The Ca\(^{2+}\) signal observed in individual fura-2-loaded hepatocytes stimulated with the \(\alpha_{1}\)-adrenergic agonist phenylephrine consisted of a variable latency period, a rapid biphasic increase in the cytosolic free Ca\(^{2+}\), followed by a period of maintained elevated cytosolic Ca\(^{2+}\) (plateau phase) that depended on the continued presence of both agonist and external Ca\(^{2+}\). Microinjection of guanosine-5'-O-(3-thiophosphate) elicited a Ca\(^{2+}\) transient with the same basic features. The Ca\(^{2+}\) transient resulting from microinjecting inositol 1,4,5-trisphosphate (Ins-1,4,5-P\(_3\)) occurred with essentially no latency period and consisted of a rapid spike that decayed back to preinjection levels within 15 s. Microinjection of inositol 1,4,5-trisphosphorothioate (thio-IP\(_3\)), a nonmetabolizable analog of Ins-1,4,5-P\(_3\), elicited a Ca\(^{2+}\) transient that was initially identical to that observed with Ins-1,4,5-P\(_3\), except that the cytosolic Ca\(^{2+}\) remained elevated. The maintained thio-IP\(_3\)-induced Ca\(^{2+}\) increase was dependent on the presence of external Ca\(^{2+}\), suggesting an activation of Ca\(^{2+}\) influx. Reintroduction of external Ca\(^{2+}\) in the presence of 5 \(\mu\)M phenylephrine to Ca\(^{2+}\)-depleted cells resulted in a 2-fold greater rate of rise in the cytosolic Ca\(^{2+}\) compared to the rate observed upon Ca\(^{2+}\) addition to cells Ca\(^{2+}\)-depleted by pretreatment with thapsigargin. The rate of Ca\(^{2+}\) rise upon Ca\(^{2+}\) addition to cells microinjected with thio-IP\(_3\) was similar to that observed with phenylephrine. Coinjection of the cells with thio-IP\(_3\) plus heparin reduced the rate of Ca\(^{2+}\) rise upon Ca\(^{2+}\) addition to that observed in thapsigargin-treated cells. These data indicate that the mechanism responsible for receptor-mediated stimulation of Ca\(^{2+}\) entry into hepatocytes involves not only capacitative Ca\(^{2+}\) entry but also an additional component mediated directly by Ins-1,4,5-P\(_3\).

The concentration of cytosolic free Ca\(^{2+}\) regulates many intracellular events. These include the control of metabolism, secretion, and muscle contraction, as well as other functions mediated by hormones, neurotransmitters, and growth factors. Hormone-mediated Ca\(^{2+}\) signaling is initiated by an agonist-induced activation of phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate into two intracellular messengers, Ins-1,4,5-P\(_3\) and diacylglycerol (1, 2). The ensuing rise in cytosolic free Ca\(^{2+}\) results from an Ins-1,4,5-P\(_3\)-mediated release of Ca\(^{2+}\) from internal stores and from an activation of Ca\(^{2+}\) influx (2, 3). The mechanism of agonist-stimulated extracellular Ca\(^{2+}\) entry has not yet been resolved, but in many cell types it is distinct from voltage-operated Ca\(^{2+}\) channels (4). Current experimental data suggests three possible mechanisms: 1) receptor-activated Ca\(^{2+}\) channels, in which receptor activation and channel opening are intimately interconnected (5, 6), perhaps via a G protein (5, 7), second messenger operated Ca\(^{2+}\) channels, which are gated either by Ins-1,4,5-P\(_3\) (8–10), Ins-1,3,4,5-P\(_4\) (11, 12), or Ca\(^{2+}\) itself (13), and 3) capacitative calcium entry, in which activation of Ca\(^{2+}\) entry is linked to emptying of the internal Ins-1,4,5-P\(_3\)-sensitive Ca\(^{2+}\) store (14, 15). It is likely that the various mechanisms operate to different extents in different cell types. In this study, we have characterized the Ca\(^{2+}\) transient elicited by phenylephrine in hepatocytes at the single cell level and subsequently attempted to determine the essential intracellular signals required to generate the various aspects of the Ca\(^{2+}\) transient. This was accomplished by activating the signal transduction process distal to receptor activation by microinjecting specific compounds that are currently thought to mediate aspects of this process.

**EXPERIMENTAL PROCEDURES**

**Hepatocyte Preparation**—Hepatocytes were isolated from livers of fed, male Sprague-Dawley rats weighing 180–220 g by collagenase digestion as described previously (16). Cells were resuspended at \(2 \times 10^5\) cells/ml in Leibowitz-15 media supplemented with 20 mM Hepes and 5.5 mM glucose. Cells were attached to poly-D-Lysine coated coverslips by incubating 3 ml of cell suspension for 1 h at 37°C, at which point the media was changed and unattached cells removed. Cells were used from 1.5 to 6 h post-plating.

