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Comparison of the Subcellular Distribution of G-Proteins in Hepatocytes In Situ and in Primary Cultures

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Abstract The subcellular localization of the heterotrimeric G-proteins in hepatocytes in situ was compared to that in hepatocytes in primary culture. The ability of various ligands to activate adenylyl cyclase (AC) in membrane preparations was also investigated. In hepatocytes in situ the G proteins were mainly localized at the plasma membrane while in hepatocytes in culture they were predominantly cytoplasmic. The localization of the G-proteins in hepatocytes in situ correlates with their role in signal transduction. In homogenates prepared from the cultured cells, ligands which stimulate AC via $G_s\alpha$ were without effect, which was consistent with the localization of $G_s\alpha$ in the cytoplasmic and nuclear compartments. The "relocalization" of the G proteins to the cytoplasm when cells are cultured suggests that transmembrane signalling may be regulated by cell differentiation and cell-cell and cell-extracellular matrix interactions. © 1996 Wiley-Liss, Inc.

Key words: hepatocytes, differentiation, tissue sections, $G_s\alpha$, $G_i\alpha$, G_β, actin, stress fibres, subcellular localization, immunofluorescence microscopy, adenylyl cyclase, antibodies

INTRODUCTION

The heterotrimeric G-proteins regulate effector systems such as AC, phospholipase C, and several ion channels [Birnbaumer et al., 1990]. Their association with the plasma membrane allows them to interact with both membrane receptors and effectors [Gilman, 1987]. Recently several groups have shown that these peptides are associated with various cellular organelles and not solely confined to the plasma membranes [Brabet et al., 1988; Gabrion et al., 1989; Wang et al., 1989; Ercolani et al., 1990; Lewis et al., 1991; Muntz et al., 1992; Stow and Dealmeida, 1993; Saffriz et al., 1994; Wilson et al., 1994]. We also found that in cultured adipocytes [McFarlane-Anderson et al., 1993; Cadrin et al., 1993], $G_s\alpha$ was mainly localized in cytoplasmic vesicular structures and in the nucleus, $G_i\alpha 2$ colocalized with F-actin, $G\beta$ colocalized with F-actin, and the Golgi apparatus and $G_i\alpha 3$ was associated with the Golgi apparatus. The finding that in adipocyte cell lines G_s was located almost exclusively in the cytoplasm and in association with the nucleus [McFarlane-Anderson et al., 1993; Cadrin et al., 1994; Wilson et al., 1994] prompted us to examine further the localization of G-proteins in tissue as compared to cultured cells.

In the liver, the G-proteins regulate the response to hormones such as catecholamines and glucagon by modulation of the AC-mediated production of cAMP. In previous studies using Northern and Western blotting we found that $G_i\alpha 2$, $G_i\alpha 3$, $G_s\alpha$, and $G\beta$ subunits were the major peptides present in the liver [McFarlane-Anderson et al., 1992; Bégin-Heick, 1994].

The purpose of this investigation was to determine 1) whether the localization of the G proteins in hepatocytes in situ was consistent with their role as plasma membrane signal transducers and 2) whether maintaining hepatocytes in primary culture affected the subcellular distribution of the peptides and their ability to stimulate AC.

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MATERIALS AND METHODS

Materials

[α-^{32}P]-ATP, [^{3}H]-cAMP, and [^{3}H]-AMP and Formula-989 scintillation fluid were obtained from NEN-Dupont Canada (Mississauga, ON). Caffeine, ATP (cat. #A-2383, prepared by the phosphorylation of adenosine), cAMP, Gp-p(NH)p, Type IV collagenase, creatine phosphate (Tris salt), creatine phosphokinase, myokinase, (−)-isoproterenol, dextran sulphate, trypsin inhibitor, leupeptin, DTT, and demecolcine were from the Sigma Chemical Co. (St. Louis, MO). GTP was from PL Biochemicals, (Milwaukee, WI). GTPγS was from Boehringer Mannheim, Montreal, QC. Cell culture reagents were from GIBCO/BRL, Life Technologies Inc. (Burlington, ON). G protein antibodies were from NEN, Dupont Canada, (Mississauga, ON). They were specific for $G_i\alpha1/G_i\alpha2$ (NEI-801), $G_s\alpha$ (NEI-805), and Gβ (NEI-807). Rhodamine-conjugated phalloidin was from Molecular Probes Inc., (Eugene, OR). FITC-goat anti rabbit antibody was from Bio/Can Scientific, (Toronto, ON).

Animals. C57Bl/6J ob/ob mice and their lean controls (+/+) were obtained from The Jackson Laboratories, Bar Harbor ME at 7–8 weeks and used in experiments at 9–12 weeks of age.

