Calcium Mobilization Evoked by Hepatocellular Swelling Is Linked to Activation of Phospholipase Cγ*

Recovery from swelling of hepatocytes and selected other epithelia is triggered by intracellular Ca\(^{2+}\) release from the endoplasmic reticulum, which leads to fluid and electrolyte efflux through volume-sensitive K\(^+\) and Cl\(^-\) channels. The aim of this study was to determine the mechanisms responsible for swelling-mediated hepatocellular Ca\(^{2+}\) mobilization. Swelling of HTC rat hepatoma cells, evoked by exposure to hypotonic medium, elicited transient increases in intracellular levels of inositol 1,4,5-trisphosphate (IP\(_3\)) and cytosolic Ca\(^{2+}\). The latter was attenuated by inhibition of phospholipase C (PLC) with U73122 and by IP\(_3\) receptor blockade with 2-aminoethoxydiphenyl borate, but it was unaffected by ryanodine, an inhibitor of intracellular Ca\(^{2+}\)-induced Ca\(^{2+}\) release channels. Hypotonic swelling was associated with a transient increase in tyrosine phosphorylation of PLCγ, with kinetics that paralleled the increases in intracellular IP\(_3\) levels and cytosolic Ca\(^{2+}\). Confocal imaging of HTC cells exposed to hypotonic medium revealed a swelling-induced association of tyrosine-phosphorylated PLCγ with the plasma membrane. These findings suggest that activation of PLCγ by hepatocellular swelling leads to the generation of IP\(_3\) and stimulates discharge of Ca\(^{2+}\) from the endoplasmic reticulum via activation of IP\(_3\) receptors. By extension, these data support the concept that tyrosine phosphorylation of PLCγ represents a critical step in adaptive responses to hepatocellular swelling.

Hepatocytes undergo dynamic alterations in cell volume in response to changing metabolic demands. For example, under physiological conditions, postprandial uptake of amino acids by hepatocytes results in significant swelling, with subsequent recovery of cell volume (1). Physiological recovery of cell volume requires the opening of plasma membrane K\(^-\) and Cl\(^-\) channels with consequent fluid and electrolyte efflux (2). This process provides a safeguard against irreversible swelling, which, in pathological states, is manifested by ballooning degeneration of hepatocytes (3). Despite the importance of volume-sensitive ion channel regulation in maintenance of normal liver function, the mechanisms that couple hepatocellular swelling to channel activation remain largely undefined. We have recently shown that K\(^-\) and Cl\(^-\) channel opening evoked by hepatocellular swelling is triggered by an increase in cytosolic \([\text{Ca}^{2+}]_{i}\) and that this increase is critical for volume recovery (4). The initial increase in \([\text{Ca}^{2+}]_{i}\), results from Ca\(^{2+}\) mobilization from thapsigargin-sensitive intracellular stores, which suggests the involvement of the endoplasmic reticulum in this process. How Ca\(^{2+}\) is mobilized from the endoplasmic reticulum in response to hepatocellular swelling is yet unknown.

In extrahepatic tissues, diverse modes of volume-sensitive Ca\(^{2+}\) release from the endoplasmic reticulum exist. In airway epithelium, swelling leads to generation of inositol 1,4,5-trisphosphate (IP\(_3\)) and Ca\(^{2+}\) discharge through IP\(_3\) receptors (5). It would be anticipated that swelling-mediated IP\(_3\) generation is a consequence of cleavage of plasma membrane phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC). In support of a role for PLC, it has been shown in arterial endothelium that inhibition of PLC prevents swelling-induced increases in \([\text{Ca}^{2+}]_{i}\) (6). Four major families of PLC isoforms (β, γ, δ, and ε) have been recognized, each with distinct modes of activation. In general, PLCβ is stimulated in response to activation of heterotrimeric G protein-coupled receptors, PLCγ is stimulated by tyrosine kinases, PLCδ is stimulated by localized increases in \([\text{Ca}^{2+}]_{i}\), and PLCε is stimulated by activation of the monomeric G protein Ras (7). The nature of swelling-activated PLC isoforms has not yet been elucidated.