**Measurement of Cytosolic Free Ca\(^{2+}\)**—Cells attached to coverslips were transferred to a Leiden cell chamber and incubated in 3 ml of modified Hank’s buffer consisting of 20 mM Hepes, 5.4 mM KC\(_1\), 4.2 mM NaHCO\(_3\), 0.44 mM KH\(_2\)PO\(_4\), 0.33 mM NaHPO\(_4\), 1.3 mM Ca\(_{1}\), 1.0 mM MgCl\(_2\), and 5.5 mM glucose at pH 7.4, unless otherwise noted in the text. The chamber was maintained at 30°C on the microscope stage with a Medical Systems TC-102 temperature controller. Approximately 20 cells/coverslip were loaded with fura-2 free acid via pressure microinjection. A fine tip micropipette (\(R_i = 50–60\) M\(\Omega\)) was filled with a solution of 10 mM fura-2 free acid in 125 mM KCl and impaled into the hepatocyte. The content of the micropipette was introduced into the cell by applying a 20 psi pressure pulse for 200 ms using a General Valve Picospritzer. This injection protocol delivered approximately 50–150 femtoliters, determined using radioisotope, which represents approximately 1–3% of the total cell volume.

The fluorescence of a dye-loaded cell was imaged using a 100X
Nikon Fluor objective and a Nikon Diaphot epifluorescence microscope illuminated with alternating (100 Hz) 340 and 380 nm (6-nm half-band width) excitation light. Fluorescence emission was collected at 520 nm (50-nm half-band width) by a photomultiplier tube. Output from the fluorometer was digitized, ratioed, and analyzed by an IBM AT clone based analysis system (Indec Systems). Calibration of the fluorescence signal followed the method of Grynkiewicz et al. (17) and was performed in situ by addition of 10 μM of ionomycin (R_{max}), followed by addition of 5 mM EGTA, pH 8.0 (R_{min}). Values obtained were 15, 0.4, and 8.6 for R_{max}, R_{min}, and β, respectively. A K_d of 224 nM was used (17).

In certain experiments, the fura-2-loaded cells were reimpaled with another fine tip micropipette containing either Ins-1,4,5-P_3, thio-IP_3', Ins-1,3,4,5-P_4, or thio-IP_3, in 125 mM KCl and the contents introduced into the cell as discussed above. Agonists were delivered to the cell surface via a perfusion pipette (10 μm inner diameter) located approximately 30 μm from the cell by applying a 1-psi pressure pulse for various durations.

Materials—Ins-1,4,5-P_3 and ionomycin were obtained from Calbiochem, Thio-IP_3 and thio-IP_3' were from Du Pont-New England Nuclear. Fura-2 was from Molecular Probes. Ins-1,3,4,5-P_4 was synthesized as described by Cerdan et al. (18). Collagenase was obtained from Worthington Chemicals, Leibowitz L-15 culture media and phenylephrine were from Sigma, and GTPγS was from Boehringer Mannheim. All other chemicals were reagent grade or better. Thin wall borosilicate glass with filament (1.0 mm outer diameter X 0.75 mm inner diameter) was obtained from A-M Systems.

RESULTS

The Hormone-induced Ca^{2+} Transient—Fig. 1 illustrates characteristic responses of individual fura-2-loaded hepatocytes exposed to increasing concentrations of phenylephrine to activate the α_1-adrenergic receptors in the plasma membrane. A threshold concentration of hormone had to be exceeded in order to elicit any change of the cytosolic free Ca^{2+}, which varied between individual cells (compare cell A versus B, Fig. 1). In the cell of Fig. 1A, 0.2 μM phenylephrine produced no effect, while 0.4 μM, after a long delay, produced a submaximal increase of peak Ca^{2+} followed by a brief plateau phase. As the concentration of phenylephrine applied to the cell was raised further, the Ca^{2+} peak height continued to increase, the latency period before the initial rapid Ca^{2+} rise continued to shorten, but the level of the sustained Ca^{2+} increase during the plateau phase remained relatively unchanged. In other cells, exemplified by the cell in Fig. 1B, the peak height of the Ca^{2+} spike became maximal at a lower phenylephrine concentration than that required to produce the shortest latency period. The latency period is thought to represent the time required to generate sufficient Ins-1,4,5-P_3 to bind to and activate the Ins-1,4,5-P_3 receptor (2, 19). The pattern obtained from cell B suggests that the slowest steps for agonist-induced production of Ins-1,4,5-P_3 may occur prior to phospholipase C activation, at the level of the receptor–G protein interaction. At all phenylephrine concentrations, the cytosolic free Ca^{2+} returned to the prestimulated resting level of about 125 nM Ca^{2+} within 30 s after terminating agonist delivery from the perfusion pipette. This type of hormone response is similar to that observed with angiotensin II stimulation of single N1E-115 neuroblastoma cells (20) and many other cell types after agonist stimulation. A small portion of the hepatocytes responded upon phenylephrine stimulation with repetitive spikes in the cytosolic free Ca^{2+} concentration, as has been observed by others (21–23).

