IP₃ receptor blockade fails to prevent intracellular Ca²⁺ release by ET-1 and α-thrombin

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 Departments of 1Pediatrics and 3Medicine and 4Cardiovascular Research Institute, University of California, San Francisco, California 94143; and 2Department of Molecular Neurobiology, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Mathias, Robert S., Katsuhiro Mikoshiba, Takayuki Michikawa, Atsushi Miyawaki, and Harlan E. Ives. IP₃ receptor blockade fails to prevent intracellular Ca²⁺ release by ET-1 and α-thrombin. Am. J. Physiol. 274 (Cell Physiol. 43): C1456–C1465, 1998.—The effect of inositol 1,4,5-trisphosphate (IP₃) receptor blockade on platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), endothelin-1 (ET-1), or α-thrombin receptor-mediated intracellular Ca²⁺ release was examined using fura 2 microspectrofluorometry in single Chinese hamster ovary cells and myoblasts. Blockade of the IP₃ receptor was achieved by microinjection of heparin or monoclonal antibody (MAb) 18A10 into the IP₃ type 1 receptor. Heparin completely inhibited Ca²⁺ release after flash photolysis with caged IP₃ and after exposure to PDGF and FGF. In contrast, heparin failed to block Ca²⁺ release after α-thrombin and ET-1. Application of ligand, IP₃ levels were five- to sevenfold higher for α-thrombin than for ET-1 or PDGF. IP₃ levels after PDGF and ET-1 were comparable. Similar to heparin, MAb 18A10 blocked Ca²⁺ release after PDGF but failed to block Ca²⁺ release after ET-1 or α-thrombin. These data suggest that the mechanisms of Ca²⁺ release by tyrosine kinase and certain 7-transmembrane receptors may differ. Although both receptor types use the IP₃-signaling system, the ET-1 and α-thrombin receptors may have a second, alternative mechanism for activating Ca²⁺ release.

Platelet-derived growth factor; endothelin-1; intracellular microinjection of heparin or monoclonal antibody 18A10; Chinese hamster ovary cells; inositol 1,4,5-trisphosphate

Calcium signaling in mesenchymal cells is typically a biphasic process: in the first phase, Ca²⁺ is released into the cytoplasm from intracellular organelles; in the second phase, Ca²⁺ enters the cytoplasm from outside the cell. Inositol 1,4,5-trisphosphate (IP₃), formed by hydrolysis of phosphatidylinositol 4,5-bisphosphate, is believed to be the major mediator of Ca²⁺ release from the intracellular stores (3). IP₃ is released into the cytoplasm and binds to specific receptors located on intracellular Ca²⁺-storing organelles, which activate channel opening and release Ca²⁺ into the cytoplasm (3). IP₃ is produced after activation of tyrosine kinase and 7-transmembrane receptors. Although 7-transmembrane and tyrosine kinase receptors produce IP₃ by different isoforms of phospholipase C (51, 54), it has generally been assumed that the IP₃ formed by both signaling pathways acts similarly.

It has recently been found that there are at least three isoforms of the IP₃ receptor (IP₃R) (37). Some of these receptors may be found within a single cell (40, 45, 58) and may localize to different structures within the same cell (16, 45). This raises the possibility of functional heterogeneity among IP₃R isoforms. It is possible that the proximity of the IP₃R to different signal transduction systems could be an important determinant of the various cellular responses associated with IP₃ generation.

In this study we examined the effect of microinjected heparin and an IP₃R1 monoclonal antibody (MAb 18A10) on release of Ca²⁺ stores after activation of tyrosine kinase or 7-transmembrane receptors. We find that Ca²⁺ release by tyrosine kinase receptors [platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF)] is blocked by heparin and MAb 18A10 to the IP₃R1, suggesting that the pathway involves heparin-sensitive IP₃R1. In contrast, Ca²⁺ release by certain 7-transmembrane [endothelin-1 (ET-1) and α-thrombin] receptors is not blocked by heparin or MAb 18A10. These findings indicate that there may be alternative mechanisms for Ca²⁺ release in addition to the traditional IP₃-IP₃R pathway and that 7-transmembrane receptors may activate one or more such alternative systems for Ca²⁺ release.