A second pathway for swelling-mediated Ca\(^{2+}\) mobilization, described in corneal epithelium, involves Ca\(^{2+}\) release through ryanodine receptors (8), although the mechanisms responsible for this remain undefined. A third pathway, reported in vascular smooth muscle cells, involves processes that are independent of both IP\(_3\) and ryanodine receptors (9). However, the underlying mechanisms for swelling-mediated Ca\(^{2+}\) mobilization in these cells have not been further delineated. In hepatocytes, prolonged exposure to hypotonic solution or amino acids (which would tend to increase cell volume) is associated with a small increase in intracellular IP\(_3\) levels (10). Although this raises the possibility that hepatocellular swelling evokes Ca\(^{2+}\) mobilization via activation of IP\(_3\) receptors, it remains to be determined whether this mechanism is involved, and if so, which isoform of PLC is responsible for formation of IP\(_3\).

In the present study, we have addressed these unresolved issues concerning the origins of volume-sensitive hepatocellular Ca\(^{2+}\) signaling. Here we report in HTC rat hepatoma cells that swelling evoked by hypotonic challenge elicits Ca\(^{2+}\) mobilization by a sequence of events that involve: (a) activation of

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* This work was supported in part by National Institutes of Health Grants DK047849 and DK056644 (to S. D. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked \"advertisement\" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: \([\text{Ca}^{2+}]_{i}\), cytosolic \([\text{Ca}^{2+}]\); IP\(_3\), inositol 1,4,5-trisphosphate; PLC, phospholipase C; SES, standard extracellular solution; HES, hypotonic extracellular solution; 2-ABP, 2-aminoethoxydiphenyl borate; FAK, focal adhesion kinase; PT 3-kinase, phosphatidylinositol 3-kinase; pY-PLCγ, tyrosine-phosphorylated PLCγ.

This paper is available on line at http://www.jbc.org
PLCγ (b) generation of IP₃, and (c) activation of IP₃ receptors. We propose that PLCγ is a pivotal mediator of adaptive responses to increases in liver cell volume and serves a critical role in protection against organ level injury that results from pathological swelling.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture—** HTC rat hepatoma cells were cultured as previously described (4). Cell swelling was elicited by exposure to hypotonic medium. Prior to study, cells were incubated in standard extracellular solution (SES, ~300 mosM). SES contained (in mm): 140 NaCl, 4 KCl, 1 CaCl₂, 2 MgCl₂, 1 KH₂PO₄, 10 glucose, and 10 HEPES (pH 7.4). The hypotonic solution (HES, ~240 mosM) was identical to SES, except that the concentration of NaCl was 84 mm.

**Materials and Reagents—** Acetoxymethyl esters of fluo-3 and Fura Red were obtained from Molecular Probes. Ryonadine, U73122, and U75345 were purchased from Calbiochem. Monoclonal phosphotyrosine antibody (clone 4G10) and monoclonal paxillin antibody (clone 5H11) were purchased from Upstate Biotechnology, and phosphotyrosine-specific (Tyr-783) PLCγ rabbit antibody was from Cell Signaling. Unless otherwise indicated, all other chemicals were obtained from Sigma.

**Measurement of IP₃—** IP₃ levels were measured in cultured HTC cells by radioreceptor assay (Amersham Biosciences). Briefly, cells grown on 10-cm plates were washed twice with Ca²⁺- and Mg²⁺-free phosphate-buffered saline (pH 7.4) and then removed with nonenzymatic Cell Stripper (Mediatech). Suspended cells were washed once with SES. Following the wash, the cells were centrifuged at 260 × g and then resuspended in SES. Aliquots of 3.6 × 10⁶ cells in SES were placed in 1.5-ml centrifuge tubes and incubated in a 37 °C water bath for 10 min before swelling with HES. At specific times following hypotonic exposure, cellular IP₃ was extracted by the addition of an equal volume of ice-cold 10% perchloric acid followed by neutralization with an equal volume mixture of freon and tri-n-octylamine (11). Cellular IP₃ was measured according to the manufacturer’s directions.