Internal Ca^{2+} mobilization appears to predominate initially over the phase of Ca^{2+} entry, as illustrated by the data in Fig. 2, which show results obtained by varying the exposure time of the cell to 2.5 μM phenylephrine from 2 to 60 s. The peak height of the Ca^{2+} transient was not appreciably affected by the exposure time, but the secondary Ca^{2+} plateau phase could not be readily distinguished unless the hormone was delivered for a time longer than 15 s. In addition, the initial phase of the Ca^{2+} transient was not affected by a decrease in the extracellular Ca^{2+} concentration to about 1 μM just prior to hormone addition, while the plateau phase was abolished by removing extracellular Ca^{2+} (data not shown). This reflects an increased rate of Ca^{2+} entry into the cells consequent to receptor stimulation. In hepatocytes, the plateau phase of the Ca^{2+} transient is not affected by voltage-dependent Ca^{2+} channel antagonists or low concentrations of inhibitory cations (19). These data suggest, therefore, that while the two processes of intracellular Ca^{2+} mobilization and Ca^{2+} entry are receptor-mediated, they have different kinetic, temporal, and sensitivity characteristics.

Post-receptor Stimulation of Ca^{2+} Mobilization—Many of the receptors associated with Ca^{2+} signaling couple to and

![Fig. 1. Effect of increasing phenylephrine concentration on changes of the cytosolic free Ca^{2+} level in two individual fura-2-loaded hepatocytes. The cells in A and B were stimulated every 3 min for 60 s with an increased phenylephrine concentration from perfusion pipettes located near the cell's surface. Phenylephrine concentrations used are assigned next to each trace. A similar experimental protocol was performed on six different cells.](image1)

![Fig. 2. Effect of increasing the duration of agonist stimulation on the cytosolic free Ca^{2+} concentration. Every 3 min the cell was stimulated with 2.5 μM phenylephrine for an increased length of time of 2, 5, 15, 30, and then 60 s. The experiment was performed on six individual cells with similar results.](image2)
activate phospholipase C via a GTP-binding protein (1, 2). The non-hydrolyzable analog of GTP, GTPγS, has been used extensively to investigate the presence and role of G proteins in receptor coupling since its binding to the GTP site of the α-subunit results in a long term activation of the G-protein (24). Fig. 3 shows the results from three separate cells in which GTPγS was rapidly introduced into a 2 μm-loaded hepatocyte through pressure microinjection. The resulting Ca2+ transients were associated with a variable latency period, an abrupt increase of Ca2+ to a peak value, followed by a sustained period of elevated Ca2+. In one of the cells illustrated, the intracellular free Ca2+ exhibited a damped oscillatory behavior. These data illustrate that the basic features of the hormone-induced Ca2+ transient (latency of the response, intracellular Ca2+ release, and stimulated Ca2+ entry) were reproduced by a direct activation of a G protein(s) and represent activation of post-receptor events.

In contrast to the sustained increase in the cytosolic free Ca2+ induced by microinjection of GTPγS, Fig. 4 (trace A) shows that microinjection of Ins-1,4,5-P3 caused a rapid increase of Ca2+, which then decayed back to the resting Ca2+ level within 10–15 s. The height of the peak Ca2+ reached by pressure microinjection of a maximal concentration of Ins-1,4,5-P3 was similar to the peak height of the initial Ca2+ spike observed with a maximal amount of phenylephrine. The increase in the cytosolic free Ca2+ occurred in less than 400 ms, the minimum time resolution of the instrumentation. It has been shown in stop-flow experiments using permeabilized hepatocytes that release of internal Ca2+ stores was initiated within 20 ms of presenting Ins-1,4,5-P3 to its receptor (25). The almost immediate increase of Ca2+ observed with Ins-1,4,5-P3 injections showed that the latency period observed with hormone addition or GTPγS microinjection was caused by a combination of the time required for activation of the G-protein and for the GTP-bound α-subunit to activate one of the isoforms of phospholipase C. These data support the argument that the phospholipase C activity has to be increased to a point where the rate of production of Ins-1,4,5-P3 exceeds its rate of metabolism, thereby allowing a critical concentration to be produced for binding to the Ins-1,4,5-P3 receptor with opening of Ca2+ channels in intracellular vesicles (19, 26).

By analogy with studies involving the addition of Ins-1,4,5-P3 to permeabilized hepatocytes (27), the rapid return of the Ins-1,4,5-P3-elicited Ca2+ transient to prestimulation levels (Fig. 4, trace A) reflects its rapid metabolism to Ins-1,4-P2 by the cellular 5-phosphomonoesters and to Ins-1,3,4,5-P4 by the Ins-1,4,5-P3 3-kinase, thereby terminating its effect in causing the opening of the Ca2+ channel of the Ins-1,4,5-P3 receptor. Microinjection of Ins-1,3,4,5-P4, at 10-fold higher concentrations than Ins-1,4,5-P3, had no effect on the cytoplasmic free Ca2+ concentration, which is in agreement with the inability of Ins-1,3,4,5-P4 to release Ca2+ from liver microsomes (28). Similar to Ins-1,4,5-P3, the injected Ins-1,3,4,5-P4 is rapidly degraded by its conversion to Ins-1,3,4-P3 by the cellular 5-phosphomonoesters.

