]i$, concentration of cytoplasmic free calcium; Ins-1,3,4-P_3, inositol 1,3,4-trisphosphate; Ins-1,3,4,5-P_4, inositol 1,3,4,5-tetrakisphosphate; Ins-1,4,5-P_3, inositol 1,4,5-trisphosphate; MAb, monoclonal antibody; T3/Ti, T cell antigen receptor.

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scribed (18), were washed extensively, resuspended at 1.5×10^7 cells/ml in Hepes-buffered normal saline, and then incubated at 37°C with or without C305 (10 μg/ml). At the end of the incubation period, cells were sedimented for 10 s in an Eppendorf 5414 microfuge. After aspiration of the medium, 1 ml of ice-cold 10% (wt/vol) trichloroacetic acid was added to the cellular pellet, and samples were incubated for 10 min on ice. After removal of insoluble material by a 900 g centrifugation for 10 min, the supernatant was extracted with 6 vol of diethyl ether and then neutralized. Inositol polyphosphates were separated by a modification of the HPLC method of Irvine et al. (4, 7) using a Whatman Partisal 10 SAX column (0.46 × 25 cm, 10-μm particle size) and a guard column packed with Whatman Pellicular anion exchanger. Following sample injection, the column was washed for 10 min with water and then subjected to increasing concentrations of ammonium formate buffer (adjusted to pH 3.7 with phosphoric acid) with a flow rate of 1.2 ml/min. Following a linear gradient to 0.8 M ammonium formate over 30 min, Ins-1,3,4-P_3 and Ins-1,4,5-P_3 were sequentially eluted in 0.8 M ammonium formate and collected in 0.5-min fractions. After 18 min at 0.8 M, the concentration of ammonium formate was increased to 2 M by a linear gradient over 15 min, and $InsP_4$ was then collected in 1-min fractions. ^3H radioactivity was quantified by liquid scintillation counting in Aquasol (New England Nuclear). Under these conditions peak elution times for Ins-1,3,4-P_3, Ins-1,4,5-P_3, and $InsP_4$ were 48, 52, and 75 min, respectively, from the time of sample injection. Ins-1,4,5-P_3 in samples was identified on the basis of identity with the elution time of the [^3H]Ins-1,4,5-P_3 standard. As described (7, 19), Ins-1,3,4-P_3 was identified by an elution time intermediate between adenosine triphosphate and Ins-1,4,5-P_3. To determine the elution time of $InsP_4$, we prepared samples containing $InsP_4$ by adding [^3H]Ins-1,4,5-P_3 to saponin-permeabilized Jurkat cells by the method of Stewart et al. (6). As it has not been demonstrated that phosphorylation of Ins-1,4,5-P_3 occurs on the 3 position under these conditions, we will refer to this product as simply $InsP_4$.

Partial purification of Ins-1,4,5-P_3 kinase. Ins-1,4,5-P_3 kinase was partially purified from Jurkat cells by the method of Hansen et al. (5). 10^8 Jurkat cells were incubated for 10 min at 37°C in 1 ml of Hepes-buffered saline solution alone or with either MAb C305 (10 μg/ml) or phorbol myristate acetate (PMA) (50 ng/ml). Cells were sedimented for 15 s in an Eppendorf 5414 centrifuge, resuspended in 400 μl of ice-cold buffer containing 5 mM Tris, pH 7.5, 1 mM EGTA, 2 mM $MgCl_2$, 2 mM dithiothreitol, and 5 mM sodium pyrophosphate, and then homogenized with 65 strokes in a Potter Elvehjem tissue grinder. Following the addition of 100 μl of 1 M sucrose and the removal of particulate material by a 10-min centrifugation in an Eppendorf 5414 microfuge, the homogenate was subjected to centrifugation at 100,000 g for 90 min. The resulting supernatant was fractionated with ammonium sulfate, and a 23–40% fraction dialyzed overnight against 10 mM Tris/HCl, pH 7.5, 2 mM $MgCl_2$, and 2 mM dithiothreitol.

