Regulation of F-actin and Endoplasmic Reticulum Organization by the Trimeric G-protein G\textsubscript{i2} in Rat Hepatocytes

IMPLICATION FOR THE ACTIVATION OF STORE-OPERATED Ca\textsuperscript{2+} INFLOW

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The roles of the heterotrimeric G-protein, G\textsubscript{i2}, in regulating the actin cytoskeleton and the activation of store-operated Ca\textsuperscript{2+} channels in rat hepatocytes were investigated. G\textsubscript{i2} was principally associated with the plasma membrane and microsomes. Both F-actin and G\textsubscript{i2} were detected by Western blot analysis in a purified plasma membrane preparation, the supernatant and pellet obtained by treating the plasma membrane with Triton X-100, and after depolymerization and repolymerization of F-actin in the Triton X-100-insoluble pellet. Actin in the Triton X-100-soluble supernatant co-precipitated with G\textsubscript{i2} using either anti-G\textsubscript{i2} or anti-actin antibodies. The principally cortical location of F-actin in hepatocytes cultured for 0.5 h changed to a pericanalicular distribution over a further 3.5 h. Some G\textsubscript{i2} co-localized with F-actin in the plasma membrane. Pretreatment with pertussis toxin ADP-ribosylated 70–80% of G\textsubscript{i2} in the plasma membrane and microsomes, prevented the redistribution of F-actin, caused redistribution and fragmentation of the endoplasmic reticulum, and inhibited vasopressin-stimulated Ca\textsuperscript{2+} influx. It is concluded that (i) a significant portion of hepatocyte G\textsubscript{i2} associates with and regulates the arrangement of, cortical F-actin and the endoplasmic reticulum and (ii) either or both of these regulatory roles are likely to be required for normal vasopressin activation of Ca\textsuperscript{2+} influx.

In most nonexcitable and in some excitable cells, depletion of the inositol 1,4,5-trisphosphate (InsP\textsubscript{3})-sensitive intracellular Ca\textsuperscript{2+} stores in the endoplasmic reticulum (ER) activates a Ca\textsuperscript{2+} influx pathway, a process known as store-operated Ca\textsuperscript{2+} influx or capacitative Ca\textsuperscript{2+} entry (1). Although it has been widely accepted that the key event initiating the opening of store-operated Ca\textsuperscript{2+} channels (SOCs) in the plasma membrane is the decrease in the concentration of Ca\textsuperscript{2+} in the lumen of the ER, neither the mechanism that couples these two events nor the structures of SOCs are well understood (2). The results of recent experiments indicate that an essential prerequisite for the activation of SOCs is the close association between regions of the ER and the plasma membrane (3). It is proposed that this association is maintained by cytoskeletal elements such as the F-actin (4). There is evidence that, in some cell types, disassociating of the F-actin cytoskeleton (5), stabilization of the F-actin cytoskeleton (6), or inhibition of myosin light chain kinase (7) blocks Ca\textsuperscript{2+} influx via SOCs while leaving Ca\textsuperscript{2+} release from the intracellular stores unaffected (but see Ref. 8).

Hepatocytes are polarized epithelial cells in which the F-actin cytoskeleton is distributed around the cortex, with a high concentration at the pericanalicular (apical) region (9). This cortical F-actin may play a role in maintaining subregions of the ER close to the plasma membrane (4). Evidence, including results obtained with a microinjected inhibitory anti-G\textsubscript{i2} antibody, indicates that the activation of SOCs in hepatocytes requires the trimeric G-protein G\textsubscript{a} (10) and a brefeldin A-sensitive protein, possibly a monomeric G-protein (11). It has been reported that some G\textsubscript{a} co-localizes with F-actin in hepatocytes in primary culture (12). Moreover, studies with other cell types have provided evidence for an association between G\textsubscript{a} and F-actin (13–15), and have suggested a potential role for G\textsubscript{a} in organization of the F-actin cytoskeleton (16–18). On the basis of these observations, we proposed that G\textsubscript{a} may regulate arrangement of the actin cytoskeleton and the arrangement of the ER by which both the intimate plasma membrane-ER association is achieved and the communication between different parts of the ER is maintained and allows the activation of SOCs.