**Mechanism of Hormone-stimulated Ca2+ Entry**—During hormone-stimulated Ca2+ signaling, Ins-1,4,5-P3 is being continuously produced and, in hepatocytes with hormones such as vasopressin and phenylephrine, its concentration is maintained elevated for as long as the receptor remains occupied (29). As shown in Fig. 5, increased levels of Ins-1,4,5-P3 were maximal within 5 s after addition of 10 μM phenylephrine to a suspension of [3H]myo-inositol-labeled hepatocytes. Following the initial rise, however, Ins-1,4,5-P3 levels decreased to a new steady-state level within 30 s that was 30% of the peak level. It is during this period of an agonist-generated increase...
in the steady-state level of cellular Ins-1,4,5-P$_3$ that the plateau phase of the Ca$^{2+}$ transient occurs. The question arises whether the continued presence of reduced Ins-1,4,5-P$_3$ levels in hepatocytes plays a role other than maintaining a depletion of the Ins-1,4,5-P$_3$-sensitive Ca$^{2+}$ stores. Metabolism of Ins-1,4,5-P$_3$ can be circumvented by using the non-hydrolyzable analog of Ins-1,4,5-P$_3$, thio-IP$_3$. In hepatocytes, this compound is only slightly less potent than Ins-1,4,5-P$_3$ as a Ca$^{2+}$ mobilizing agent, and it is not a substrate for either the 5-phosphomonoesterase or the Ins-1,4,5-P$_3$-3-kinase. Fig. 4, trace B, shows results obtained by microinjecting thio-IP$_3$ into hepatocytes incubated in 1.3 mM Ca$^{2+}$-containing medium. Introduction of thio-IP$_3$ caused a similar rapid increase in the cytosolic free Ca$^{2+}$ as observed with Ins-1,4,5-P$_3$, reaching peak levels equivalent to maximal hormone stimulation. Unlike addition of Ins-1,4,5-P$_3$, however, the intracellular Ca$^{2+}$ level remained elevated after addition of thio-IP$_3$. Fig. 6 shows that upon removal of extracellular Ca$^{2+}$ by microinjection of thio-IP$_3$ into a cell bathed in normal Ca$^{2+}$ medium, the thio-IP$_3$-induced rise of the cytosolic free Ca$^{2+}$ concentration returned toward resting values. Reintroduction of 1.3 mM external Ca$^{2+}$ by turning off the perfusion pipette containing 3 mM EGTA in Ca$^{2+}$-free Hank's buffer resulted in a subsequent increase of the cytosolic Ca$^{2+}$ concentration as Ca$^{2+}$ entered the cell. These data suggest, therefore, that thio-IP$_3$ was stimulating Ca$^{2+}$ entry into the cell.

The data in Fig. 7 provide additional support for a thio-IP$_3$-stimulated Ca$^{2+}$ influx. Representative results from two separate hepatocytes are shown in which the cells were first exposed for 10 min to nominally Ca$^{2+}$-free medium (approximately 1 µM) and then exposed to 25 µM phenylephrine for 90 s in order to deplete the Ins-1,4,5-P$_3$-sensitive Ca$^{2+}$ stores. Separate experiments showed that a subsequent stimulation of these Ca$^{2+}$-depleted cells with phenylephrine did not elicit an increase in the cytosolic free Ca$^{2+}$ concentration. After the cytosolic free Ca$^{2+}$ had returned to resting levels, the cell was microinjected with thio-IP$_3$. This caused an immediate small increase of the cytosolic free Ca$^{2+}$, which presumably reflects the residual amount of Ca$^{2+}$ in the Ins-1,4,5-P$_3$-sensitive Ca$^{2+}$ stores in this experiment. Fifteen s after the injection of thio-IP$_3$, the cell was exposed to 1.3 mM Ca$^{2+}$. The cytosolic free Ca$^{2+}$ increased rapidly and leveled off at about 600 nM (cf. Fig. 6). In contrast, microinjection of thio-IP$_3$ produced no effect by itself on the cytosolic free Ca$^{2+}$, and there was no increase in the cytosolic free Ca$^{2+}$ upon readdition of Ca$^{2+}$ to the medium. The result of the thio-IP$_3$ study on Ca$^{2+}$ entry was similar to that obtained in the controls, which consisted of microinjecting 125 mM KCl followed by Ca$^{2+}$ addition to the medium (data not shown). Hence, the stimulated Ca$^{2+}$ entry observed with thio-IP$_3$ appears to be specific to this Ins-1,4,5-P$_3$ analog.