Assay for Ins-1,4,5-P_3 kinase activity. The assay for Ins-1,4,5-P_3 kinase activity was performed according to published methods (4, 5). Assays were performed at 37°C in a final volume of 40 μl and were initiated by the addition of 10 μg of the Ins-1,4,5-P_3 kinase preparation. The reaction buffer contained [^3H]Ins-1,4,5-P_3 as indicated, 50 mM Tris, pH 7.5, 5 mM ATP, 2 mM sodium pyrophosphate, 5 mM EGTA, and $CaCl_2$ to give the indicated [Ca^{2+}]. As noted previously, this concentration of sodium pyrophosphate minimizes residual Ins-1,4,5-P_3 phosphomonoesterase activity without inhibiting Ins-1,4,5-P_3 kinase activity (5). The reaction was terminated by the addition of 1 ml of ice-cold 10% trichloroacetic acid. [^3H]Ins-1,4,5-P_3 and $InsP_4$ were separated either by a modification of the HPLC method described above or by elution from Dowex 1-X8 columns in formate form (100–200 mesh; Bio-Rad Laboratories) in, respectively, 20 ml of 0.8 M ammonium formate plus 0.1 M formic acid and 10 ml of 2 M ammonium formate plus 0.1 M formic acid (4).

Results

To confirm that the Ins-1,4,5-P_3 kinase is active in Jurkat cells following perturbation of T3/Ti, we stimulated intact [^3H]inositol-labeled Jurkat cells and resolved the extracted [^3H]inositol polyphosphates by HPLC (Fig. 1). The addition of C305, a MAb with specificity for T3/Ti on Jurkat (17), led to prompt increases in [^3H]Ins-1,4,5-P_3 and [^3H]$InsP_4$. The level of [^3H]Ins-1,4,5-P_3 reached a peak by 60 s and then fell over the succeeding minute to a plateau that remained elevated for > 15 min. [^3H]$InsP_4$ increased for ~ 5 min to steady-state level that was substantially higher than that of [^3H]Ins-1,4,5-P_3. After a 30-s lag, an increase in [^3H]Ins-1,3,4-P_3 was detected. Because the only known source of $InsP_4$ and Ins-1,3,4-P_3 requires Ins-1,4,5-P_3 kinase activity (4–6), these results indicate that, following stimulation of T3/Ti on Jurkat cells, a substantial proportion of the Ins-1,4,5-P_3 generated is phosphorylated to $InsP_4$.

Permeabilized Jurkat cells have Ins-1,4,5-P_3 kinase activity (6). As has been observed in rat brain and liver, this enzyme is soluble and can be partially purified by ammonium sulfate fractionation of the supernatant obtained from an ultracentrifugation of cell homogenates (4, 5). The Ins-1,4,5-P_3 kinase partially purified from resting Jurkat cells in this manner had a maximum velocity (V_{max}) of 0.09 nmol/min/mg protein and an apparent Michaelis constant (K_m) of 0.2 μM (Fig. 2). The latter value is in approximate agreement with the reported K_m (0.6 μM) of the Ins-1,4,5-P_3 kinase activity of rat brain cytosol (4). To determine whether the $InsP_4$ pathway is regulated as a result of stimulating T3/Ti, we compared the kinetics of Ins-1,4,5-P_3 kinase obtained from resting Jurkat cells and from cells stimulated by C305 (Fig. 2, *A* and *B*). Within 10 min, stimulation of T3/Ti by the MAb led to a twofold increase in the V_{max} of the Ins-1,4,5-P_3 kinase without a detectable change in K_m. A comparable increase in kinase activity was observed after treating Jurkat cells for 10 min with PMA, an activator of protein kinase C (Fig. 2, *A* and *C*). We observed similar T3/Ti- and PMA-induced increases in the Ins-1,4,5-P_3 kinase activity of unfractionated Jurkat cytosol (not shown).