**Measurement of [Ca²⁺]i—** [Ca²⁺], was determined in individual cells by dual wavelength microfluorimetry, using the Ca²⁺-sensitive fluorescent dye fluo-3 and Fura Red as described previously (4). Measurements of fluo-3 and Fura Red fluorescence emission intensity were acquired every 10 s, from which the ratio R of fluo-3 to Fura Red fluorescence was calculated. Changes in [Ca²⁺], were inferred from changes in the relative fluorescence ratio, calculated by dividing R at each time point by R₀, the mean fluorescence ratio averaged over the initial 2 min of data acquisition. For studies involving inhibitors, comparisons were made with same day controls.

**Immunoprecipitation and Immunoblot Analysis—** Studies were performed on cells that were plated onto 10-cm dishes and cultured overnight. Prior to hypotonic exposure, cells were equilibrated with SES for 10 min at room temperature. Following this, SES was removed and replaced with either 10 ml of SES (basal) or 10 ml of HES (hypotonic). At designated times, the solutions were replaced with 500 μl of ice cold lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 0.25% sodium deoxycholate, 1% Nonidet P-40) plus 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM sodium orthovanadate, and complete protease inhibitor mixture (Roche Molecular Biochemicals). The plates were left on ice for 10 min, and the lysates were harvested. Protein concentration was determined by Bradford reagent (Bio-Rad).

Immunoprecipitations with phosphotyrosine antibody were performed according to the manufacturer’s directions, with minor modifications. Phosphotyrosine antibody (3 μl) was bound to 50 μl of a slurry of washed Protein A-agarose beads by rotating the mixture for 1 h at 4 °C. Subsequently, 1 ml of whole cell lysate (1 mg of protein) was added to the beads and gently rocked overnight at 4 °C. The beads were washed five times with lysis buffer, resuspended in Laemmli sample buffer, and heated at 100 °C for 5 min. The proteins were resolved by SDS electrophoresis on 10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with PLCγ (Tyr-783) antibody, as recommended by the manufacturer. Proteins were detected by exposure to horseradish peroxidase-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratory) and enhanced chemiluminescence (Super Signal, Pierce), and densitometric analysis was performed using an Alpha Image 2000 (Alpha Innotech).

**Immunocytochemistry and Confocal Fluorescence Microscopy—** Studies were performed in cells that were seeded onto glass coverslips and cultured overnight. After equilibration with SES for 10 min at room temperature, SES was replaced with either SES (basal) or HES (hypotonic). At designated times, cells were fixed with 4% paraformaldehyde in TBS (Tris-buffered saline, 25 mM, pH 7.4) for 10 min, then permeabilized for 10 min with 0.2% Triton X-100, 300 mM sucrose in TBS, and gently rocked for 1 h with 1% bovine serum albumin and 10% normal donkey serum (Jackson ImmunoResearch Laboratories). Coverslips were washed twice with TBS and rocked overnight at 4 °C with PLCγ (Tyr-783) antibody and in selected studies with paxillin antibody. Coverslips were washed three times with TBS and rocked for 1 h at room temperature with Cy3-labeled donkey anti-rabbit IgG (Fab’), and/or Cy5-labeled donkey anti-mouse IgG (Fab’), (Jackson ImmunoResearch Laboratories). Coverslips were washed three times with TBS and then mounted on slides with Aqua Polymount (Polysciences). Prepared slides were analyzed on a Bio-Rad MRC 1024 ES laser scanning confocal microscope as previously described (12).

**RESULTS**

**Hepatocellular Swelling Elicits Increases in Intracellular IP₃ Levels—** We have previously reported that hepatocellular swelling evoked by hypotonic challenge produces a transient increase in [Ca²⁺], that peaks at 1–2 min and falls to basal values within 10 min (4). In addition, in cultured hepatocytes, prolonged exposure to hypotonic solution causes modest increases in intracellular levels of IP₃ (10). Although these findings raise the possibility that swelling-activated elevations in intracellular IP₃ levels lead to intracellular Ca²⁺ mobilization, the kinetcs of IP₃ generation and the temporal relation to increases in [Ca²⁺], have not been delineated. It has thus remained unresolved whether IP₃ is a volume-sensitive signaling molecule for intracellular Ca²⁺ mobilization.