In contrast to the lack of enhanced Ca$^{2+}$ entry in Ca$^{2+}$-depleted cells, readdition of Ca$^{2+}$ in the presence of 5 µM phenylephrine produced a stimulation of Ca$^{2+}$ influx. Fig. 8 shows mean results of a series of experiments in which intracellular Ca$^{2+}$ stores were first depleted by perfusion of cells.

**Fig. 6.** Effect of removing external Ca$^{2+}$ on the Ca$^{2+}$ transient elicited by microinjection of thio-IP$_3$. At 15 s, the cell was microinjected with thio-IP$_3$ as described in Fig. 4. At 75 s, the external Ca$^{2+}$ was removed by perfusing the cell with Ca$^{2+}$-free Hank's buffer containing 3 mM EGTA via a perfusion pipette. At 195 s, the perfusion pipette was turned off, and external Ca$^{2+}$ in the bath was returned to the cell. The trace shown was representative of six experiments.

**Fig. 7.** Specificity of thio-IP$_3$ promoted Ca$^{2+}$ entry in Ca$^{2+}$-depleted hepatocytes. Cells were partially depleted of internal Ca$^{2+}$ by first incubating the cell in nominally Ca$^{2+}$-free medium for 10 min (free Ca$^{2+}$ < 1 µM as measured by a Ca$^{2+}$-sensitive electrode). The cell was then impaled with the injection micropipette and subsequently stimulated (t = 15 s) with a maximal dose of phenylephrine (25 µM) for 90 s via a perfusion pipette to deplete the internal Ins-1,4,5-P$_3$-sensitive Ca$^{2+}$ store. Thirty s later (t = 135 s), the contents of the micropipette were introduced into the cell by a 200-ms, 20 psi pressure pulse. Fifteen s later (t = 150 s), external Ca$^{2+}$ was reintroduced to the cell via a second perfusion pipette for a period of 90 s. In trace A, the micropipette contained 60 µM thio-IP$_3$ in 125 mM KCl. In trace B, the micropipette contained 50 µM thio-IP$_3$ in 125 mM KCl. Microinjection of 125 mM KCl produced results identical to trace B (data not shown). Traces shown are representative of 12, six and four experiments performed with thio-IP$_3$, thio-IP$_4$, and KCl, respectively.

**Fig. 8.** Enhancement of the rate of increase in the cytosolic free Ca$^{2+}$ upon readdition of external Ca$^{2+}$ to Ca$^{2+}$-depleted hepatocytes by phenylephrine. Cells were depleted of internal Ca$^{2+}$ either by the procedure described in Fig. 7 (circles, squares) or by incubation with 500 nM thapsigargin in nominally Ca$^{2+}$-free media for 10 min (triangles). External Ca$^{2+}$ was then reintroduced by perfusing the cells with modified Hank's buffer containing either 1.3 mM Ca$^{2+}$ (circles, triangles) or 1.3 mM Ca$^{2+}$ plus 5 µM phenylephrine (squares) at t = 15 s (arrow) for 75 s from a perfusion pipette. The average rate of increase in the cytosolic free Ca$^{2+}$ was, for circles, 38 ± 8 nmol/min, n = 17, for squares, 254 ± 50 nmol/min, n = 8, and for triangles, 124 ± 18 nmol/min, n = 27.

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with phenylephrine or thapsigargin under Ca\(^{2+}\)-free conditions followed by readdition of Ca\(^{2+}\) at the arrow (t = 15 s). When Ca\(^{2+}\) was reintroduced in the presence of 5 \(\mu\)M phenylephrine (Fig. 8, squares), the rate at which cytosolic free Ca\(^{2+}\) increased was stimulated more than 6-fold compared to control cells in which Ca\(^{2+}\) was added in the absence of phenylephrine (Fig. 8, circles). These data indicate that events associated with receptor activation were necessary for stimulated Ca\(^{2+}\) entry. One hypothesis for receptor-stimulated Ca\(^{2+}\) entry, the capacitative model, proposes that Ca\(^{2+}\) entry is enhanced when the Ins-1,4,5-P\(_3\)-sensitive Ca\(^{2+}\) store is emptied. Since the thio-IP3 injection studies demonstrated that there was a small amount of residual Ca\(^{2+}\) in the Ins-1,4,5-P\(_3\)-sensitive Ca\(^{2+}\) stores in some experiments, it was possible that this Ca\(^{2+}\) was adequate to diminish activation of Ca\(^{2+}\) entry. To deplete the residual Ca\(^{2+}\) in the Ins-1,4,5-P\(_3\)-sensitive Ca\(^{2+}\) store, the cells were placed in nominally Ca\(^{2+}\)-free medium containing 500 \(\mu\)M thapsigargin. Thapsigargin is an inhibitor of the Ca\(^{2+}\)-ATPase that pumps Ca\(^{2+}\) into the Ins-1,4,5-P\(_3\)-sensitive Ca\(^{2+}\) stores and causes them to gradually lose their stored Ca\(^{2+}\) (31). Under these conditions the cytosolic Ca\(^{2+}\) initially increased as Ca\(^{2+}\) was released from the Ca\(^{2+}\) stores and then fell to below the normal resting value with establishment of a new basal level within 7 min (data not shown). Addition of phenylephrine after thapsigargin to cells bathed in Ca\(^{2+}\)-free medium failed to elicit an increase in cytosolic free Ca\(^{2+}\), indicating that the thapsigargin treatment completely emptied the agonist-sensitive Ca\(^{2+}\) store. Upon readdition of external Ca\(^{2+}\) to thapsigargin-treated cells, (Fig. 8, triangles), the rate at which the cytosolic Ca\(^{2+}\) increased was greater than that observed with control cells (Fig. 8, circles), but less than that observed after Ca\(^{2+}\) addition in the presence of phenylephrine.