Experimental Procedures

Materials. Unless otherwise specified, all chemicals were purchased from Sigma Chemical, including ET-1 (E-9262), low-molecular-weight heparin (H-5271), MAb to IgG₂a (M-9144), and a polyclonal antibody to IgG (I-5381). Fura 2 and Ca²⁺-green 1 were purchased from Molecular Probes (Eugene, OR). BSA was fatty acid-poor fraction V from Miles (Kankakee, IL). Highly purified human α-thrombin was generously supplied by Dr. John Fenton (State Health Department, Albany, NY). PDGF-BB was purchased from Boehringer Mannheim (Indianapolis, IN). Recombinant human basic FGF (bFGF) was generously supplied by Chiron (Emeryville, CA). Caged IP₃ was purchased from Calbiochem (San Diego, CA). IP₃R type-specific MAb (18A10, KM1083, and KM1082) were generated as previously described (17).

Preparation of cell lines. CHO-PDGF cells were stable transfectants of Chinese hamster ovary (CHO) cells containing the cDNA for PDGF-BB receptor (12). CHO-PDGF/FGF cells were stable transfectants containing the chimeric receptor composed of the PDGF receptor extracellular domain ligated to the FGF receptor transmembrane-cytoplasmic domain (11). M-FGF were stable transfectants in rat L6 myoblasts containing the cDNA for FGF receptor (44). CHET-B cells were stable transfectants of CHO-K1 cells containing the cDNA for ET-1 receptor (32).

Cell culture. CHO-PDGF and CHO-PDGF/FGF cells were grown in Ham's F-12 medium; M-FGF and CHET-B cells were grown in DMEM in a humidified atmosphere of 5% CO₂-95% air at 37°C. All media contained 10% (vol/vol) fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 U/ml).
Medium for stable transfectants was supplemented with 400 μg/ml G418. Culture medium was changed every 2–3 days until cells were confluent. CHO-PDGf and CHO-PDGf/FGF cells were prepared as described by Monka et al. (40). Cells were homogenized in a solution containing 250 mM sucrose, 5 mM Tris·HCl (pH 8.0), 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 0.01 mM pepstatin A, 0.01 mM leupeptin, and 0.01 mM E-64 in a chilled glass-Teflon Potter homogenizer with 10 strokes at 1,000 rpm. The supernatants were centrifuged at 105,000 g for 5 min at 4°C. The supernatants were resuspended in a 50 mM Tris·HCl (pH 8.0), 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 0.01 mM pepstatin A, 0.01 mM leupeptin, and 0.01 mM E-64 and used as total cell lysates. Protein concentrations were measured with a Bio-Rad (Richmond, CA) protein assay with BSA as the standard.

Western blot analysis. Twenty micrograms of total cell lysates were resolved by 5% SDS-PAGE and electroblotted onto a Hybond enhanced chemiluminescence membrane (Amersham). The blot was processed through sequential incubations with blocking solution (5% skim milk and 0.1% Tween 20 in PBS) and IP-R type-specific antibodies [IP-R1- (4C11, 10A6, and 18A10), IP-R2- (KM1083), or IP-R3-specific antibodies (KM1082)]. Secondary antibodies were anti-rat IgG for IP-R1-specific antibodies, anti-mouse IgG for KM1082 and KM1083, horseradish peroxidase-linked F(ab)2 fragment (Amersham) of anti-rat IgG (for 18A10), or anti-mouse IgG for KM1082 and KM1083. Immunodetection signals were visualized by the enhanced chemiluminescence Western blotting system (Amersham). The intensities of the bands from immunoblotting were measured on a Macintosh computer with use of NIH Image (version 1.61) software.

Results. Microinjected heparin inhibits release of Ca2+ stores after photolysis of caged IP3. Ca2+ green (0.1 mM) and caged IP3 (0.375 mM) were microinjected into CHO-PDGf cells in the absence or presence of heparin (5–20 mg/ml injectate concentrations) to determine whether microinjected heparin was capable of blocking IP3-mediated Ca2+ release. The microinjected volume was 6 × 10−14 liter, ~2% of cell volume (see Experimental Procedures). Photorelease of IP3 was achieved with flashes of ultraviolet light, as described in Experimental Procedures. Cells in nominally Ca2+-free medium were exposed to multiple flashes (~10 of 0.5- to 1-s duration (Fig. 1A; n = 8). Each flash caused a single Ca2+ spike. Heparin, at injectate concentration of 10 mg/ml, blocked Ca2+ release in nominally Ca2+-free medium after flash photolysis of caged IP3 (Fig. 1B; n = 5).
Heparin blocks release of Ca^{2+} stores by activation of tyrosine kinase receptors. To illustrate the two phases of Ca^{2+} mobilization, a single CHO-PDGF cell microinjected with fura 2-pentapotassium salt was exposed to 25 ng/ml PDGF-BB in nominally Ca^{2+}-free medium (Fig. 2A). PDGF caused a delayed and transient increase in Ca^{2+} to 765 ± 210 nM. On addition of 2 mM Ca^{2+} to the medium, there was a second, sustained increase in Ca^{2+} to 1,445 ± 296 nM (n = 16). As observed previously in vascular smooth muscle cells (25), microinjected low-molecular-weight heparin (5 mg/ml in the injectate) completely blocked Ca^{2+} release from intracellular stores after exposure to PDGF (Fig. 2B). Although there is variability in the appearance of the sustained Ca^{2+} increase after the addition of 2 mM Ca^{2+} to the medium, on average the peak Ca^{2+} concentration (1,298 ± 200 nM) was unaffected by heparin (n = 12).