To investigate this further, we determined the time course of IP₃ generation in response to hepatocellular swelling. As shown in Fig. 1, exposure of HTC cells to hypotonic medium elicited a transient increase in intracellular IP₃ levels. The increase in IP₃ levels occurred within 30 s, and these levels approached basal values within 10 min. These kinetics are consistent with the concept that IP₃ elicits intracellular Ca²⁺ mobilization in response to hepatocellular swelling.

**Hypotonic—** Hypotonic

**Swelling-evoked Ca²⁺ Mobilization Requires IP₃ Receptor Activation—** The results above suggest that if hepatocellular swelling elicits intracellular Ca²⁺ mobilization in response to increased intracellular levels of IP₃, then such Ca²⁺ increases would be dependent on activation of IP₃ receptors. To test this, we determined the effect of 2-aminoethoxydiphenyl borate (2-APB), an IP₃ receptor inhibitor, on changes in [Ca²⁺], evoked by hypotonic challenge. As shown in Fig. 2A, 2-APB markedly attenuated swelling-induced increases in [Ca²⁺]. However, it...
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Fig. 2. Swelling-induced intracellular Ca\(^{2+}\) mobilization involves activation of IP\(_3\) receptors. Cells on coverslips were loaded at room temperature with the Ca\(^{2+}\)-sensitive dyes fluo-3 and Fura Red (see "Experimental Procedures") and subsequently exposed to hypotonic solution (40% reduction in NaCl concentration). [Ca\(^{2+}\)]\(_i\), was measured using ratiometric laser scanning confocal microfluorimetry, and changes in [Ca\(^{2+}\)]\(_i\), were inferred from the ratio of fluo-3 to Fura Red fluorescence (see "Experimental Procedures"). A, effects of the IP\(_3\) receptor inhibitor 2-APB (50 μM) on swelling-induced changes in [Ca\(^{2+}\)]\(_i\). (Red Ratio). The left panel depicts a representative cell. In the presence of 2-APB (gray bar), exposure to hypotonic solution (open bar) did not result in a change in [Ca\(^{2+}\)]\(_i\), [Ca\(^{2+}\)]\(_i\), subsequently rose following 2-APB removal. The right panel shows summary data of the means ± S.E. of peak [Ca\(^{2+}\)]\(_i\), (Peak Ratio) following exposure to hypotonic solution in the absence (control, Ctl, n = 90 cells) versus presence (n = 90 cells) of 2-APB. B, effects of ryanodine (100 μM in 0.1% dimethyl sulfoxide) on swelling-induced changes in [Ca\(^{2+}\)]\(_i\). The left panel shows in a representative cell the effects on [Ca\(^{2+}\)]\(_i\), (Red Ratio) of exposure to hypotonic solution (open bar) in the presence of ryanodine (gray bar). The right panel depicts summary data of the means ± S.E. of peak [Ca\(^{2+}\)]\(_i\), (Peak Ratio) following exposure to hypotonic solution in the absence (control, Ctl, 0.1% dimethyl sulfoxide, n = 74 cells) versus presence (n = 81 cells) of ryanodine (Ryan).

is possible that the effects of 2-APB were not confined to intracellular Ca\(^{2+}\) mobilization because 2-APB has been suggested to inhibit store-dependent Ca\(^{2+}\) influx in addition to IP\(_3\) receptor blockade (13). To clarify this, we examined, in the nominal absence of extracellular Ca\(^{2+}\), the effects of 2-APB on swelling-mediated changes in [Ca\(^{2+}\)]\(_i\). Under these conditions, contributions to such changes in [Ca\(^{2+}\)]\(_i\), that arose from Ca\(^{2+}\) influx would be disabled. In the nominal absence of extracellular Ca\(^{2+}\), 2-APB ablated increases in [Ca\(^{2+}\)]\(_i\), evoked by hypotonic challenge (peak relative fluorescence ratio 1.15 ± 0.06, n = 46 in the presence of 2-APB versus 3.66 ± 0.31, n = 45 in its absence, p < 0.05). These findings indicate that 2-APB prevented intracellular Ca\(^{2+}\) mobilization elicited by hepatocellular swelling and are consistent with a role for IP\(_3\) receptors in this process.