The aims of the present experiments were to elucidate the role of G\textsubscript{a} in the activation of SOCs in hepatocytes by investigating the intracellular distribution of G\textsubscript{a} and F-actin, the association of Ga\textsubscript{a} with F-actin, and the requirement for Ga\textsubscript{a} in regulation of the arrangement of F-actin and in the activation of SOCs. The results indicate that a significant proportion of the cellular Ga\textsubscript{a} is associated with F-actin and regulates F-actin organization (especially the cortical actin layer near the canalicular membrane) and the arrangement of the ER. To our knowledge, this is the first demonstration of the role of Ga\textsubscript{a} in regulating the arrangement of F-actin in an epithelial cell type. Taken together with previous evidence that the normal function of Ga\textsubscript{a} is required for the activation of SOCs in rat hepatocytes (10), these observations suggest that Ga\textsubscript{a} either through regulation of cortical F-actin organization and/or arrangement of the ER, allows the normal activation of SOCs.

EXPERIMENTAL PROCEDURES

Materials—Affinity-purified rabbit polyclonal anti-G\textsubscript{a} antibody, raised against the C-terminal decapeptide (KENLKDCCGLF) of the \(\alpha\)-subunit of transducin, was kindly provided by Dr. Michael Crouch.

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The abbreviations used are: InsP\textsubscript{3}, inositol 1,4,5-trisphosphate; SOC, store-operated Ca\textsuperscript{2+} channel; ER, endoplasmic reticulum; DIOC\textsubscript{6}(3), 3,3′-dihexyloxacarbocyanine iodide; PAGE, polyacrylamide gel electrophoresis; F-actin, filamentous actin; G-actin, globular actin.
G_{i2} and F-actin Interaction in Hepatocytes

(John Curtin School of Medical Research, Australian National University, Canberra, Australia). Although this antibody detects both G_{i1} and G_{i2}, liver does not express detectable G_{i1} (19, 20), so that the G-protein detected by this antibody in the present experiments is G_{i2}. Peptides KENLIDCGLF and QLNLKEYNL, synthesized as described (Ref. 10), were provided by Dr. Kenneth F. Karas (Pertussis Institute of Medical Research, Victoria, Australia). Purified phosphoprotein phosphatases 1 and 2A were kind gifts from Dr. Alistair Sim (University of Newcastle, Australia). Pertussis toxin, affinity-purified rabbit polyclonal anti-actin antibody, goat anti-rabbit IgG conjugated to alkaline phosphatase, actin standard for Western blotting, protein A-Sepharose, Triton X-100, nitro blue tetrazolium, and bromochloroindolyl phosphate were from Sigma, and Texas Red-X phallolidin, 3,3'-dihexyloxacarbocyanine iodide (DiOC(6)(3)), fura-2, and goat anti-rabbit IgG conjugated to Alexa(TM) 488 were from Molecular Probes, Inc. (Eugene, OR). Recombinant G_{i2} protein was from Calbiochem (Alexandria, Australia). All other chemicals and materials were of the highest grade commercially available.

Western Blot Analysis of G_{i2} and Actin—SDS-PAGE was performed on 12% polyacrylamide resolving gels with the Laemmli discontinuous buffer system (21), and the resolved proteins were electrotransferred to nitrocellulose membranes by the method of Towbin et al. (22). Membranes were blocked with 1× glycine containing 5% (w/v) nonfat milk powder, 5% (v/v) fetal calf serum, and 1% (w/v) ovalbumin for 1 h at room temperature, then washed three times (5 min each) in PBS containing 0.1% (v/v) Tween 20, 0.1% (w/v) nonfat milk powder, and 0.1% (v/v) ovalbumin dissolved in 137 mM NaCl, 2.7 mM KCl, 8 mM Na_{2}HPO_{4}, and 1.4 mM KH_{2}PO_{4} (pH 7.2). Membranes were incubated overnight at 4 °C with either anti-G_{i2} antibody (1:200 dilution in the above wash buffer) or anti-actin antibody (1:100 dilution) or, in some cases, both antibodies together followed by incubation with secondary antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase, 1:1000 dilution) for 2 h at room temperature and finally developed for 5 min in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM MgCl_{2} containing 0.33 mg/ml nitro blue tetrazolium and 0.16 mg/ml bromochloroindolyl phosphate. Quantitation of the bands was performed on a Bio-rad model GS-700 imaging densitometer driven by the Molecular Analyst software package (Bio-Rad). SDS-PAGE in the presence of 6M urea was conducted as described by Komatsu et al. (23).

Subcellular Fractionation and Marker Enzyme Assays—Rat livers were homogenized in a medium containing 250 mM sucrose, 5 mM HEPES/KOH (pH 7.4), and 1 mM EGTA (homogenization medium), supplemented with 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml pepstatin A (protease inhibitors) and subcellular fractions were prepared as described (5) for 5 min, then washed three times (5 min each) in PBS containing 0.1% (v/v) Tween 20 and 1% (w/v) bovine serum albumin (Twee dilution) and incubated overnight at 4 °C with anti-G_{i2} antibody (5 µg/ml in Tween solution). Thereafter, cells were washed six times with the Tween solution, incubated with secondary antibody (Alexa(TM) 488-conjugated goat anti-rabbit IgG, 1:100 dilution in Tween solution), and washed twice with Tween solution and four times with phosphate-buffered saline before the coverslips were mounted on slides in 50% glycerol in phosphate-buffered saline.