Similar observations were made with bFGF in single M-FGF cells. bFGF (5 ng/ml) caused a delayed and transient increase in Ca^{2+} to 282 ± 56 nM in nominally Ca^{2+}-free medium and a second, sustained increase in Ca^{2+} to 657 ± 104 nM on addition of 2 mM Ca^{2+} to the medium (Fig. 2C; n = 5). As for PDGF, microinjected low-molecular-weight heparin (5 mg/ml injectate concentration) blocked the first phase (Ca^{2+} mobilization) but not the second phase of Ca^{2+} mobilization (influx) in an M-FGF cell after exposure to bFGF (Fig. 2D; n = 5). Finally, we examined Ca^{2+} transients after activation of a chimeric PDGF/FGF receptor. As observed with PDGF and FGF, the chimeric receptor (see EXPERIMENTAL PROCEDURES) elicited two phases of Ca^{2+} mobilization after exposure to PDGF, but only the first phase was blocked by microinjected heparin (data not shown). Taken together, the data suggest that tyrosine kinase receptor-mediated release of Ca^{2+} stores occurs via heparin-sensitive IP_{3}R but that Ca^{2+} entry from the medium is regulated by a different mechanism.

Heparin does not block release of Ca^{2+} stores after activation by ET-1 and a-thrombin. On addition of ET-1 (1 nM) to CHO cells in nominally Ca^{2+}-free medium, there was a transient increase in Ca^{2+} to 833 ± 168 nM that developed more rapidly than in the tyrosine kinase receptors (Fig. 3A). The lowest concentration of ET-1 that generated reproducible Ca^{2+} transients was 1 nM (approximately twice the receptor dissociation constant). Subsequent addition of 2 mM Ca^{2+} to the medium resulted in a second, prolonged increase in Ca^{2+} to 1,883 ± 437 nM (n = 8). In contrast to what was observed with tyrosine kinase receptors, microinjected heparin (5 mg/ml injectate concentration) failed to block ET-1-induced release of Ca^{2+} from intracellular stores (980 ± 105 nM; Fig. 3B). The sustained, second phase of Ca^{2+} entry was also unaffected by heparin (1,724 ± 263 nM, n = 10).

In view of the unexpected failure of heparin to block Ca^{2+} release with ET-1 in nominally Ca^{2+}-free medium, additional controls were performed with the same medium containing 1 mM EGTA to ensure that the first Ca^{2+} transient was indeed due to Ca^{2+} release. Extracellular Ca^{2+} concentration, measured with fura 2-pentapotassium, was 1.5 µM in nominally Ca^{2+}-free solutions and 116 nM after addition of 1 mM EGTA. Despite the dramatically lower extracellular Ca^{2+} after addition of EGTA to the medium, heparin again failed to block Ca^{2+} release (Fig. 3C).

Further studies were performed using a-thrombin, a ligand to a 7-transmembrane receptor that is native to the CHO cell. a-Thrombin (1 U/ml) caused a transient increase in Ca^{2+} to 933 ± 231 nM in nominally Ca^{2+}-free medium and a second, transient increase in Ca^{2+} to 971 ± 302 nM on addition of 2 mM Ca^{2+} to the medium (Fig. 3D; n = 13). As for ET-1, microinjected heparin (5 mg/ml injectate concentration) failed to block a-thrombin-induced release of Ca^{2+} from intracellular stores (889 ± 167 nM) or Ca^{2+} entry (850 ± 152 nM, n = 7; Fig. 3E).