In addition to IP\(_3\) receptors, it is possible that swelling-mediated intracellular Ca\(^{2+}\) mobilization involved ryanodine receptors, as has been reported in corneal epithelial cells (8). We therefore determined the effect of ryanodine, which blocks ryanodine receptors, on changes in [Ca\(^{2+}\)]\(_i\), evoked by hypotonic exposure. In contrast to 2-APB, ryanodine did not inhibit swelling-mediated increases in [Ca\(^{2+}\)]\(_i\), (Fig. 2B). This observation suggests that ryanodine receptors do not play a role in Ca\(^{2+}\) mobilization evoked by hepatocellular swelling, and it is consistent with the absence of expression of known ryanodine receptor isoforms in hepatocytes (14).

Swelling-evoked Ca\(^{2+}\) Mobilization Requires Activation of PLC—The findings thus far support the concept that hepatocellular swelling elicits intracellular Ca\(^{2+}\) mobilization via generation of IP\(_3\) and activation of IP\(_3\) receptors. It would thus be predicted that swelling-mediated increases in [Ca\(^{2+}\)]\(_i\), would require stimulation of phospholipase C, which cleaves IP\(_3\) from phospholipid precursors. To test this, we applied the phospholipase C inhibitor U73122 and determined its effect on changes in [Ca\(^{2+}\)]\(_i\), evoked by hypotonic challenge. U73122 prevented such increases in [Ca\(^{2+}\)]\(_i\), (Fig. 3). By contrast, U73343, an inactive analog of U73122, did not prevent swelling-mediated [Ca\(^{2+}\)]\(_i\), increases (Fig. 3).

Although these findings are consistent with a role for PLC, U73122 has been suggested to inhibit store-dependent Ca\(^{2+}\) influx in addition to inhibition of PLC (15). To minimize contributions arising from Ca\(^{2+}\) influx, we examined, in the nominal absence of extracellular Ca\(^{2+}\), the effects of U73122 on swelling-mediated changes in [Ca\(^{2+}\)]\(_i\). Under these conditions, U73122 ablated increases in [Ca\(^{2+}\)]\(_i\), evoked by hypotonic challenge (peak relative fluorescence ratio 1.08 ± 0.01, n = 69 in the presence of U73122 versus 1.43 ± 0.08, n = 41 in its absence, p < 0.05). These findings indicate that U73122 prevented intracellular Ca\(^{2+}\) mobilization elicited by hepatocellular swelling and are consistent with a requirement for PLC in this process.

Swelling Elicits Activation of PLCγ—Activation of PLC by cell swelling has been implicated in airway and intestinal epithelium and in vascular endothelium (5, 6, 16), but neither the mechanisms of activation nor the responsible PLC isoforms have been determined. In liver, selected downstream responses to cell swelling, such as endosomal alkalization and stimulation of bile flow, have been linked to activation of tyrosine kinases (17, 18). We therefore reasoned that PLCγ, an effector of both receptor and non-receptor tyrosine kinase-mediated pathways, could be involved in volume-sensitive hepatocellular Ca\(^{2+}\) signaling. When activated by tyrosine kinases, PLCγ undergoes tyrosine phosphorylation and becomes associated with the plasma membrane (19, 20). With this in mind, we tested whether hepatocellular swelling elicited time-dependent changes in the abundance or localization of tyrosine-phosphorylated PLCγ.

Extracts of HTC cells, taken before and after exposure to hypotonic medium, were analyzed for abundance of tyrosine-phosphorylated PLCγ by immunoprecipitation with anti-phosphotyrosine antibody and subsequent immunoblotting with anti-tyrosine-phosphorylated PLCγ. Increases in tyrosine-phosphorylated PLCγ were evident within 1 min after exposure to hypotonic medium and had fallen to basal values within 10 min (Fig. 4).
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lar staining for tyrosine-phosphorylated PLC
within the plane of adherent membrane), under
i.e. A
sence microscopy (Fig. 5). As shown in Fig. 5
following swelling, we performed confocal immunofluores-
/H9253
positively for tyrosine-phosphorylated PLC
with rapid activated PLC
was found to be in a diffuse intracellular pattern, consistent
ody, the focal adhesion structures labeled by PLC
/adhesion protein paxillin (21). Localization of tyrosine-
mation. The onset of increased PLC
phosphorylation paralleled
the onset of IP 3 generation, and these events preceded the
the focal adhesion structures labeled by PLC
progressively enlarged over the following 8 min, a time at which
(21). Localization of tyrosine-
phosphorylated PLC
related to focal adhesion protein paxillin (21). Localization of tyrosine-
phosphorylated PLC
was detected in peripheral structures that also labeled for the focal
hesion protein paxillin (21). Localization of tyrosine-
phosphorylated PLC
in focal adhesions is consistent with observations by others (22). Upon exposure to hypotonic medi-
plasma membrane labeling of pY-PLC
that recognized the focal adhesion protein paxillin (right panels). B, images of cells stained for pY-PLC
were taken 4 μm above the base. Plasma membrane labeling of pY-PLC
can be seen in the cell at the lower right of the panel that corresponds to 1 min after hypotonic exposure.