The statistical significance of the changes in cytosolic free Ca\(^{2+}\) are more clearly seen from the data in Table I, which shows the mean rate of change of the cytosolic Ca\(^{2+}\) over 10-s intervals following Ca\(^{2+}\) addition to Ca\(^{2+}\)-depleted cells exposed to different conditions. In the thapsigargin-treated cells (column II) the rate of increase of cytosolic free Ca\(^{2+}\) was maximal about 60 s after Ca\(^{2+}\) addition, while with phenephrine treatment (column III) the rate was maximal about 40 s, and thereafter declined to a rate comparable to that observed after thapsigargin treatment. Thus, the primary effect of phenylephrine relative to thapsigargin was to enhance the rate of Ca\(^{2+}\) entry over the time interval from 25 to 55 s after Ca\(^{2+}\) addition. The tailing off of the phenylephrine-induced rate of Ca\(^{2+}\) entry may indicate negative feed-

### Table I

| Time after Ca\(^{2+}\) addition | Ca\(^{2+}\) addition only | Ca\(^{2+}\) addition following thapsigargin | Ca\(^{2+}\) addition in the presence of phenylephrine | Ca\(^{2+}\) addition to thio-IP3, injected cells | Ca\(^{2+}\) addition to thio-IP3, plus heparin injected cells |
|-----------------|----------------|----------------|----------------|----------------|----------------|
| s               | nM/min         | nM/min         | nM/min         | nM/min         | nM/min         |
| 5-15            | 11 ± 4         | 31 ± 7         | 17 ± 10        | 104 ± 40\(^a\) | 12 ± 8         |
| 15-25           | 35 ± 7         | 74 ± 11\(^a\)  | 117 ± 29\(^b\) | 250 ± 100\(^b\) | 11 ± 8\(^a\)   |
| 25-35           | 42 ± 10        | 116 ± 18\(^a\) | 263 ± 93\(^b\) | 262 ± 85\(^b\) | 96 ± 28\(^a\)  |
| 35-45           | 40 ± 8         | 122 ± 19\(^b\) | 270 ± 85\(^b\) | 275 ± 51\(^b\) | 156 ± 58\(^b\) |
| 45-55           | 33 ± 8         | 133 ± 20\(^b\) | 239 ± 74\(^b\) | 245 ± 45\(^b\) | 188 ± 50\(^b\) |
| 55-65           | 28 ± 10        | 156 ± 26\(^b\) | 193 ± 73\(^b\) | 215 ± 58\(^b\) | 163 ± 71\(^b\) |
| 65-75           | 35 ± 11        | 155 ± 26\(^b\) | 159 ± 49\(^b\) | 212 ± 62\(^b\) | 128 ± 43\(^b\) |

\(^a\)Signifies \(p < 0.05\) relative to Column I.

\(^b\)Signifies \(p < 0.05\) relative to Column II.
FIG. 9. Inhibition by heparin of the thio-IP₃-enhanced rate of increase in the cytosolic free Ca²⁺ following readdition of external Ca²⁺ to Ca²⁺-depleted hepatocytes. Cells were depleted of internal Ca²⁺ by the procedure described in Fig. 7. Subsequently the cells were microinjected with either thio-IP₃ (circles) or thio-IP₃ plus heparin (squares). External Ca²⁺ was then reintroduced by perfusing the cells with modified Hank’s buffer containing either 1.3 mM Ca²⁺ at t = 15 s (arrow) for 75 s from a perfusion pipette. The average rate of increase in the cytosolic free Ca²⁺ was, for circles, 254 ± 50 nmol/min, n = 12, and for squares, 138 ± 43 nmol/min, n = 8. The micropipette contained either 100 µM thio-IP₃ in 125 mM KCl (circles) or 100 µM thio-IP₃ plus 10 mg/ml heparin in 125 mM KCl (squares).