To demonstrate that the observed differences in Ca^{2+} transients between 7-transmembrane and tyrosine kinase receptors were not due to cell variability, we
examined Ca\(^{2+}\) mobilization by PDGF and \(\alpha\)-thrombin in a single CHO-PDGF cell microinjected with fura 2 in the absence of heparin (Fig. 4A). PDGF (25 ng/ml) and \(\alpha\)-thrombin (1 U/ml) caused Ca\(^{2+}\) release and Ca\(^{2+}\) entry (n = 10). In single cells microinjected with heparin (5 mg/ml injectate concentration), there was a complete block of Ca\(^{2+}\) release from intracellular stores after the addition of PDGF (25 ng/ml; Fig. 4B; n = 8). However, Ca\(^{2+}\) release after \(\alpha\)-thrombin (1 U/ml) was intact. Ca\(^{2+}\) entry from the medium was intact for both ligands. We then examined the effect of microinjected heparin on Ca\(^{2+}\) stores after photorelease of caged IP\(_3\) and activation of PDGF and \(\alpha\)-thrombin receptors in single CHO-PDGF cells. In Fig. 4C, heparin, at injectate concentrations of 10 mg/ml, blocked Ca\(^{2+}\) release in nominally Ca\(^{2+}\)-free medium after flash photolysis of caged IP\(_3\) (0.375 nM; 4 single flashes of 0.5- to 4-s duration) or PDGF (25 ng/ml) but failed to block Ca\(^{2+}\) release after \(\alpha\)-thrombin receptor (1 U/ml; n = 5).

IP\(_3\) formation after activation of tyrosine kinase and 7-transmembrane receptors. Because heparin is a competitive inhibitor of the IP\(_3\)R, the results in Figs. 2–4 might be explained by increased production of IP\(_3\) in response to activation of 7-transmembrane receptors. We therefore determined the level of IP\(_3\) formation after the addition of PDGF, ET-1, or \(\alpha\)-thrombin (Table 1). Indeed, with \(\alpha\)-thrombin, IP\(_3\) levels were elevated 7.5-fold from baseline at 15 s, higher than the values observed for PDGF (1.5-fold at 60 s). However, with ET-1 the increase in IP\(_3\) level was comparable to that produced by PDGF, as demonstrated by ANOVA of the data in Table 1.

Assessment of competitive inhibition of Ca\(^{2+}\) release by microinjected heparin. To further assess the competitive inhibition of Ca\(^{2+}\) release by heparin, we varied the microinjected heparin concentration before stimulation at fixed agonist concentration (Table 2) or held heparin constant and reduced the \(\alpha\)-thrombin concentration to the minimum necessary to elicit Ca\(^{2+}\) transients (see below and Fig. 5).

As the microinjected heparin concentration was increased from 0.01 to 1.0 mg/ml (injectate concentration), the percentage of cells that produced a Ca\(^{2+}\) response to PDGF (25 ng/ml) declined in a dose-dependent manner. Half-maximal inhibition of Ca\(^{2+}\) release was 0.1–0.3 mg/ml heparin; nearly complete blockade was observed at heparin concentrations >1.0 mg/ml. In contrast, microinjected heparin failed to block \(\alpha\)-thrombin-induced release of Ca\(^{2+}\) stores at concentrations as high as 80 mg/ml, ~400-fold higher than those producing detectable inhibition of PDGF-induced Ca\(^{2+}\) release. Because the level of IP\(_3\) formation by ET-1 was more comparable to that produced by PDGF (Table 1), we examined the dose-response relationship for inhibition of Ca\(^{2+}\) transients by microinjected heparin with ET-1 as the agonist. As for \(\alpha\)-thrombin, microinjected heparin failed to block ET-1-induced release of Ca\(^{2+}\) stores at concentrations as high as 100 mg/ml, ~500-fold higher than those producing detectable inhibition of PDGF-induced Ca\(^{2+}\) release. The percentage of cells responding to ET-1 in the presence of microinjected heparin (5–100 mg/ml) was not different from the percentage of cells responding after microinjection with fura 2 alone (~90%).

To further assess the inhibition of Ca\(^{2+}\) release by heparin, \(\alpha\)-thrombin concentration was reduced at a fixed concentration of microinjected heparin. The lowest concentration of \(\alpha\)-thrombin that elicited reproducible Ca\(^{2+}\) transients in this system (data not shown) was 0.1 U/ml (0.7 nM). At this concentration, \(\alpha\)-
thrombin caused a transient increase in Ca\(^{2+}\) to 839 ± 619 nM (n = 14) in nominally Ca\(^{2+}\)-free medium (Fig. 5A). As for PDGF (Fig. 1A), and unlike higher concentrations of \(\alpha\)-thrombin, this increase was significantly delayed after agonist exposure. After heparin injection (5 mg/ml), 0.1 U/ml \(\alpha\)-thrombin elicited Ca\(^{2+}\) release transients (772 ± 202 nM, n = 17) that were not different from the transients in control cells microinjected with fura 2 alone (Fig. 5B). The percentage of cells responding to \(\alpha\)-thrombin (0.1 U/ml) in the presence of microinjected heparin (23 of 29 cells, ~80%) was also not different from the percentage of cells responding after microinjection with fura 2 alone (16 of 19 cells). Similar results were obtained in six cells microinjected with 80 mg/ml heparin (data not shown). Microinjection of nonspecific antibodies (IgG2a or a polyclonal antibody to IgG) had no effect on PDGF-mediated release of Ca\(^{2+}\) stores (data not shown).