Primary culture of hepatocytes. Hepatocytes were isolated by the two-step method described by Berry et al. [1991]. The essential feature of the two-step approach, originally described by Seglen [1976], is that the liver is flushed with a calcium-free medium before perfusing collagenase in a medium containing calcium. Isolated hepatocytes were grown on fibronectin-coated glass coverslips (for immunofluorescence) or culture dishes (for biochemical assays) in serum-free William's E medium containing dexamethasone (10 μM) and insulin (10 nM), as described [Cadrin et al., 1995]. The experiments were performed on 48 h cultures.

Liver Membrane Preparation

Membranes from whole liver were prepared by differential centrifugation, as previously described [McFarlane-Anderson et al., 1992]. Membranes from freshly isolated hepatocytes and primary-cultured hepatocytes were prepared as follows. Freshly isolated hepatocytes were centrifuged at 1000*g*, resuspended in five times their volume of 0.25 M sucrose containing 5 mM Tris.HCl, pH 7.4 and homogenized in a Polytron and rehomogenized in a hand-held homogenizer. The homogenate was centrifuged at 1500*g* for 15 min and the resulting supernatant at 11,500 g for 15 min. After three washes in sucrose-Tris buffer, the final pellet was resuspended in the storage buffer (10 mM KPO_4 buffer at pH 7.4, containing 1 mM EDTA, 1 mM DTT, 40 μM leupeptin, and 1 μg/ml trypsin inhibitor) at a concentration of 2 mg protein/ml. Cultured hepatocytes were scraped from the culture plates and processed as described above. The isolated membranes were rapidly frozen in a mixture of methanol and solid CO_2 and stored at −70°C.

Immunofluorescence microscopy. Cultured hepatocytes and 5 μm liver cryosections on coverslips were fixed for 10 min in ethanol (−20°C) and rinsed in PBS as described previously [McFarlane-Anderson et al., 1993]. For G-protein labelling, coverslips were incubated for 45 min with the appropriate primary antibody, diluted 1/50 in PBS containing 0.5% skim milk. After rinsing in PBS, they were incubated with an FITC conjugated goat-anti rabbit IgG (1/25). For visualisation of F-actin, fixed cells were incubated with rhodamine-phallodin (1/20) for 20 min at room temperature and rinsed in PBS. For double labelling, the samples were stained with rhodamine-phalloidin as described above and then processed for G-protein labelling. After staining, coverslips were mounted (0.1% p-phenylene diamine in 50% glycerol/PBS) on glass slides and observed by conventional epifluorescence microscopy on a Zeiss Axiophot Photomicroscope (Zeiss, Germany). For controls, the first antibody was omitted from the labelling reaction. No staining was observed.

Adenylyl Cyclase Assay

Fractions were tested as described in detail by Bégin-Heick and Welsh [1988], using 0.1 mM ATP and 5 mM $MgCl_2$.

Protein Determination

Protein content was determined by the Coomassie Blue method, using bovine serum albumin as standard.

RESULTS

Microscopy

Hepatocytes in situ. Double fluorescence labelling with rhodamine-phalloidin for the de-

tection of actin and the different anti-G proteins showed that $G_i\alpha 2$, $G_i\alpha 3$, $G_s\alpha$, and $G\beta$ colocalized with actin at the plasma membrane delimitating the normal polygonal shape of hepatocytes (Fig. 1A and B, for example). However, each peptide had a specific membrane and subcellular distribution as described below. $G_i\alpha 2$ (Fig. 2A) showed an intense staining on the sinusoid side of hepatocytes. The presence of a diffuse cytoplasmic labelling was also observed. $G_i\alpha 3$ (Fig. 2B) appeared to be uniformly distributed along the plasma membrane and was present around the bile canaliculi. A punctate cytoplasmic staining close to the nucleus and nuclear staining were also noted. $G_s\alpha$ (Fig. 2C) was uniformly distributed along the plasma membrane. However, there was also some diffuse cytoplasmic and nuclear staining. $G_s\alpha$ densely stained thread-like structures localized at the exterior of the hepatocytes, as determined by confocal microscopy (not shown); the nature of these structures is not known. $G\beta$ (Fig. 2D) showed a dense staining around the bile canaliculi and a punctate cytoplasmic pattern close to the nucleus.