Discussion

The data presented in this paper demonstrate that the basic features of the Ca²⁺ transient elicited by α₁-adrenergic stimulation in hepatocytes can be related to the generation of Ins-1,4,5-P₃. The latency period, which is the aspect of the Ca²⁺ transient most affected by changes in agonist concentration, results from the time required for ligand-bound receptor to activate its G-protein, which in turn activates a subtype of phospholipase C to the point where the amount of Ins-1,4,5-P₃ rises to a level that can discharge the Ca²⁺ from internal stores. This discharge of the internal Ca²⁺ stores appears as a rapid spike of increased cytosolic free Ca²⁺ that immediately decays to prestimulation levels if the agonist is present for less than 15 s. Since direct microinjection of Ins-1,4,5-P₃ produces a similar rapid increase in Ca²⁺ which then returns to basal levels, the rise of cytosolic Ca²⁺ during the initial activation phase would appear to simply follow the hormone-induced production of Ins-1,4,5-P₃. If the presence of agonist is maintained, the initial Ca²⁺ increase decays to a new plateau level that is significantly higher than the initial resting level of Ca²⁺. This plateau phase is relatively independent of agonist concentration but is dependent on the presence of extracellular Ca²⁺ and returns to the resting level when agonist is removed. Injection of either GTPγS, which presumably sustains an enhanced formation of Ins-1,4,5-P₃ by maintaining the G-protein/phospholipase C interaction in an activated state, or thio-IP₃, also produce a plateau phase. These data indicate a role for Ins-1,4,5-P₃ in maintaining elevated levels of cytosolic free Ca²⁺, the important point being that activation of the hormone-sensitive Ca²⁺ entry mechanism via the α₁-adrenergic receptor requires the continued formation of Ins-1,4,5-P₃. Ins-1,4,5-P₃ enhances Ca²⁺ entry not only as a consequence of it mobilizing Ca²⁺ from internal stores, thereby triggering a capacitative type mechanism, but also by an apparent specific activation of a Ca²⁺ channel in the plasma membrane. This conclusion is based on the assumption that thio-IP₃ mimics the action of Ins-1,4,5-P₃. One mechanism for the action of Ins-1,4,5-P₃ on Ca²⁺ entry would be through an Ins-1,4,5-P₃-gated Ca²⁺ channel located in the plasma membrane. There have been several reports that suggest the presence of these channels in several cell types. These include electrophysiological data obtained in patch-clamped T cells (8) and the observation that Ins-1,4,5-P₃ can induce Ca²⁺ release from purified plasma membrane vesicles from liver (33) and platelets (34, 35). These data were further supported by the observation that antipeptide antibodies to amino acid sequences of the cerebellum Ins-1,4,5-P₃ receptor reacted with protein in the plasma membranes of Purkinje cells (36). However, it now appears that the basis for the immunoreactivity results from a close apposition of the internal Ins-1,4,5-P₃ receptors to the plasma membrane (37–39). Similarly, purified rat liver plasma membranes positively react to polyclonal antibodies raised against the COOH terminus sequence of the Ins-1,4,5-P₃ receptor but again, it is not yet clear that this does not represent contamination of the plasma membrane preparation with Ins-1,4,5-P₃ receptor from microsomes (see Ref. 39). Hence, it is clearly premature to conclude that hepatocyte plasma membranes contain an integral Ins-1,4,5-P₃ receptor/Ca²⁺ channel.

If hepatocytes possess both plasma membrane and internal Ins-1,4,5-P₃ receptors, there must be differences in their regulatory properties, since there is a distinct temporal difference between mobilization of internal stores and activation of extracellular Ca²⁺ entry. At present there is insufficient data to conclude that distinct subsets of Ins-1,4,5-P₃ receptor exist in hepatocytes. However, the properties of the Ins-1,4,5-P₃ receptor can be quite different between different cell types. For example, in brain (32, 40) and uterus membranes (41), Ins-1,4,5-P₃ binding to its receptor is inhibited by Ca²⁺, while in adrenal cortex membranes (41), Ca²⁺ has no effect on Ins-1,4,5-P₃ binding and in liver membranes (42) Ca²⁺ enhances Ins-1,4,5-P₃ binding. Both high and low affinity Ins-1,4,5-P₃-binding sites have been characterized in liver (43) and adrenal cortex membranes (44). In liver, it appears that it is the hormone-induced rise in Ca²⁺ that converts the Ins-1,4,5-P₃ receptor from the low to the high affinity state (45). The basis for the differential effects of Ca²⁺ is not yet known but may be related to the presence of the regulatory Ca²⁺-binding protein calmodin (45) or to the phosphorylation state of the receptor (46, 47). Hence, the temporal difference between activation of internal Ins-1,4,5-P₃ receptors and plasma membrane Ins-1,4,5-P₃ receptors could result from their association with different regulatory proteins, which results in a requirement for different ancillary factors to induce activation of the Ca²⁺ channel.