In marked contrast, microinjected MAb 18A10 (0.75–2.2 mg/ml injectate concentration) in CHET-B cells failed to block ET-1-induced release of Ca\(^{2+}\) stores. ET-1 raised Ca\(^{2+}\) to 1,573 ± 509 nM in nominally Ca\(^{2+}\)-free medium and to 2,036 ± 303 nM after the addition of 2 mM Ca\(^{2+}\) (Fig. 6C; n = 8). Likewise, microinjected MAb 18A10 at similar concentrations failed to block \(\alpha\)-thrombin-induced release of Ca\(^{2+}\) stores, raising Ca\(^{2+}\)
distinguishes the Ca$^{2+}$ responses after activation of tyrosine kinase and 7-transmembrane receptors.

IP$_3$R isoform profile is similar in CHO cells expressing PDGF or ET-1 receptor. To determine whether the failure of microinjected heparin or MAb 18A10 to block Ca$^{2+}$ release after activation of the ET-1 or α-thrombin receptor was due to different IP$_3$R isoform profiles in cells expressing the PDGF (CHO-PDGF) or ET-1 receptor (CHET-B), total cell lysates from CHO-PDGF and CHET-B cells were analyzed by immunoblot with use of MAb to IP$_3$R1, IP$_3$R2, and IP$_3$R3 (Fig. 7). Densitometry of the relevant bands revealed that the isoform expression was nearly identical in the two cell lines. With the band density for CHO-PDGF cells set arbitrarily at 1.0, the density for CHET-B cells was 0.74, 0.93, and 1.18 for IP$_3$R1, IP$_3$R2, and IP$_3$R3, respectively.

**DISCUSSION**

The role of IP$_3$ as a major second messenger in the release of Ca$^{2+}$ from intracellular stores was first shown in permeabilized cells (49) and later in intact cells (6, 25, 26). IP$_3$, formed by hydrolysis of phosphatidylinositol 4,5-bisphosphate, is released into the cytoplasm, where it binds to a receptor on Ca$^{2+}$-storing organelles (3). After binding, Ca$^{2+}$ is released from the stores into the cytoplasm (13). Although 7-transmembrane and tyrosine kinase receptors produce IP$_3$ by different mechanisms and with slightly different time courses (41), it has generally been assumed that the IP$_3$ formed by both signaling pathways acts similarly.

Surprisingly, microinjected heparin failed to block release of Ca$^{2+}$ stores in single CHO cells after activation of the native α-thrombin receptor or a transfected ET-1 receptor. These findings with 7-transmembrane receptors contradict earlier studies in which heparin has been shown to block 7-transmembrane receptor-mediated release of Ca$^{2+}$ stores. Three distinct experimental methods have been used in this earlier work: permeabilized cells after the addition of heparin to the medium (23, 27, 42), cells microinjected with low-molecular-weight heparin (4, 8, 18, 26), and cells internally perfused with heparin during patch-clamp experiments (6, 52). In permeabilized cells, it is possible that alternative signaling molecules to IP$_3$ that are involved in Ca$^{2+}$ release were not produced or leaked from the cells during the experiment. Loss of such an alternative molecule could then render the Ca$^{2+}$ release process dependent on IP$_3$.

Using direct microinjection techniques, several investigators found that microinjected heparin blocked Ca$^{2+}$ release by cholecystokinin (18), bradykinin (26), isoproterenol (8), and tert-butyl hydroperoxide (4). It is notable that, in many of these reports (4, 8, 26), heparin concentrations >100 mg/ml were used for microinjection. This concentration is three orders of magnitude higher than that needed to block Ca$^{2+}$ release by PDGF in the present study. With the assumption that the injectate volume was 2% of cell volume (see experimental procedures), the heparin concentration we observed for half-maximal inhibition of PDGF-induced Ca$^{2+}$ release (0.2 mg/ml) corresponds to an intracellular protein concentration of 10$^{-6}$ M, which is below the threshold of detection for most heparin-binding proteins (5, 18).