Confocal microscopy was performed using a Bio-Rad MRC-1000 laser-scanning confocal microscope system in combination with a Nikon Diaphot 300 inverted microscope and a × 40 NA 1.15 water immersion objective lens. The excitation and emission wavelengths were set at 520/10 and 605/35 nm, respectively, for Texas Red-X, and at 488/10 and 522/32 nm, respectively, for DiOC(6)(3) and Alexa(TM) 488. To standardize the fluorescence intensity measurements among experiments, the time of image capturing, the image intensity gain, the image enhancement, and the fluorescence intensity measurements among experiments, the time of image capturing, the image intensity gain, the image enhancement was maintained between the experiments. The activities of the marker enzymes 5'-nucleotidase (plasma membrane) and glucose-6-phosphatase (ER) were determined as described previously (31). Negative controls for ER and F-actin staining were carried out systematically by omitting DiOC(6)(3) and Texas Red-X phalloidin, respectively. Determination of the location of G_{i2} by immunofluorescence was performed as described previously (10). Controls were performed by omitting either the primary antibody or the secondary antibody or both and by incubating the primary antibody with excess blocking peptide before use.

For double labeling of F-actin and G_{i2} in the same cell, F-actin staining was first performed as described above. The cells were then washed with phosphate-buffered saline containing 0.05% (v/v) Tween 20 and 1% (w/v) bovine serum albumin (Twee solution) and incubated overnight at 4 °C with anti-G_{i2} antibody (5 µg/ml in Tween solution). Thereafter, cells were washed six times with the Tween solution, incubated with secondary antibody (Alexa(TM) 488-conjugated goat anti-rabbit IgG, 1:100 dilution in Tween solution), and washed twice with Tween solution and four times with phosphate-buffered saline before the coverslips were mounted on slides in 50% glycerol in phosphate-buffered saline.

Quantitative examination of the captured images was performed using CoMOS (Bio-Rad) image analysis software. To quantitate F-actin distribution, for each experimental condition, 60 hepatocyte doublets were randomly selected from the images obtained from three separate cell preparations (20 doublets from each preparation), and the fluorescence (pixels) in the total doublet and in the pericanalicular area was measured. The fluorescence in the pericanalicular area was expressed as a percentage of the total doublet fluorescence. This percentage indicates the relative amount of F-actin in the bile canaliculus and hence the degree of reorganization of F-actin during primary culture (cf. Ref. 32). To avoid the subjectivity of this measurement, it was verified that the elliptical area designated as "pericanalicular area" occupied 9.95 ± 0.06% (mean ± S.E., n = 60) of the total area of control doublets and 9.92 ± 0.06% (mean ± S.E., n = 60) of the total area of pertussis toxin-treated doublets, respectively.

Electron Microscopy—Pelelts (3000 × g for 2 min) of the plasma membrane fraction (~1 mg) were fixed in 1% (w/v) glutaraldehyde in 25 mM HEPES buffer (pH 7.4) for 30 min on ice. After washing the sample with 25 mM HEPES buffer, the samples were postfixed with 1% (w/v)OsO_{4} in the same HEPES buffer for 1 h. From freshly isolated intact hepatocytes (pellet centrifugation at 80 × g for 30 s) were fixed for 2 h at room temperature in a solution containing 1% (w/v)OsO_{4} and 0.1% Na_{2}HPO_{4}/NaH_{2}PO_{4} (pH 7.4). Fixed samples were dehydrated by stepwise exposure to increasing concentrations of ethanol (50, 75, 85, and 95%, and 100% (v/v)) and embedded in Durcupan with propylene oxide as an intermediate transition medium. The ultrathin sections were cut on
FIG. 1. Western blot analysis of Goα12 present in whole liver homogenate (A), plasma membrane and cytosolic fractions (B), and after treatment with phosphoprotein phosphatases (C). A, whole liver. Samples of rat liver homogenate were subjected to SDS-PAGE (15 μg of protein/lane) and Western blot analysis using anti-Goα12 antibody (lane 1), anti-Gi2 antibody mixed with immunizing peptide KENLKDCEGLF (100 μg/ml) (lane 2), or anti-Gi2 antibody mixed with an unrelated decapetide QLNLKEYNLV (100 μg/ml) (lane 3). The results shown are from one of three experiments that gave similar results. B, plasma membrane and cytosolic fractions. Lane 1, plasma membrane fraction (15 μg of protein); lane 2, plasma membrane fraction mixed with recombinant Goα12 (2 μl); lane 3, cytosolic fraction (15 μg of protein); lane 4, cytosolic fraction mixed with recombinant Goα12 (2 μl). Samples were subjected to SDS-PAGE and Western blotting using anti-Gi2 antibody. The results shown are from one of three experiments that gave similar results. C, the effect of treatment of the liver cytosolic fraction with phosphoprotein phosphatases on the mobility of the 43-kDa Goα12 band. The cytosolic extract was treated with phosphoprotein phosphatase 1 or 2A as described under "Experimental Procedures." Samples were subjected to SDS-PAGE and Western blot analysis using anti-Gi2 antibody as the probe. Lane 1, cytosolic extract; lane 2, cytosolic extract treated with phosphoprotein phosphatase 1; lane 3, cytosolic extract treated with phosphoprotein phosphatase 2A; lane 4, cytosolic extract plus recombinant Gi2; lane 5, cytosolic extract treated with phosphoprotein phosphatase 1 plus recombinant Goα12; lane 6, cytosolic extract treated with phosphoprotein phosphatase 2A plus recombinant Goα12. The results shown are from one of two experiments that gave similar results.