Hepatocytes in primary cultures. As seen by phase-contrast microscopy, primary hepatocytes growing on fibronectin-coated coverslip formed a compact cell monolayer typical of hepatocytes differentiating in culture [Cadrin et al., 1995]. The pattern of G-protein distribution differed from that observed in situ in that there was an increase in cytoplasmic staining. In some cells, $G_i\alpha 2$ (Fig. 3A,B) was localized at the plasma membrane, while in others, the pattern colocalized with actin stress fibres (Fig. 3C,D,E). Cytoplasmic staining was seen in all cells (Fig. 3B,C). $G_i\alpha 3$ was almost entirely cytoplasmic and appeared to be associated with the Golgi apparatus (Fig. 4A,B). When the hepatocytes were stained for $G\beta$ (Fig. 4C,D) some $G\beta$ staining was localized in the plasma membrane but there was also dense cytoplasmic staining. On the other hand, $G_s\alpha$ showed no plasma membrane staining but the cytoplasm and nucleus were strongly labelled (Fig. 4E,F).

Adenylyl Cyclase Activity

To determine whether the localization of G-proteins in hepatocytes in situ and in cultured hepatocytes was associated with alterations in transducing capacity, we assayed AC in membrane fractions prepared from liver and from cultured and freshly isolated hepatocytes. In addition, because of the unique localization of $G_s\alpha$ with the cytoplasm and nuclear structures in cultured hepatocytes, we assessed the enzyme activity in crude homogenates of primary-cultured and freshly isolated hepatocytes. The results of these experiments are shown in Table I. The ability of various nucleotides, ligands, and activators to stimulate basal AC activity was similar in membranes isolated from whole liver and in homogenate and membrane preparations from freshly isolated hepatocytes. In contrast, in primary-cultured cell homogenates, neither glucagon nor aluminum fluoride, which activate AC via G_s, increased activity to a level comparable to that obtained in homogenates or membrane preparations of freshly isolated cells. Furthermore, membrane preparations from

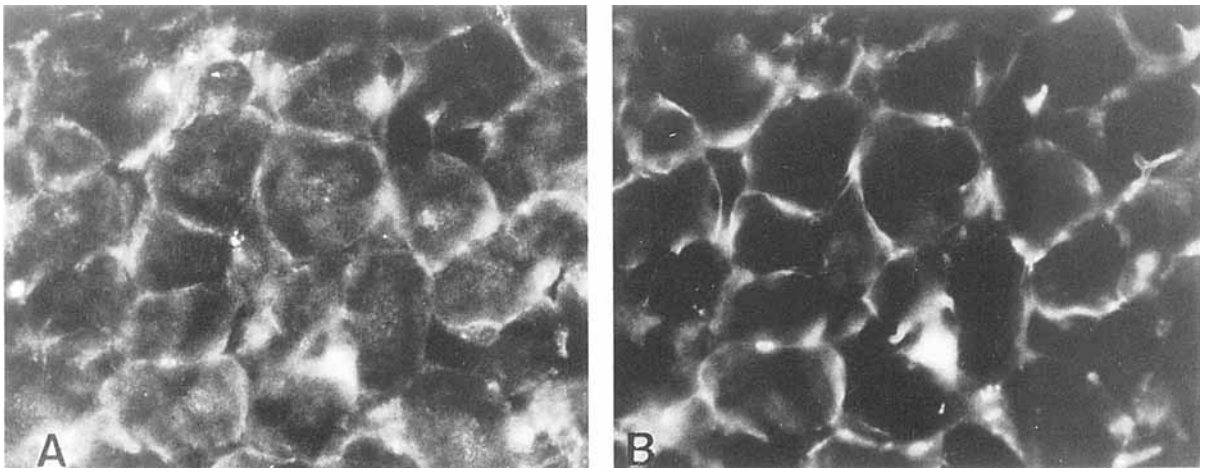

Fig. 1. Liver section double stained for the detection of $G_i\alpha 2$ **(A)** and actin **(B)**. Note that the peripheral staining of $G_i\alpha$ colocalizes with actin.