**Fig. 4.** Effect of microinjected heparin on Ca$^{2+}$ transients in single CHO cells expressing PDGF receptor. Before Ca$^{2+}$ measurements, single CHO-PDGF cells were microinjected with 5 mM fura 2 (A), fura 2 and 5 mg/ml heparin (B), or 0.1 mM Ca$^{2+}$ green, 0.375 mM caged IP$_3$, and 10 mg/ml heparin (C). Twenty to 30 min later, Ca$^{2+}$ transients were measured in single cells after sequential additions of PDGF (25 ng/ml) and α-thrombin (1 U/ml) in nominally Ca$^{2+}$-free medium followed by addition of 2 mM Ca$^{2+}$ (A and B) or after multiple single flashes of ultraviolet light (0.5- to 4-s duration; C). Medium Ca$^{2+}$ concentration was varied between "0" mM (solid line) and 2 mM (filled bar). Traces are representative of 5-10 experiments under each condition.

To 414 ± 55 nM in nominally Ca$^{2+}$-free medium and to 920 ± 145 after the addition of 2 mM Ca$^{2+}$ (data not shown). To demonstrate that the distinction between tyrosine kinase and 7-transmembrane receptors could be observed in a single cell, a CHO-PDGF cell was sequentially exposed to PDGF and α-thrombin after microinjection of MAb 18A10 (0.75 mg/ml injectate concentration). PDGF-induced release of Ca$^{2+}$ stores was completely blocked, but α-thrombin-induced Ca$^{2+}$ release was not (Fig. 6D). α-Thrombin raised Ca$^{2+}$ to 489 ± 167 nM in nominally Ca$^{2+}$-free medium and to 967 ± 241 nM on readdition of Ca$^{2+}$ to the medium (n = 5). Ca$^{2+}$ entry from the medium was unaffected by MAb 18A10 for either ligand. Thus MAb 18A10, like heparin,
Finally, it is possible that the mechanisms by which inhibition of Ca\(^{2+}\) release at high concentrations of heparin may be due to a mechanism other than interference with the IP\(_3\) receptor, such as binding of Ca\(^{2+}\) (27). Artifactual blockade of Ca\(^{2+}\) release is a concern in microinjection studies, where cellular damage is possible. We noted that ~10% of cells failed to respond to ET-1 at any dose of heparin (Table 2), possibly because of damage from the microinjection. By performing a dose-response relationship and comparing the findings with the results using PDGF, we were able to show that the apparent inhibition of Ca\(^{2+}\) release in 10% of the cells by heparin was indeed an artifact. We also found that injectate concentrations >100 mg/ml caused intracellular vacuolization and poor Ca\(^{2+}\) responsiveness in general. Finally, it is possible that the mechanisms by which 7-transmembrane receptors release Ca\(^{2+}\) stores vary from system to system. In some systems this process may depend entirely on IP\(_3\), whereas in the system we studied there may be additional mechanisms.

One obvious explanation for the failure of heparin (a competitive inhibitor) to block Ca\(^{2+}\) transients in response to 7-transmembrane receptors could be the lower concentration of 4 µg/ml, very close to the heparin inhibition constant of 5–15 µg/ml when Ca\(^{2+}\) release was measured in response to IP\(_3\) in permeabilized cells (23, 42). This raises the possibility that inhibition of Ca\(^{2+}\) release at high concentrations of heparin may be due to a mechanism other than interference with the IP\(_3\) receptor, such as binding of Ca\(^{2+}\) (27). Artifactual blockade of Ca\(^{2+}\) release is a concern in microinjection studies, where cellular damage is possible. We noted that ~10% of cells failed to respond to ET-1 at any dose of heparin (Table 2), possibly because of damage from the microinjection. By performing a dose-response relationship and comparing the findings with the results using PDGF, we were able to show that the apparent inhibition of Ca\(^{2+}\) release in 10% of the cells by heparin was indeed an artifact. We also found that injectate concentrations >100 mg/ml caused intracellular vacuolization and poor Ca\(^{2+}\) responsiveness in general. Finally, it is possible that the mechanisms by which 7-transmembrane receptors release Ca\(^{2+}\) stores vary from system to system. In some systems this process may depend entirely on IP\(_3\), whereas in the system we studied there may be additional mechanisms.

