Evidence That the Pertussis Toxin-sensitive Trimeric GTP-binding Protein G_{i2} Is Required for Agonist- and Store-activated Ca^{2+} Inflow in Hepatocytes

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The role of a trimeric GTP-binding protein (G-protein) in the mechanism of vasopressin-dependent Ca^{2+} influx in hepatocytes was investigated using both antibodies against the carboxyl termini of trimeric G-protein α subunits, and carboxyl-terminal α-subunit synthetic peptides. An anti-G_{i1-2α} antibody and a G_{i2α} peptide (Ile345–Phe355), but not a G_{i3α} peptide (G_{i3α}, Ile344–Phe354), inhibited vasopressin- and thapsigargin-stimulated Ca^{2+} influx, had no effect on vasopressin-stimulated release of Ca^{2+} from intracellular stores, and caused partial inhibition of thapsigargin-stimulated release of Ca^{2+}. An anti-G_{qα} antibody also inhibited vasopressin-stimulated Ca^{2+} influx and partially inhibited vasopressin-induced release of Ca^{2+} from intracellular stores. Immunofluorescence measurements showed that G_{i2α} was distributed throughout much of the interior of the hepatocyte as well as at the periphery of the cell. By contrast, G_{i3α} was found principally at the cell periphery. It is concluded that the trimeric G-protein, G_{i2}, is required for store-activated Ca^{2+} influx in hepatocytes and acts between the release of Ca^{2+} from the endoplasmic reticulum (presumably adjacent to the plasma membrane) and the receptor-activated Ca^{2+} channel protein(s) in the plasma membrane.

Receptor-activated calcium channels (RACCs) are present in most non-excitable and in some excitable animal cells and are responsible for allowing the influx of Ca^{2+} to specific regions of the cytoplasmic space and the refilling of intracellular Ca^{2+} stores (most likely a region of the endoplasmic reticulum) (1–3). For a number of cell types it has been shown that agonist-receptor complexes open at least two types of RACCs differing in selectivity for divalent cations (3). The mechanism(s) by which RACCs are opened is poorly understood (1–3). The hypothesis presently favored is the store-operated (capacitative) mechanism in which an increase in inositol 1,4,5-trisphosphate (InsP_3) and the release of Ca^{2+} from a region of the InsP_3-sensitive store are prerequisites for channel activation (1–3). This hypothesis is based, in part, on the observation that thapsigargin, which inhibits the endoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase causing the release of Ca^{2+} from this organelle, leads to a stimulation of Ca^{2+} influx (2). The results of a variety of experimental approaches have implicated the InsP_3 receptor (4), a mobile intracellular messenger (5–7), a monomeric G-protein (8, 9), a trimeric G-protein (10–14), protein phosphorylation (15), and/or elements of the cytoskeleton (16) in the mechanism that couples the release of Ca^{2+} from the endoplasmic reticulum to activation of the plasma membrane Ca^{2+} channels.