There are conflicting reports as to the effect of the concentration of [Ca^{2+}] on Ins-1,4,5-P_3 kinase activity from other cells in vitro (4, 22). In studies of Ins-1,4,5-P_3 kinase from Jurkat

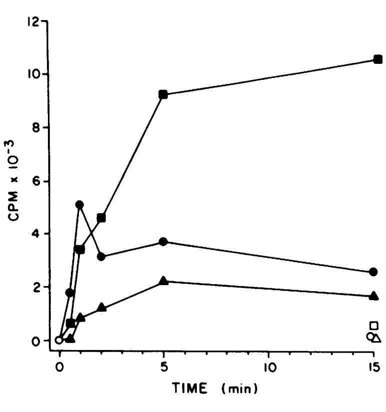

Figure 1. Time course of the increases in Ins-1,4,5-P_3 (*closed circles*), $InsP_4$ (*closed boxes*), and Ins-1,3,4-P_3 (*closed triangles*) after the addition of a T3/Ti MAb to Jurkat cells. C305, a MAb with specificity for T3/Ti, was added at time 0 to [^3H]inositol-labeled Jurkat cells, and aliquots containing 1.5 × 10^7 cells removed subsequently for each data point. The indicated time points are the intervals from the addition of C305 to the lysis of cells in trichloroacetic acid. Inositol polyphosphate levels from 1.5 × 10^7 unstimulated cells incubated concomitantly for 15 min are shown (*open circles, open boxes, open triangles*). [^3H]Inositol polyphosphates were extracted, separated by HPLC, and quantified as described in Methods. Results are expressed as the change in cpm from unstimulated cells at time 0. The levels of Ins-1,4,5-P_3, $InsP_4$, and Ins-1,3,4-P_3 in these unstimulated cells were 89, 618, and 66 cpm, respectively.

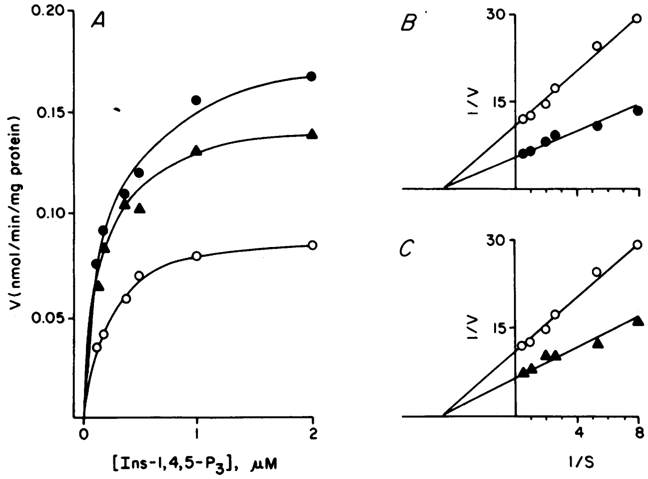

Figure 2. The relationship between kinase activity (V) and substrate concentration (S) for Ins-1,4,5-P_3 kinase prepared from unstimulated Jurkat cells (*open circles*); Jurkat cells stimulated for 10 min with C305, a T3/Ti MAb (*closed circles*), and Jurkat cells treated for 10 min with PMA (50 ng/ml) (*closed triangles*). (*A*) Ins-1,4,5-P_3 kinase was assayed as described in Methods with $CaCl_2$ added to give a calculated free Ca^{2+} concentration of 0.5 μM (23). After 3 min, the reaction was terminated by the addition of trichloroacetic acid, and Ins-1,4,5-P_3 and $InsP_4$ were separated by HPLC. (*B* and *C*) Lineweaver-Burk plots of the data shown in (*A*).

cells, we observed a stimulatory effect on kinase activity when $[Ca^{2+}]$ was increased from 0.1 μM to 1 μM with a maximal effect at 0.5 μM (Fig. 3). This effect of $[Ca^{2+}]$ was observed with Ins-1,4,5-P_3 kinase prepared both from resting cells and from cells exposed to C305. Increasing $[Ca^{2+}]$ to > 1 μM had an inhibitory effect on kinase activity (Fig. 3).

Discussion

The addition of a T3/Ti MAb to the T cell line, Jurkat, leads to substantial, sustained increases in Ins-1,4,5-P_3, $InsP_4$, and Ins-1,3,4-P_3. Studies of the kinetics of Ins-1,4,5-P_3 kinase partially purified from Jurkat cells demonstrate that the V_{max} of this enzyme is increased approximately twofold within 10 min of stimulating T3/Ti. These results establish that the activity of the Ins-1,4,5-P_3 kinase can be regulated as a consequence of receptor stimulation.