FIG. 6. Proposed model for hepatocellular volume-sensitive Ca 2
signaling. Hepatocellular swelling elicits the activation of a
tyrosine kinase, which in turn activates PLCγ. PLCγ activation leads to
generation of IP 3 , which triggers intracellular Ca 2
release from the endoplasmic reticulum (ER) via activation of IP 3 receptors. The resulting
increase in [Ca 2
], are required for activation of K + and Cl -
channels, which leads to volume recovery.

FIG. 5. Effects of swelling on the localization of tyrosine-phosphorylated PLCγ (pY-PLCγ) in HTC cells. Cells on coverslips were
exposed at room temperature to hypotonic solution for the times indicated and fixed as described under “Experimental Procedures.” The
acellular distribution of pY-PLCγ by laser confocal fluorescence microscopy of cells stained with phosphotyrosine PLCγ1 (Tyr-783) antibody. A, at
the base of the group of cells depicted, pY-PLCγ (left panels) was found in peripheral structures that co-immunostained with antibody that
recognized the focal adhesion protein paxillin (right panels). B, images of cells stained for pY-PLCγ were taken 4 μm above the base.

DISCUSSION

This study tested whether PLC could act as a trigger for Ca 2
mobilization evoked by hepatocellular swelling. We
found that hypotonic swelling of HTC cells elicited a transient increase in IP 3 levels, and swelling-evoked increases in
[Ca 2
] were attenuated by the PLC inhibitor U73122 and by the
IP 3 receptor blocker 2-APB. Moreover, hypotonic exposure led to increased tyrosine phosphorylation of PLCγ and
plasma membrane localization, consistent with PLCγ activation. The onset of increased PLCγ phosphorylation paralleled
the onset of IP 3 generation, and these events preceded the
increase in [Ca 2
], evoked by cell swelling. Collectively, these
findings support the hypothesis that Ca 2
mobilization evoked by hepatocellular swelling involves a cascade of events that include: (a) activation of PLCγ, (b) production of
IP₃, and (c) release of Ca²⁺ from the endoplasmic reticulum via activation of IP₃ receptors (Fig. 6). Because Ca²⁺ mobilization is essential for volume recovery after hepatocellular swelling, these data suggest that PLCγ is a critical mediator of cell volume regulation in liver.

Several important caveats merit comment. First, the compound U73122 has been reported to have effects on [Ca²⁺], that are independent of inhibition of PLC, in particular inhibition of store-operated Ca²⁺ influx (15). Second, 2-APB has also been reported to inhibit store-operated Ca²⁺ influx and thus appears to have effects on [Ca²⁺], that are independent of IP₃ receptors. However, the results of control experiments performed in the absence of extracellular Ca²⁺ demonstrate that U73122 and 2-APB each inhibited Ca²⁺ mobilization (and not Ca²⁺ influx per se) and are thus consistent with blockade of PLC activation and IP₃ receptors, respectively. This interpretation is further supported by two independent assays of PLC activation: measurement of IP₃ levels and tyrosine phosphorylation of PLCγ. It should be noted that although the onset of the increase in tyrosine-phosphorylated PLCγ paralleled the initial increase in IP₃ levels, it is evident that the kinetics of tyrosine phosphorylation are not exactly identical to those of swelling-mediated changes in intracellular levels of IP₃. The most likely explanation for this is that the IP₃ assays and analysis of tyrosine-phosphorylated PLCγ were not performed under strictly comparable conditions (e.g. temperature, substrate adherence), and these differences may have influenced the kinetics of PLCγ phosphorylation and IP₃ generation. Nonetheless, the most parsimonious explanation of our findings is that intracellular Ca²⁺ mobilization evoked by swelling results from activation of IP₃ receptors in response to PLCγ-mediated production of IP₃.