In hepatocytes, agonists that employ RACCs include vasopressin, adrenaline, angiotensin II, and epidermal growth factor (11–13). It has been shown previously that pretreatment of hepatocytes with pertussis toxin, or the microinjection of GDPβS, inhibits stores-operated, as well as vasopressin-stimulated, Ca^{2+} influx. These treatments did not affect the release Ca^{2+} from intracellular stores. This suggested that in addition to G_{i1α}, a slowly ADP-ribosylated pertussis toxin-sensitive trimeric G-protein is required for activation of the hepatocyte RACC(s) (11–13). Since the two pertussis toxin-sensitive trimeric G-proteins present in hepatocytes at detectable levels are G_{i2} and G_{i3} (17, 18), it was considered likely that one of these G-proteins is involved in activation of the hepatocyte RACC(s) (11, 12). The aim of the present experiments was to identify the trimeric G-proteins involved in store-operated Ca^{2+} influx in hepatocytes. The approach employed used antibodies generated against peptides corresponding to a region of the carboxyl termini of G_{i2} (19–21) and synthetic peptides corresponding to specific regions of the carboxyl termini of G_{i3} (22, 23). These antibodies and peptides have been shown by others to inhibit the activation of trimeric G-proteins (19–23). Thapsigargin-stimulated Ca^{2+} influx is inhibited by an anti-G_{i1-2α} antibody and by a G_{i2α} peptide, indicating that the trimeric G-protein G_{i2α} controls store-operated Ca^{2+} influx in hepatocytes. Immunofluorescence experiments indicate that the G_{i2α} polypeptide is located in the interior of the hepatocyte and is not restricted in intracellular location to the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials—A rabbit polyclonal anti-G_{i1-2α} antibody, which recognizes G_{i1α}, G_{i1α}, and G_{i2α}, was raised against the peptide KENLKD-
CGLF, a region of the carboxyl amino acid sequence for Gi3, and a rabbit polyclonal anti-Gi3, antibody, which recognizes Gi4 and Gi11, was raised against the peptide QLNLKEYNLV, a common region of the carboxyl amino acid sequence for Gi3 and Gi11. The antibodies were prepared using peptides linked to keyhole limpet hemocyanin, affinity-purified as described previously (24), and routinely stored at −70 °C in Tris-glycine buffer, pH 8.3. The abilities of the antibodies to recognize Gi2a, (anti-Gi2a,1) and Gi2a,12, respectively, in extracts of hepatocytes were confirmed by Western blot analysis. The anti-Gi2a,1 antibody detected a single band with an apparent molecular mass of 42 kDa and the anti-Gi2a,12 antibody a single band with an apparent molecular mass of 43 kDa. The peptides against which antibodies were raised, and the peptides IKNNLKDCGLF (Gi2a,1 Ile344–Phe354, peptide G i2a,12) and IKNNLKECGLY (Gi3a,1 Ile344–Phe354, peptide G i3a,1), corresponding to the carboxyl termini of Gi2a,1 and Gi3a,1, respectively, were synthesized (as free COOH) by the Merrifield solid-phase synthesis procedure using an Applied Biosystems 430A synthesizer and were analyzed by quantitative amino acid analysis and mass spectrometry, as described previously (25). Solutions of peptides Gi2a,1 and Gi3a,1 were prepared fresh each day by dissolving the peptide in a solution of 10 mM fura-2 in 125 mM KCl to give a concentration of 12 mM peptide in the microinjection tip (estimated intracellular concentration 160 μM).

Fluorescein isothiocyanate (FITC)-conjugated rabbit IgG was obtained from Sigma-Aldrich, Castle Hill, New South Wales, Australia; and donkey anti-rabbit IgG (Cy3) from Jackson, West Grove, PA. Other reagents were obtained from sources described previously (11).

Microinjection of Fluorescent Dyes, Antibodies, and Peptides to Hepatocytes and Measurement of Ca2+ Inflow—The isolation of hepatocytes, attachment of hepatocytes to coverslips coated with collagen, the microinjection of fura-2, antibodies, and peptides, and measurement of the fluorescence of single hepatocytes loaded with fura-2 were conducted as described previously (11). The dilution factor for the microinjection of reagents to hepatocytes, determined previously (12), was approximately 75-fold. Antibodies were concentrated in a buffer composed of 27 mM Tris-glycine buffer, pH 8.3, 54 mM Na2HPO4, and 26 mM KH2PO4 (adjusted to pH 7.3 by addition of KOH) (phosphate buffer) (26) using a Centricron-10 concentrator (Amicon, Beverly, MA) at 4,000 × g for 30 min, stored at 4 °C, and used within 1–8 days. The final concentration of antibody in the microinjection pipette tip ranged from 2.5 to 3.0 mg/ml (estimated intracellular concentration 30–40 μg/ml). Antibodies were coinjected with fura-2 (10 μM in the pipette tip, estimated intracellular concentration 130 μM) at the concentrations indicated in the figure legends. In control experiments, the Gi2a,1 antibody (2.2–3.0 mg/ml in the pipette tip) was mixed with blocking peptide (KMNKLDCGLF, 1.7 mg/ml in the pipette tip) and coinjected with fura-2. Peptides Gi2a,1 (Ile344–Phe354) and Gi3a,1 (Ile344–Phe354) were dissolved in phosphate buffer, mixed with fura-2 to give 12 mM peptide and 10 mM fura-2 in the micropipette tip, and introduced to the cytoplasmic space of hepatocytes by microinjection. The fluorescence of FITC-conjugated rabbit IgG injected into hepatocytes was measured using excitation and emission wavelengths of 490 and 540 nm, respectively. Immunofluorescence—Freshly isolated hepatocytes (106 cells/50-mm plastic Petri dish) were cultured for 24 h in William’s Medium E, fixed with 4% (v/v) paraformaldehyde, and permeabilized using Triton X-100 as described by Liewenont et al. (27). After washing twice with 0.05% (v/v) Tween in PBS (Tweens-PBS), the cells were incubated overnight with a given affinity-purified rabbit anti-Gi, antibody (50 μg/ml) or with a given anti-Gi, antibody which had been mixed with a molar excess of appropriate blocking peptide. The cells were washed five to six times with Tweens-PBS to remove primary antibody and then incubated with donkey anti-rabbit IgG antibody conjugated to indocarbocyanine (Cy3) (1 in 200). Immunofluorescence was detected using a Nikon Eclipse E2000 microscope, a × 60 or × 100 oil immersion objective lens and a Bio-Rad MRC 1000 scanning confocal imaging system incorporating a krypton-argon laser.