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Table 1. Time course for agonist-mediated IP\(_3\) formation

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Basal</th>
<th>5 s</th>
<th>15 s</th>
<th>30 s</th>
<th>60 s</th>
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<td>PDGF</td>
<td>0.25 ± 0.02</td>
<td>0.27 ± 0.02</td>
<td>0.35 ± 0.02*</td>
<td>0.40 ± 0.03*</td>
<td>0.40 ± 0.03*</td>
</tr>
<tr>
<td>α-Thrombin</td>
<td>0.28 ± 0.03</td>
<td>0.85 ± 0.27*</td>
<td>2.29 ± 0.31*</td>
<td>2.03 ± 0.40*</td>
<td>0.71 ± 0.07*</td>
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<tr>
<td>ET-1</td>
<td>0.24 ± 0.02</td>
<td>0.31 ± 0.07</td>
<td>0.32 ± 0.04</td>
<td>0.40 ± 0.06*</td>
<td>0.44 ± 0.07*</td>
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</tbody>
</table>

Values are means ± SE. Platelet-derived growth factor (PDGF, 25 ng/ml), endothelin-1 (ET-1, 1 nM), and α-thrombin (1 U/ml) were added for indicated times to cells expressing PDGF, ET-1, or native α-thrombin receptor. Inositol 1,4,5-trisphosphate (IP\(_3\)) levels were determined as described in EXPERIMENTAL PROCEDURES. Each value is representative of 3–7 experiments. All time points in each experiment were performed in duplicate or triplicate. For PDGF at 5 s and PDGF at 15 s, values are representative of 1 experiment (performed in triplicate). *For PDGF, basal vs. 30 and 60 s were not significantly different (P < 0.05), and PDGF and ET-1 at 30 and 60 s were not significantly different (P > 0.33) by unpaired t-test or ANOVA.

Table 2. Dose response of heparin blockade of agonist-induced release of intracellular Ca\(^{2+}\) stores

<table>
<thead>
<tr>
<th>Injectate Heparin, mg/ml</th>
<th>PDGF</th>
<th>α-Thrombin</th>
<th>ET-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>100 (4)</td>
<td>100 (11)</td>
<td>88 (8)</td>
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<td>0.1</td>
<td>62 (8)</td>
<td>90 (10)</td>
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<td>90 (10)</td>
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<tr>
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<td>90 (20)</td>
<td>90 (10)</td>
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<td>11 (9)</td>
<td>90 (10)</td>
<td>90 (10)</td>
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<td>5.0</td>
<td>7 (27)</td>
<td>100 (11)</td>
<td>89 (19)</td>
</tr>
<tr>
<td>10.0</td>
<td>11 (9)</td>
<td>92 (12)</td>
<td>92 (12)</td>
</tr>
<tr>
<td>40.0</td>
<td>90 (10)</td>
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</tr>
<tr>
<td>60.0</td>
<td>90 (20)</td>
<td>90 (12)</td>
<td>90 (10)</td>
</tr>
<tr>
<td>80.0</td>
<td>100 (5)</td>
<td>100 (5)</td>
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</tr>
<tr>
<td>100.0</td>
<td>88 (8)</td>
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<td>88 (8)</td>
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</table>

Values are expressed as percentage of cells responding to ligand in presence of indicated microinjected heparin (injectate concentration); number of single cell experiments at each concentration is shown in parentheses. Low-molecular-weight heparin was microinjected into Chinese hamster ovary cells expressing PDGF, ET-1, or α-thrombin receptor. Twenty to 30 min later, intracellular Ca\(^{2+}\) response was measured in nominally Ca\(^{2+}\)-free medium after exposure to PDGF (25 ng/ml), α-thrombin (1 U/ml), or ET-1 (1 nM).

Fig. 5. Microinjected heparin fails to inhibit Ca\(^{2+}\) transients even at reduced α-thrombin concentration. Before Ca\(^{2+}\) measurements, single CHO-PDGF cells were microinjected with 5 mM fura 2 (A) or fura 2 and 5 mg/ml heparin (B). Twenty to 30 min later, Ca\(^{2+}\) transients were measured in single CHO-PDGF cells after addition of α-thrombin (0.1 U/ml) in nominally Ca\(^{2+}\)-free medium followed by addition of 2 mM Ca\(^{2+}\). Medium Ca\(^{2+}\) concentration was varied between 0 mM (solid line) and 2 mM (filled bar). Traces are representative of 14–17 experiments under each condition.
muscle cells (28) and fibroblasts (9). On the other hand, as we found, other investigators observed that \( \alpha \)-thrombin increased IP₃ levels by four- to sixfold (7, 24, 56). Because we were unable to block \( \text{Ca}^{2+} \) release after ET-1 with heparin concentrations that were nearly three orders of magnitude higher than those that did block \( \text{Ca}^{2+} \) release with PDGF, it seems unlikely that the failure of heparin to block \( \text{Ca}^{2+} \) release after activation of ET-1 receptors is simply due to the competitive interaction between IP₃ and heparin at the IP₃ receptor. Although for \( \alpha \)-thrombin it also seems unlikely that the high levels of IP₃ produced after activation of this receptor can explain our results, we cannot rule out the possibility that the greater IP₃ formation after \( \alpha \)-thrombin was in fact sufficient to overcome the heparin blockade of the IP₃ receptor.