Molecular cloning studies of the Ins-1,4,5-P₃ receptor have indicated that it is related to the other major intracellular Ca²⁺ release channel, the ryanodine receptor (48, 49). The ryanodine receptor possesses a large NH₂-terminal domain (50), forming the foot structure that interacts with the dihydropyridine receptor (the L-type Ca²⁺ channel) of the plasma membrane in skeletal muscle excitation-contraction coupling. Since the Ins-1,4,5-P₃ receptor also possesses a large cytoplasmic NH₂-terminal domain, it has been suggested (51) that it might interact with plasma membrane proteins involved in regulating Ca²⁺ entry in an analogous fashion. Information relating the Ca²⁺ content of the internal Ca²⁺ store could then be sensed by the luminal domain of the Ins-1,4,5-P₃ receptor and then transmitted to the plasma membrane to signal Ca²⁺ entry. This hypothesis is supported by the observation that a

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significant portion of the 1,4,5-P₃ receptors are located in the subplasma membrane space (33, 38, 39). This postulated protein interaction not only provides a mechanism for capacitative Ca²⁺ entry, but also provides a mechanism for the putative interaction of cytosolic regulatory molecules, like Ins-1,4,5-P₃, to coordinate internal and extracellular Ca²⁺ mobilization. Whereas internal Ca²⁺ release would require only Ins-1,4,5-P₃ maximal stimulation of extracellular Ca²⁺ entry would require both the release of internal Ca²⁺ and the presence of Ins-1,4,5-P₃, as observed in this paper. Depleted internal Ca²⁺ stores in the absence of Ins-1,4,5-P₃ would not necessarily be an effective signal for activating Ca²⁺ entry. That is, the conformational changes in the large cytoplasmic domain induced by Ins-1,4,5-P₃ binding would not only cause opening of the intrinsic Ca²⁺ channel of the Ins-1,4,5-P₃ receptor, but also modify the interaction of this domain with associated plasma membrane proteins, thus promoting Ca²⁺ entry. Hence, this scheme would not require actual integral internal Ca²⁺ stores in the absence of Ins-1,4,5-P₃ would not lead to a significant stimulation of Ca²⁺ entry. Only upon activation of the factors, but from our study it appears that Ins-1,4,5-P₃ is one of the factors.

Despite intense research by many laboratories to elucidate the mechanism that underlies hormone-stimulated Ca²⁺ entry, no simple consensus has emerged. This is not surprising, since studies on single cells have demonstrated that the Ca²⁺ signal generated by activation of different receptor systems is quite diverse, being capable of differentially activating internal mobilization relative to extracellular Ca²⁺ entry, and coordinating these responses to generate Ca²⁺ waves, repetitive Ca²⁺ spikes, and gradients of Ca²⁺ within the cytosolic compartment (see Ref. 54). In endothelial (55) and parotid cells (15), the Ca²⁺ status of the internal stores appears to be a major factor, whereas in hepatocytes (52, this study), hormone-generated Ins-1,4,5-P₃ is essential, and in lacrimal cells (12), the presence of both Ins-1,4,5-P₃ and Ins-1,3,4,5-P₃ appears to be necessary to activate Ca²⁺ entry. Given the diversity of receptor systems coupled to Ca²⁺ signaling, the variety of responses that can be elicited from the same cell and the probable complex network of cross-talk that exists between these systems, it is likely that additional external factors will be found that regulate and coordinate internal Ca²⁺ mobilization and extracellular Ca²⁺ entry.

REFERENCES

1. Berridge, M. J., and Irvine, R. F. (1989) Nature 341, 197–205
2. Williamson, J. R., and Monck, J. R. (1989) Annu. Rev. Physiol. 51, 107–124
3. Joseph, S. K., and Williamson, J. R. (1989) Arch. Biochem. Biophys. 273, 1–15
4. Hallim, J. J., and Rink, T. J. (1989) Trends Pharmacol. Sci. 10, 8–10
5. Penner, H., Matthews, G., and Neher, E. (1987) Nature 324, 499–504
6. Mahaut-Smit, M. P., Sage, S. O., and Rink, T. J. (1990) J. Biol. Chem. 265, 10479–10486
7. Hughes, B. P., Crofts, J. N., Auld, A. M., Read, L. C., and Barratt, G. J. (1989) Biochem. J. 248, 911–918
8. Kuro, M., and Gardiner, R. G. (1987) Nature 325, 301–304
9. Ehrlich, B. E., and Watras, J. (1989) FEBS Lett. 262, 5–9
10. Snyders, R. W., Krause, K. H., and Welsh, M. L. (1988) J. Biol. Chem. 263, 4959–4975
11. Ehrlich, B. E., and Watras, J. (1989) J. Biol. Chem. 264, 4976–4979
12. Thomas, A. P., Alexander, J., and Williamson, J. R. (1984) J. Biol. Chem. 259, 5574–5584
13. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
14. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
15. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
16. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
17. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
18. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
19. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
20. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
21. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
22. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
23. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
24. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
25. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
26. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
27. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
28. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
29. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
30. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
31. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
32. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
33. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
34. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
35. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
36. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
37. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
38. Czajkowski, G., Pienoe, M., and Teis, R. Y. (1985) J. Biol. Chem. 260, 3440–3450