RESULTS

Hepatocytes loaded with anti-Gi1,2a antibody exhibited a substantial inhibition of vasopressin-stimulated Ca2+ influx compared with control hepatocytes (Fig. 1a, solid line, cf. Fig. 1b). As reported previously (8, 11, 12, 19, 26), there was some heterogeneity in the responses given by individual hepatocytes. Details of the total number of cells tested and the numbers of cells yielding a given type of response are set out in Table I. In the majority of cells tested the ability of vasopressin to release Ca2+ from intracellular stores was not affected by microinjection of the antibody (Table I). Pretreatment of the anti-Gi1,2a antibody with the peptide against which the antibody was raised (the blocking peptide) prevented the inhibition of vasopressin-stimulated Ca2+ influx (Fig. 1a, broken line, Table I). The anti-Gi1,2a antibody also inhibited thapsigargin-stimulated Ca2+ influx and caused some inhibition of thapsigargin-stimulated release of Ca2+ from intracellular stores (Fig. 1c, Table I). No inhibition of vasopressin-stimulated Ca2+ influx was observed in cells loaded with an anti-Gi2a,1 antibody raised against a peptide which corresponded to a region near the amino terminus of Gi2a,1 (results not shown).

The effects of the anti-Gi1,2a antibody were compared with those of an antibody against Gi11a,1. This inhibited vasopressin-stimulated Ca2+ influx in most cells tested and caused partial inhibition of the vasopressin-stimulated release of Ca2+ from intracellular stores (Fig. 1d, Table I), as shown previously for hepatocytes by Yag et al. (19). The possibility that the microinjected anti-Gi11a,1 antibody was incompletely distributed in the cytoplasmic space was tested by microinjecting FITC-conjugated rabbit IgG to hepatocytes. The fluorescence signal diffused evenly in recipient cells within 5 min following the microinjection of the antibody, indicating that antibody microinjected to an hepatocyte is distributed throughout the cell within 5 min following its microinjection (results not shown).

The microinjection of peptide Gi2a,1 (Ile345–Phe355) inhibited vasopressin-stimulated Ca2+ influx in almost all cells tested but had no effect on the ability of vasopressin to release Ca2+ from intracellular stores (Fig. 2a, Table I). By contrast, microinjection of peptide Gi3a,1 (Ile344–Phe354) at the same concentration as that employed for peptide Gi2a,1 caused no inhibition of either Ca2+ influx or Ca2+ release from intracellular stores induced by vasopressin (Fig. 2a, Table I). Neither peptide caused an activation of Ca2+ influx in the absence of vasopressin or thapsigargin (results not shown). Thapsigargin-stimulated Ca2+ influx was also inhibited by peptide Gi2a,1. Complete inhibition was observed in 55% of the cells tested (Fig. 2b, cf. Fig. 2c, Table I). Peptide Gi2a,1 caused little or no inhibition of thapsigargin-stimulated Ca2+ influx (Fig. 2b, cf. Fig. 2c, Table I). However, both peptides caused some inhibition of thapsigargin-induced release of Ca2+ from intracellular stores (Fig. 2b, cf. Fig. 2c, Table I). In 7 out of 11 cells (peptide Gi2a,1) and in 3 out of the 4 cells (peptide Gi3a,1) the inhibition of the thapsigargin-induced release of Ca2+ from intracellular stores by the peptide was associated with an inhibition of thapsigargin-induced Ca2+ influx. This suggests there may be some correlation between the effects of the peptides on the release of Ca2+ from intracellular stores and their effects on Ca2+ influx.