A second potential explanation for our findings is that microinjected macromolecules may have differential access to IP₃R associated with the two receptor classes. In nasal epithelial cells, apical and basolateral purinergic receptors appear to access distinct \( \text{Ca}^{2+} \) stores (43). Using CHO cells, Chun et al. (10) showed that ET-1 receptors are localized to caveolae, which have been proposed to contain isolated signal transduction systems (53). Although some doubt has been cast on this signaling role for caveolae (48), Fujimoto et al. found a \( \text{Ca}^{2+} \) pump (15) and an IP₃R-like protein (16) in caveolae. Thus it appears likely that some cell types may have isolated pools of \( \text{Ca}^{2+} \)-signaling molecules that theoretically could be inaccessible to microinjected reagents.

A final possible explanation for our results is that 7-transmembrane receptors could regulate release of \( \text{Ca}^{2+} \) stores via an IP₃-independent mechanism. Babich et al. (1) demonstrated that parathyroid hormone (PTH) and \( \alpha \)-thrombin mobilized \( \text{Ca}^{2+} \) from intracellular stores but that only \( \alpha \)-thrombin caused detectable IP₃ formation. Seuwen and Boddeke (47) also found that PTH raised \( \text{Ca}^{2+} \) but failed to generate IP₃ in HEK cells expressing the PTH receptor. Although the production of IP₃ in these studies may have been below the detection limit for the assay used, it is notable that internal perfusion of heparin failed to block PTH-mediated release of \( \text{Ca}^{2+} \) stores in this system (47). Other receptors, including neuropeptide (36), \( \alpha_{2A} \)-
adrenergic (33), cholecystokinin analog (46), prostacyclin (55), purinergic (14), glucagon-like peptide (21), and isoproterenol (57), have been found under certain circumstances to cause mobilization of Ca\textsuperscript{2+} stores in the absence of measurable IP\textsubscript{3} formation. Taken together, these data raise the possibility of an IP\textsubscript{3}-independent mechanism for the mobilization of Ca\textsuperscript{2+} stores under certain conditions.

A novel second messenger that might be involved in Ca\textsuperscript{2+} release by 7-transmembrane receptors is cyclic ADP-ribose (cADPR), an NAD\textsuperscript{+} metabolite. cADPR introduced by direct addition to homogenates or permeabilized cells or by microinjection into intact cells has recently been found to cause Ca\textsuperscript{2+} release in neurosecretory cells (29), sea urchin eggs (30), and epithelial cells (2). Furthermore, addition of 8-amino-cADPR, a known antagonist of cADPR, to sea urchin egg homogenates blocks cADPR-induced Ca\textsuperscript{2+} release (31). The role of cADPR in Ca\textsuperscript{2+} mobilization after activation of 7-transmembrane receptors has not been determined.

The results of this study show that release of Ca\textsuperscript{2+} stores by PDGF and FGF, the receptors of which belong to the tyrosine kinase family, is blocked by heparin and an MAb to IP\textsubscript{3}R1. Strikingly, these maneuvers do not block Ca\textsuperscript{2+} release after exposure to ET-1 or α-thrombin, the receptors of which belong to the 7-transmembrane family. For PDGF and ET-1, this difference is not due to different quantities of IP\textsubscript{3} released after activation of the receptors or to differences in IP\textsubscript{3}R isoform profile. The simplest conclusion that can be drawn from these data is that, in the systems we examined, certain 7-transmembrane receptors are capable of activating Ca\textsuperscript{2+} by a signaling system that differs spatially or chemically from the IP\textsubscript{3}-signaling system as utilized by tyrosine kinase receptors. Although it seems certain that IP\textsubscript{3} plays an important role in Ca\textsuperscript{2+} release by all the receptors we examined, it may be that an alternative signaling system comes into play under certain physiological conditions. Future work is required to identify the spatial or chemical nature of this alternative Ca\textsuperscript{2+}-signaling system.

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