an ultramicrotome, stained with aqueous uranyl acetate and Reynolds’s lead citrate, and examined with a JEOI 1200 EX transmission electron microscope.

Measurement of Ca2+ Inflow—Cytoplasmic free Ca2+ concentrations ([Ca2+]cyt) and initial rates of Ca2+ inflow (measured using a Ca2+-add-back protocol) in rat hepatocytes loaded with fura-2 by microinjection were determined using fluorescence microscopy (31).

RESULTS

Nature and Distribution of Goα12 in Rat Liver Subcellular Fractions—When rat liver homogenates were subjected to Western blot analysis, two forms of Goα12, with apparent molecular masses of 41 and 43 kDa, were detected (Fig. 1A). The plasma membrane fraction contained predominantly the 41-kDa band, which co-migrated with recombinant Goα12 (Fig. 1B, lanes 1 and 2), while the cytosolic fraction contained predominantly the 43-kDa band (Fig. 1B, lanes 3 and 4). Treatment of the dissociation of loosely bound proteins from liver microsomal membranes (34). Phosphorylated Goα12 was principally found in the cytosolic fraction with phosphoprotein phosphatase 1 converted the 43-kDa form of Goα12 to a form that co-migrates with recombinant Goα12 (Fig. 1C, lanes 1, 2, and 5). By contrast, treatment with phosphoprotein phosphatase 2A did not alter the mobility of the 43-kDa band (Fig. 1C, lanes 1, 3, and 6). These results indicate that (i) the 41-kDa form of Goα12 (subsequently referred to as Goα12) corresponds to the form of Goα12 (nonphosphorylated) normally detected in most cell types and (ii) the species of Goα12 with an apparent molecular mass of 43 kDa (subsequently referred to as phosphorylated Goα12) is a phosphorylated form of Goα12 (cf. Ref. 33).

Goα12 was found in the plasma membrane, the nuclear-plasma membrane, and the heavy and light microsomal fractions of the liver (Fig. 2A) but was barely detectable in the cytosolic fraction. The amount of Goα12 associated with the microsomes was estimated to be 40% of total cellular Goα12 (41-kDa) was the predominant form of Goα12 found in the plasma membrane and the nuclear plasma membrane fractions. Phosphorylated Goα12 was principally found in the cytosolic fraction, but some was also associated with the heavy and light microsomes (Fig. 2A). In order to determine how tightly Goα12 is associated with the microsomal membranes, the microsomes were treated with KCl, which has been shown to cause the dissociation of loosely bound proteins from liver microsomal membranes (34). Phosphorylated Goα12, but not the non-phosphorylated form, could be removed from microsomes by treat-
Evidence for the Association of \( G_{i2} \) and F-actin in a Purified Rat Liver Plasma Membrane Fraction—It has been previously shown that a purified liver plasma membrane fraction (prepared in a manner similar to that described above) contains F-actin, which is attached to the plasma membrane (35). Experiments were undertaken to determine whether \( G_{i2} \) is associated with this plasma membrane-attached actin. First, the quality of the plasma membrane fraction was further assessed by electron microscopy (Fig. 4). This showed numerous

extended sheets of membrane (large arrow), the presence of small vesicles adherent to some sheets (small arrows), and numerous other vesicles of varying size. The preparation was largely free of mitochondria and nuclei.