Since the results of the experiments conducted with anti-Gi2a,1, antibodies and site-specific Gi antibodies suggest that Gi2a,1 is required for the activation of Ca2+ influx, the intracellular location of Gi2a,1 was investigated by immunofluorescence, using anti-Gi1,2a antibody as the primary antibody and anti-rabbit IgG antibody coupled to the fluorescent dye Cy3. In most cells, immunofluorescence, which was dependent on anti-Gi1,2a antibody, was found to be distributed throughout the cytoplasmic space as well as in most parts of the cell periphery (Fig. 3a). This distribution is seen more clearly at higher magnification (Fig. 3b). The fluorescence signal given by anti-Gi1,2a antibody was not observed when the anti-Gi1,2a antibody was omitted or when this antibody was pretreated with the blocking peptide (results not shown). A pattern of immunofluorescence similar to that given by anti-Gi1,2a antibody was observed when an anti-Gi3a,1 antibody was employed as the primary antibody (results not shown). In contrast to the results obtained with the anti-Gi1,2a antibody, when anti-Gi1,2a antibody was employed,
the fluorescence signal was largely confined to the periphery of the cell, adjacent to the plasma membrane (Figs. 3, c and d). The fluorescence given by anti-Gq-11a antibody was abolished when anti-Gi1–2a antibody was omitted or when this antibody was pretreated with blocking peptide (results not shown). The cells labeled with the anti-Gi1–2a antibody exhibited a much more defined location of immunofluorescence at the cell periphery than that exhibited by cells labeled with the anti-Gq-11a antibody (Fig. 3c, cf. Fig. 3a). These results indicate that while Gq11a is distributed in the plasma membrane and in various

TABLE I

Effects of anti G1–2a and anti-Gi1–2a antibodies on vasopressin- and thapsigargin-stimulated Ca2+ influx: summary of the number of cells tested and the responses given by individual cells.

The experiments were performed as described under "Experimental Procedures" and in the legends to the figures. Traces which are representative of the predominant effect of each agent under test are shown in the figures.

| Fig. | Agonist | Agent under test | Total number of cells tested | Ca2+ influx | Release of Ca2+ from intracellular stores |
|------|---------|------------------|-----------------------------|-------------|----------------------------------------|
|      |         |                  |                             | Normal | Reduced | None or very slow | Normal | Reduced | None or very slow |
| 1a   | Vasopressin | Anti G1–2a antibody | 26 | 4 | 8* | 14 | 20 | 6 | 0 |
|      |         | Anti G1–2a antibody + blocking peptide | 8 | 6 | 1 | 1 | 8 | 0 | 0 |
| 1b   | Vasopressin | None | 26 | 21 | 2 | 3 | 22 | 4 | 0 |
| 1c   | Thapsigargin | Anti G1–2a antibody | 10 | 2 | 0 | 8 | 5 | 5 | 0 |
|      |         | None | 9 | 8 | 1 | 0 | 7 | 2 | 0 |
| 1d   | Vasopressin | Anti Gi1–2a antibody | 7 | 2 | 0 | 5* | 2b | 5b | 0 |
|      |         | None | 3 | 3 | 0 | 0 | 3 | 0 | 0 |
| 2a   | Vasopressin | Gi1–2a peptide | 18 | 4 | 7 | 7 | 14 | 0 | 4 |
| 2b   | Thapsigargin | Gi1–2a peptide | 10 | 9 | 1 | 0 | 9 | 1 | 0 |
|      |         | Gi3–2a peptide | 20 | 8 | 1 | 11 | 8 | 7 | 5 |
| 2c   | Thapsigargin | None | 15 | 11 | 2 | 2 | 10 | 4 | 1 |

* Two cells showed oscillations in [Ca2+]i following the addition of extracellular Ca2+ (Ca2+o). (Oscillations were never observed in control cells treated with vasopressin.)

b Of those cells which showed "normal" or reduced Ca2+ release, five cells showed a delayed (2 min) Ca2+ release.