Precedent for swelling-mediated activation of PLC exists in airway and intestinal epithelium as well as vascular endothelium (5, 6, 16). In the latter two tissues, volume-sensitive PLC activation appears to involve autocrine stimulation of P2Y purinergic receptors. Although it is known that P2Y receptors can activate PLCb and PLCγ (23, 24), the identity of PLC isoforms that mediate volume-sensitive purinergic Ca²⁺ signaling has not been elucidated. As we have shown that swelling-mediated increases in [Ca²⁺], in HTC cells are independent of purinergic receptor stimulation (4), it is likely that distinct mechanisms are involved in volume-sensitive PLC activation in liver. It should be emphasized that we have not excluded a potential role for PLCb, PLCδ, or PLCε in swelling-evoked [Ca²⁺], increases, but our findings clearly demonstrate that PLCγ is tyrosine-phosphorylated upon swelling and thus implicates its involvement in hepatocellular volume regulation. Activation of PLCγ by swelling would not only account for generation of IP₃, as we have shown in the present study, but it could be responsible for stimulation (via liberation of diacylglycerol) of protein kinase C, which mediates activation of volume-sensitive hepatocellular Cl⁻ channels (25).

There is good reason to consider PLCγ as a transducer of signaling events elicited by hepatocellular swelling as several potential upstream activators of PLCγ, including phosphatidylinositol (PD) 3-kinase (26) and tyrosine kinases (7), have been implicated in volume regulatory responses in liver. PI 3-kinase is required for volume recovery after hepatocellular swelling (27), but it is uncertain whether it is involved in swelling-mediated PLCγ activation. Specifically, activation of PLCγ by PI 3-kinase appears to be independent of tyrosine phosphorylation (26); yet tyrosine phosphorylation of PLCγ was seen in the present study. Thus, regulators of PLCγ other than PI 3-kinase warrant consideration.

Focal adhesion kinase (FAK) undergoes tyrosine phosphorylation upon hepatocellular swelling (28), but its cellular localization in response to swelling has not been delineated. If FAK is involved in volume-sensitive PLCγ activation, our observations would suggest that focal adhesions may not be the sole site of the swelling-activated interaction between these two proteins because the bulk of plasma membrane-bound tyrosine-phosphorylated PLCγ, as detected by immunofluorescence, did not appear to be present in focal adhesions during times corresponding to maximal swelling-induced tyrosine phosphorylation of PLCγ as determined by immuno blot analysis.

Members of the Src family of tyrosine kinases, which can associate with PI 3-kinase (29) as well as FAK (30), mediate volume recovery from swelling in lymphocytes (31) and play important roles in mechanotransduction (32). In lung, mechanical strain elicits Src-mediated PLCγ activation as a consequence of rearrangements of the actin cytoskeleton (33). Because dynamic actin rearrangements occur in response to hepatocellular swelling (34), this could serve as a mechanism to couple Src to PLCγ activation in liver. Whether Src kinases, FAK, or PI 3-kinase are involved in volume-sensitive PLCγ activation remains to be determined.

It is of interest that PLCγ has been found to be mislocalized or down-regulated in experimental models of liver injury (35, 36) that are associated with hepatic cellular swelling (37, 38). This raises the possibility that PLCγ, through its effects on hepatocellular volume regulation, plays a pivotal role in cytoprotection against pathological swelling. In this way, PLCγ and its upstream regulators may represent important therapeutic targets for the prevention of organ level injury in pathological conditions associated with disordered control of liver cell volume.

Acknowledgments—We thank Dr. Elisabeth Barford for helpful discussions and Drs. Joseph Brayden, Gary Mawe, and Mark Nelson for critical comments on the manuscript.

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J. Biol. Chem. 2002, 277:34030-34035.
doi: 10.1074/jbc.M205945200 originally published online July 11, 2002

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