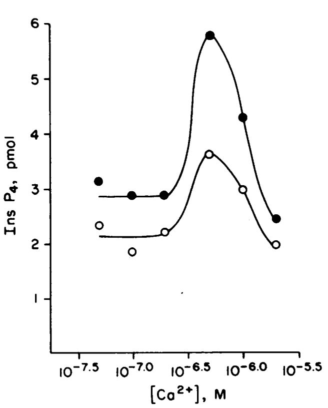

Figure 3. Effect of the concentration of free Ca^{2+} on the activity of the Ins-1,4,5-P_3 kinase prepared from unstimulated Jurkat cells (*open circles*) and Jurkat cells exposed to C305 for 10 min (*closed circles*). Ins-1,4,5-P_3 kinase activity was assayed for 5 min with sufficient $CaCl_2$ added to give the indicated free Ca^{2+} concentration (23). The concentration of [^{3}H]Ins-1,4,5-P_3 was 1 μM. [^{3}H]Ins-1,4,5-P_3 and $InsP_4$ were separated by anion exchange chromatography using Dowex 1-X8 columns in formate form.

The mechanism by which stimulation of T3/Ti leads to an increase in the V_{max} of the Ins-1,4,5-P_3 kinase is not established but presumably involves either a direct modification of the kinase (or a cofactor) or an increase in the amount of soluble kinase. The ability of PMA to mimic the effects of T3/Ti MAb on the V_{max} of the Ins-1,4,5-P_3 kinase raises the possibility that the increase in kinase activity may be a consequence of T3/Ti-mediated activation of protein kinase C. Whether the Ins-1,4,5-P_3 kinase itself is actually a substrate for protein kinase C, of course, cannot be determined until the Ins-1,4,5-P_3 kinase has been purified. Interestingly, in platelets, activated protein kinase C augments the conversion of Ins-1,4,5-P_3 to inositol-1,4-bisphosphate by phosphorylating the Ins-1,4,5-P_3 5′-phosphomonoesterase (24, 25).

Our studies, together with those of Biden and Wollheim (22), indicate that changes in $[Ca^{2+}]$ can increase Ins-1,4,5-P_3 kinase activity in vitro. Increasing $[Ca^{2+}]$ influences the activity of kinase obtained from stimulated as well as resting Jurkat cells. Maximal activity occurs at 0.5–1.0 μM free Ca^{2+}, within the range achieved by receptor stimulation of intact, quin2-loaded Jurkat cells (8–10). Following perturbation of T3/Ti on intact Jurkat cells, therefore, the activity of the Ins-1,4,5-P_3 kinase may be enhanced by two distinct mechanisms with additive effects: the receptor-stimulated increase in V_{max} and a direct effect of the receptor-mediated increase in $[Ca^{2+}]$i.

Because the activity of the Ins-1,4,5-P_3 kinase is increased as a consequence of stimulating T3/Ti, it is likely that the $InsP_4$ pathway plays an important role in signal transduction by T3/Ti. T3/Ti-mediated signaling, therefore, either may require that the levels of Ins-1,4,5-P_3 be regulated within certain limits or it may use the products of the $InsP_4$ pathway as second messengers. ~ 2 h of ligand occupancy of T3/Ti are required to commit Jurkat cells to the production of interleukin 2, suggesting that ongoing signal generation by T3/Ti is required for activation (26). Perturbation of T3/Ti by MAb leads to an increase in $[Ca^{2+}]$i, which is sustained in quin2-loaded Jurkat cells for > 30 min and which has been identified as a signal for activation (8–10). The initial peak increase in $[Ca^{2+}]$i that occurs within 60 s of the addition of T3/Ti MAb is due to the mobilization of intracellular Ca^{2+} and is presumably mediated by Ins-1,4,5-P_3 (10). The T3/Ti-mediated increase in $[Ca^{2+}]$i is then sustained by uptake of extracellular Ca^{2+}, probably through voltage-independent Ca^{2+} channels (27, 28). One possible function of the prolonged T3/Ti-mediated increase in inositol polyphosphates may be to regulate sustained increases in $[Ca^{2+}]$i. Alternatively, these compounds may activate other, as yet unidentified, signaling pathways.

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