The plasma membrane fraction was treated with 1% (w/v) Triton X-100 to solubilize membrane lipids and integral proteins and thereby to obtain, by centrifugation, a plasma membrane Triton X-100-insoluble pellet enriched in F-actin and other cytoskeletal components (15). \( G_{i2} \) and actin were detected by Western blotting in both the Triton X-100-insoluble pellet (predominantly F-actin) and the Triton X-100-soluble supernatant (predominantly G-actin) (Fig. 5). It was estimated from densitometric analysis that approximately 27 ± 3% (mean ± S.E., \( n = 4 \)) of the total plasma membrane \( G_{i2} \) and approximately 45 ± 1% (mean ± S.E., \( n = 3 \)) of the total plasma membrane actin were recovered in the plasma membrane Triton X-100-insoluble pellet.

To further test that \( G_{i2} \) associates specifically with F-actin among the various cytoskeletal components of the plasma membrane, a repolymerized F-actin fraction was prepared from the plasma membrane Triton X-100-insoluble pellet by a two-step depolymerization-polymerization procedure (15). Analysis by SDS-PAGE and Western blotting with anti-\( G_{i2} \) and anti-actin antibodies demonstrated the presence of \( G_{i2} \) in the repolymerized F-actin fraction (Fig. 5, lane 4). Approximately 44 ± 0% of the \( G_{i2} \) and 47 ± 2% of the actin in the plasma membrane Triton X-100-insoluble pellet were recovered in the final repolymerized F-actin fraction. This corresponds to 12 ± 0 and 21 ± 1% (means ± S.E., \( n = 3 \)) of the total plasma membrane \( G_{i2} \) and actin, respectively.

The idea that \( G_{i2} \) and actin associate near the plasma membrane was also investigated using a co-immunoprecipitation approach. When an anti-\( G_{i2} \) antibody was used to precipitate \( G_{i2} \) from the Triton X-100-soluble supernatant of the purified plasma membrane fraction, the precipitate was found to contain actin, identified using an anti-actin antibody and Western blot analysis (Fig. 6A). When an anti-actin antibody was used to precipitate actin from the Triton X-100-soluble supernatant of the purified plasma membrane fraction, the precipitate was found to contain \( G_{i2} \), identified using an anti-\( G_{i2} \) antibody and Western blot analysis (Fig. 6B). When a similar co-immunoprecipitation experiment was performed with a liver cytosolic fraction (which is enriched in phosphorylated \( G_{i2} \)), no co-immunoprecipitation of phosphorylated \( G_{i2} \) and actin was observed (data not shown).

Distribution of F-actin and \( G_{i2} \) in Hepatocytes in Primary
Culture—The intracellular distribution of Go\textsubscript{12} and F-actin and the interaction between these proteins was further investigated using hepatocytes attached to collagen-coated coverslips, and Texas Red-X phalloidin and immunofluorescence to detect F-actin and Go\textsubscript{12}, respectively. In freshly isolated rat hepatocytes allowed to attach to coverslips for 0.5 h, F-actin was observed around the cortex, in both single hepatocytes and in hepatocyte doublets (Fig. 7A). When cultured for a further 3.5 h, the amount of F-actin in single cells and in doublets decreased in most regions of the cortex. In single cells, areas of high F-actin remained in some small regions of the cortex. In doublets, a pronounced concentration of F-actin at the canalicular membranes was observed (Fig. 7C). This most likely corresponds to the re-establishment of F-actin polarity and cell polarity, as described previously (32, 36). Hepatocytes cultured for 4 h appeared to be more flattened and to have a larger diameter compared with cells cultured for 0.5 h (Fig. 7, compare C with A).

Substantial amounts of Go\textsubscript{12} (presumably both phosphorylated and nonphosphorylated forms) were found in the cytoplasmic space as well as at the plasma membrane of most hepatocytes examined, as shown previously (10, 12) (Fig. 7, E and G). In order to investigate the possible co-localization of Go\textsubscript{12} and F-actin, hepatocytes were double stained with Texas Red-X phalloidin and anti-G\textsubscript{0} antibody (Fig. 8, A–C). The results indicate that there are regions of the cortex where the fluorescence signals representing Go\textsubscript{12} and F-actin overlap (indicated by the orange-yellow regions in Fig. 8C).