FIG. 1. Effects of anti-G1–2a and anti-Gi1–2a antibodies on vasopressin- and thapsigargin-stimulated Ca2+ influx. The fluorescence of single cells was measured as described under "Experimental Procedures." Vasopressin (5 nM), thapsigargin (20 μM), and Ca2+ (1.3 mM) were added at the beginning of the periods indicated by the horizontal bars. Anti-G1–2a antibody (estimated intracellular concentration 30–40 μg/ml) or anti-Gi1–2a antibody (estimated intracellular concentration 30 μg/ml) was co-injected with fura-2 (estimated intracellular concentration 130 μM). a and b, inhibition by anti-G1–2a antibody of vasopressin-stimulated Ca2+ influx. a, solid trace, anti-G1–2a antibody; broken trace, anti-G1–2a antibody pretreated with blocking peptide; b, no antibody present. The solid and broken traces in a are representative of the results obtained for 1 of 14 cells (26 cells tested) and for 1 of 6 cells (8 cells tested), respectively (Table I). The trace shown in b is representative of the results obtained for 1 of 21 cells (26 cells tested) (Table I). c, inhibition by anti-Gi1–2a antibody of thapsigargin-stimulated Ca2+ influx. The solid (anti-Gi1–2a antibody present) and broken (no antibody present) traces are representative of the results obtained for 1 of 8 cells (10 cells tested) and 1 of 8 cells (9 cells tested), respectively (Table I). The solid trace in d is representative of the results obtained for 1 of 5 cells (7 cells tested), and for 1 of 3 cells (3 cells tested), respectively (Table I).
regions of the cytoplasmic space, G_{\alpha11\alpha} is located predominantly at the plasma membrane.

**DISCUSSION**

Previous studies with hepatocytes which utilized pertussis toxin, GTP-\gamma-S, and GDP/\beta-S have shown that vasopressin-de-
membrane-spanning receptors through a mechanism which does not involve $G_{q11}$ (28). However, no evidence for such a pathway in hepatocytes has so far been reported. Furthermore, $G_{q2}$ is unlikely to be involved in the activation of phosphoinositide-specific phospholipase C in hepatocytes, since first, this function of $G_{q2}$ in hepatocytes has not been reported, and second, the anti-$G_{q1,1}$ antibody and the $G_{q2}$ peptide caused no inhibition of vasopressin-stimulated release of Ca$^{2+}$ from intracellular stores. It is noteworthy that in several cells the anti-$G_{q1,1}$ antibody inhibited vasopressin-stimulated Ca$^{2+}$ influx with little effect on the release of Ca$^{2+}$ from intracellular stores. One possible explanation for this observation is that only a small region of the intracellular Ca$^{2+}$ stores (most likely the endoplasmic reticulum near the plasma membrane) is involved in activation of the plasma membrane Ca$^{2+}$ channels.

Based on the results obtained, the sequence of events emerging for the activation by vasopressin of RACCs in hepatocytes is likely to include the following steps: formation of the vasopressin-receptor complex, activation of $G_{q11}$, activation of phospholipase C, an increase in InsP$_3$ at the periphery of the cell, release of Ca$^{2+}$ from the endoplasmic reticulum in this region, activation of $G_{q2}$, and activation of one or more RACCs. Since the anti-$G_{q1,2a}$ antibody and the $G_{q2}$ peptide inhibit thapsigargin-stimulated Ca$^{2+}$ influx (in the absence of added vasopressin and hence in the absence of the formation of the vasopressin-receptor complex) activation of $G_{q2}$ would not involve its interaction with a seven-transmembrane-spanning receptor protein. One possible role of $G_{q2}$ may be to regulate the movement of Ca$^{2+}$ between components of the endoplasmic reticulum (8, 29, 30) or interaction of the endoplasmic reticulum with the plasma membrane.

The proposed role of $G_{q2}$ in store-activated Ca$^{2+}$ influx in hepatocytes does not exclude a role for a low molecular weight G-protein, as proposed for mast and mouse lacrimal acinar cells, in part, on the basis of the observation that GTP-$S$ inhibits store-activated Ca$^{2+}$ influx in these cell types (8, 9). Indeed, other studies conducted in this laboratory have also shown that a relatively high concentration of GTP-$S$ inhibits thapsigargin-stimulated Ca$^{2+}$ influx in hepatocytes. Furthermore, the action of $G_{q2}$ may be complimentary to that of a Ca$^{2+}$ influx factor (5–7).

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