Effects of the Ablation of Go\textsubscript{12} Function by Pretreatment with Pertussis Toxin on the Intracellular Distribution of F-actin, Go\textsubscript{12}, and the Endoplasmic Reticulum and the Activation of Ca\textsuperscript{2+} Inflow—In order to further elucidate the role of Go\textsubscript{12} in regulation of the arrangement of the actin cytoskeleton and to study the roles of Go\textsubscript{12} and F-actin in the activation of SOCs, the treatment of rats with pertussis toxin was used to ablate Go\textsubscript{12} function. The effectiveness of pertussis toxin treatment was assessed by determining the degree of ADP-ribosylation of Go\textsubscript{12}, using SDS-PAGE in the presence of 6 M urea to identify ADP-ribosylated Go\textsubscript{12}(23). Pertussis toxin treatment caused ADP-ribosylation of Go\textsubscript{12}, as shown by the appearance of a new band in the urea/SDS-PAGE gel with a slower mobility than that of Go\textsubscript{12} (Fig. 9). Treatment with pertussis toxin did not result in any change in the mobility of the phosphorylated (43-kDa) Go\textsubscript{12} band (results not shown). The slower band (ADP-ribosylated Go\textsubscript{12}) was observed in the plasma membrane fraction (Fig. 9A, lower panel, lane 2), the plasma membrane Triton X-100-insoluble pellet (lane 4), the plasma membrane Triton X-100-soluble supernatant (lane 6), and the heavy and light microsomal fractions (Fig. 9B). Quantitation of the bands using densitometry showed that pertussis toxin treatment resulted in ADP-ribosylation of 60, 80, and 50% of Go\textsubscript{12} in the total plasma membrane fraction, the plasma membrane Triton X-100-insoluble pellet, and the plasma membrane Triton X-100-soluble supernatant, respectively, and approximately 70% of Go\textsubscript{12} associated with the heavy plus the light microsomes.

Pertussis toxin pretreatment caused no detectable changes in the total amount of actin in the plasma membrane fraction (Fig. 9A, upper panel, compare lane 2 with lane 1). Further, since the Triton X-100-insoluble pellet contains predominantly F-actin and the Triton X-100-soluble supernatant contains mainly G-actin (6, 15), the results also indicated that pertussis toxin treatment did not change the relative distribution of the two forms of actin in the plasma membrane fraction (Fig. 9A, upper panel, compare lane 4 with lane 3 for F-actin; compare lane 6 with lane 5 for G-actin).

Cells from rats treated with pertussis toxin (pertussis toxin-treated cells) that had been cultured for 0.5 h exhibited no substantial differences in the intracellular distribution of F-actin compared with cells from vehicle-treated rats (control cells) cultured for this time (Fig. 7, compare B and A). However, the treatment with pertussis toxin prevented the redistribution of F-actin from the cortex to the bile canaliculus and other parts of the cell observed in control cells cultured for 4 h (Fig. 7, compare D and C). To quantitatively compare the differences in the distribution of F-actin in 4-h cultured doublets from control and pertussis toxin-treated rats, the pericanalicular fluorescence due to the F-actin-Texas Red-X phalloidin complex was expressed as a percentage of the total doublet fluorescence. This value was 18.87 ± 0.70% (mean ± S.E., n = 60) in control doublets compared with 11.27 ± 0.26% (mean ± S.E., n = 60) in pertussis toxin-treated doublets (p < 0.001, heteroscedastic t test). Pertussis toxin treatment also inhibited the spreading of cells observed at 4 h (Fig. 7, compare D and C). Thus, the total doublet area was 1153 ± 49 μm\textsuperscript{2} (mean ± S.E., n = 60) in control doublets compared with 936 ± 25 μm\textsuperscript{2} (mean ± S.E., n = 60) in pertussis toxin-treated doublets (p < 0.001, heteroscedastic t test).

Pertussis toxin-treated hepatocytes cultured for both 0.5 and 4 h exhibited noticeable differences in the distribution of Go\textsubscript{12} (Fig. 7, compare F and E; compare H and G). In contrast to control cells, where considerable Go\textsubscript{12} was present in the cytoplasmic space as well as at the plasma membrane, in pertussis toxin-treated cells, Go\textsubscript{12} was principally located at the plasma membrane and in the cortical region (Fig. 7, compare F and H with E and G).

Pertussis toxin-treated hepatocytes exhibited more intense staining of the ER, monitored using DiOC\textsubscript{3}(3), than that observed in control cells (Figs. 10, compare B and D with A and C). Moreover, the DiOC\textsubscript{3}(3) signal was more evenly distributed in pertussis toxin-treated cells. These differences were observed in cells cultured for both 0.5 and 4 h. Examination of the cells by electron microscopy revealed that pertussis toxin-treated hepatocytes had largely lost the regular parallel arrangement of sheets of rough ER that were observed in control hepatocytes (Fig. 11, compare B and A). These differences can be seen more clearly at higher magnification (Fig. 11, compare...
**DISCUSSION**

Role of G\textsubscript{12} in Regulating the Organization of F-actin and the Endoplasmic Reticulum—In keeping with the observations of others (37), a 43-kDa phosphorylated form of G\textsubscript{12} as well as the nonphosphorylated 41-kDa form were detected in hepatocytes. The present study has focused on G\textsubscript{12} (the 41-kDa form), which is bound to the plasma membrane and ER (microsomes), rather than on the phosphorylated 43-kDa G\textsubscript{12}, for the following reasons: (i) the phosphorylated G\textsubscript{12} is hardly detectable in the plasma membrane fraction and is only loosely associated with the microsomes, (ii) there was no evidence from co-immunoprecipitation studies of an association between actin and phosphorylated G\textsubscript{12}, and (iii) there was no evidence that the phosphorylated G\textsubscript{12} was ADP-ribosylated by pertussis toxin treatment.

The following observations indicate that G\textsubscript{12} (the 41-kDa form) associates with actin at the periphery of the hepatocyte: (i) the detection of both G\textsubscript{12} and F-actin in a Triton X-100-insoluble pellet prepared from a highly purified liver plasma membrane fraction; (ii) the detection of G\textsubscript{12} in re-polymerized actin obtained after F-actin in the plasma membrane Triton X-100-insoluble fraction was de-polymerized and re-polymerized; (iii) co-precipitation of G\textsubscript{12} and actin from the plasma membrane Triton X-100-soluble fraction using either an anti-G\textsubscript{12} antibody or an anti-actin antibody; and (iv) the observed co-localization of some G\textsubscript{12} and F-actin at the cell periphery.

The results of experiments that employed pertussis toxin to ablate the action of G\textsubscript{12} indicate that this trimeric G-protein is...
involved in regulating the organization of cortical F-actin in hepatocytes. Pertussis toxin specifically ADP-ribosylates and inactivates the α subunit of Gi1, Gi2, Gi3, Go, and transducin (38). Since neither transducin, Go, nor Gi1 is expressed at detectable levels in hepatocytes (19, 20), Gi2 and Gi3 are the only two known targets for pertussis toxin in these cells. Moreover, there is evidence that the time course for ADP-ribosylation of Gi1 by pertussis toxin treatment in vitro (72 h) is longer than that for Gi2 (24–48 h) (23). Therefore, the in vivo pertussis toxin treatment employed in this study (24 h) is likely to result chiefly in inactivation of Gi2. Moreover, urea/SDS-PAGE and Western blotting confirmed that the majority of the Gi2 on the plasma membrane, in particular the Gi2α associated with F-actin, was ADP-ribosylated and hence inactivated. It is clear from our results that this pertussis toxin treatment inhibited the redistribution of F-actin from the cortex to the bile canaliculus in hepatocyte doublets and the redistribution of F-actin to specific regions of the plasma membrane in single hepatocytes. Normally, cell polarity, which is lost during isolation of hepatocytes, can be restored within 3–4 h in monolayer culture (36). This re-establishment of cell polarity has been shown to be part of the machinery that governs the maintenance of a polarized distribution of F-actin in hepatocytes (37). The observations that pertussis toxin pretreatment prevented the spreading of hepatocytes in primary culture provides further evidence that Gi2 regulates F-actin organization, since it has been shown
that hepatocyte spreading in culture requires F-actin organization (39).

Studies with several other types of cells have also shown that Gi2 interacts with F-actin (13–15) and is likely to play a role in regulating the organization of F-actin (16–18). For example, the degree of actin polymerization in differentiating U937 cells was found to correlate well with an increase in the amount of Gaα2 at the plasma membrane (16). In human airway smooth muscle cells, it has been shown that Gaα2 is required for carbachol-induced stress fiber formation (18). In experiments employing pertussis toxin, evidence has also been obtained that the dysfunction of Goα causes a 40–50% decrease in the cortical F-actin content in chromaffin cells (40) and diminishes fMet-Leu-Phe-induced actin polymerization in neutrophils (41). Furthermore, evidence for a link between the activity of Goα, the basal concentration of intracellular cyclic AMP, and the assembly of stress fibers in primary human granulosa-lutein cells has recently been reported (42). These observations, together with our present results with hepatocytes, suggest that trimeric G-proteins such as Gi2 are involved in regulating the organization of the actin cytoskeleton in a variety of cell types.

Pertussis toxin treatment also caused fragmentation and redistribution of the ER, detected using DiOC6(3) and fluorescence microscopy and by electron microscopy. Furthermore, 40% of the total cellular Gaα2 was found to be associated with microsomes, and approximately 70% of microsome-associated Gaα2 was ADP-ribosylated by pertussis toxin treatment. These results indicate that Gi2 is likely to be directly or indirectly involved in regulating the structure and intracellular distribution of the ER in hepatocytes. Moreover, considering the evidence of Hajnoczy et al. (43) that the luminal communication between intracellular Ca2+ stores is cooperatively modulated by GTP and the cytoskeleton, an intriguing possibility is that Gi2 is involved in maintaining the luminal continuity of the ER in hepatocytes, either via the actin cytoskeleton or by interaction with other proteins.

Pertussis toxin treatment caused a noticeable redistribution of Gaα2 immunofluorescence from the cytoplasmic space to the cell periphery. This observation may reflect the redistribution of some Gaα2 from the cytoplasmic space to the cell periphery. However, others have shown, using Western blotting, that compared with native Gaα2, ADP-ribosylated Gaα2 has a higher affinity for the anti-Gaα antibody employed in the present studies (44). Therefore, some of the substantial increase in Gaα2 immunofluorescence at the cortex of pertussis toxin-treated hepatocytes may be due to an enhanced affinity of the anti-Gaα antibody for ADP-ribosylated Gaα2 (compared with native Gaα2).

Role of Actin and Gi2 in Activation of Ca2+ Inflow—Pertussis toxin treatment caused a substantial inhibition of vasopressin-induced Ca2+ inflow with little effect on vasopressin-induced release of Ca2+ from intracellular stores (present and previous (30) results). Previous studies have shown that pertussis toxin treatment completely inhibits thapsigargin-induced Ca2+ inflow without a substantial effect on thapsigargin-induced release of Ca2+ from the ER (45) and have shown that the effects of pertussis toxin can be mimicked by the microinjection of an anti-Gaα antibody or peptide corresponding to the carboxyl region of Gaα2, which inhibits Gaα function (10). These results provided substantial evidence to indicate that Gi2 (rather than Gaα2, which is also present in rat hepatocytes and can be ADP-ribosylated by pertussis toxin (20, 38)) is necessary for the activation of SOCs in rat hepatocytes (10). Moreover, the previous experiments also indicate that the ablation of Gaα2 action by pertussis toxin does not substantially affect the formation of InsP3 receptors with InsP3 receptors, the ability of InsP3 receptors to release Ca2+ from most regions of the ER, or the interaction of thapsigargin with the ER (Ca2++ Mg2+)-ATPase and the inhibition of this Ca2+ pump. (The possibility that ablation of Goα2 affects the release of Ca2+ from a small region of the ER near the plasma membrane that is central to the activation of SOCs but was not detected as a reduction in vasopressin-induced release of Ca2+ from intracellular stores cannot be excluded.)

The present results show that two of the functions of Gi2 in hepatocytes are to regulate F-actin assembly at the cortex and arrangement of the ER. It is possible that one or both of these functions is essential for the activation of SOCs. Thus, as suggested by others, the activation of SOCs may require maintenance of a region of the ER near the plasma membrane (e.g. “docking” of regions of the ER with the plasma membrane and/or the fusion of vesicles containing SOC proteins with the plasma membrane (6, 46)). In this respect, it is interesting to note that the effects of Goα2 ablation (pertussis toxin treatment) in stabilizing F-actin at the hepatocyte cortex and inhibiting SOC activation are similar to results recently reported by Patterson et al. (6). These authors showed that, in a smooth muscle cell line, the stabilization of F-actin by different procedures (treatment with jasplakinolide or calcineurin A, which induced the formation of a dense ring of F-actin around the cell periphery) also inhibited SOC activation (6).

A requirement for Gi2 in the activation of SOCs has not been reported in studies of most other mammalian cells (47). This suggests that the requirement for Gi2 in SOC activation in hepatocytes (10) reflects one or more aspects of the specific structure and function of these cells, such as maintenance (via Gi2 regulation of the actin cytoskeleton or interaction of Goα2 with another ER-associated protein) of cell polarity and/or a specific distribution of the ER throughout the cell, which is critical for the activation of SOCs. This may be due to a requirement for Goα2 in the regulation of F-actin organization that is more accentuated in hepatocytes than in other cell types. Another possibility is that, in the hepatocyte, the InsP3 receptors principally involved in inducing a decrease in Ca2+ in the lumen of the ER are located some distance from the SOCs so that normal intraluminal communication through the ER is required for SOC activation (cf. Ref. 48).

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