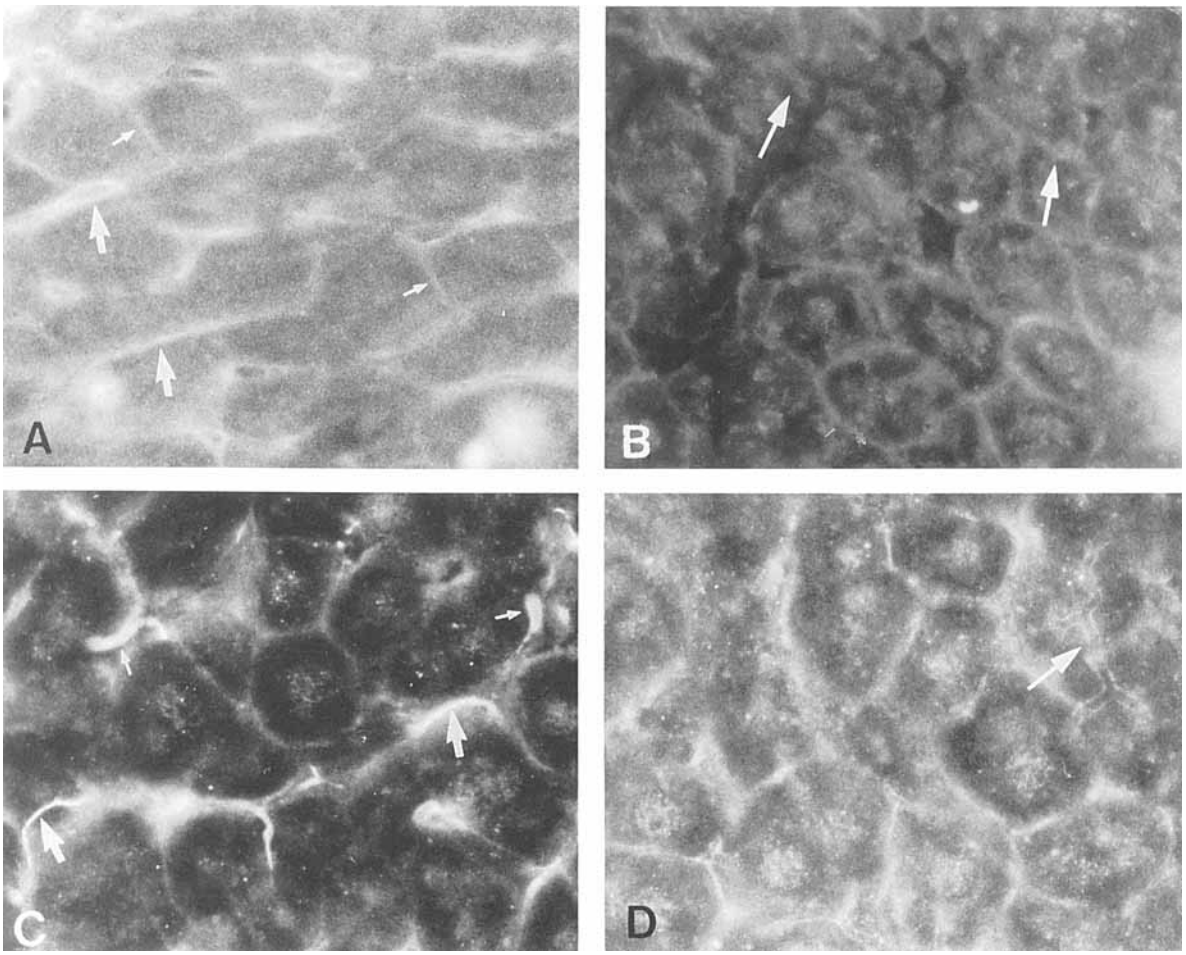

Fig. 2. Distribution of G proteins in situ in liver sections. **A:** $G_i\alpha 2$ is localized at the plasma membrane (*small arrows*). The sinusoid area shows a more intense staining (*large arrows*). **B:** $G_i\alpha 3$ is localized at the plasma membrane and shows a distinc pericanalicular distribution (*arrows*). **C:** $G_s\alpha$ is localized at the plasma membrane and present distinct pericanalicular distribution (*small arrows*). $G_s\alpha$ is also present on tread like structures localized at the exterior of the hepatocytes (*large arrows*). **D:** $G\beta$ is localised at the level of the plasma membrane present a clear pericanalicular distribution (*arrow*).

primary-cultured hepatocytes did not have detectable basal AC activity and only the combination of forskolin + Mn, which activate the catalytic subunit directly, elicited measurable activity in three out of five experiments (8.8 ± 0.8 ($n = 3$), compared to 15.5 ± 0.8 ($n = 5$) pmol/min/mg in the homogenates assayed in the same experiments). These data suggest that in primary-cultured hepatocytes, the intracellular localization of G_s largely at sites other than the plasma membrane, limits signal transduction to adenylyl cyclase when fractions enriched in plasma membranes are used for the assay.

DISCUSSION

As new G-protein peptides have been identified, it has become apparent that not all are associated with the plasma membranes. Diverse roles have since been identified for some of these proteins. A role for G-protein in the secretory process has long been advocated [Melançon et al., 1987; Bourne, 1988]. It is now well established that $G_i\alpha 3$ is involved in the control of intracellular protein transport, which correlates well with its dual location at the plasma membrane and with the Golgi system [Dealmeida et al., 1993; Pimplikar and Simon, 1993; Stow and Dealmeida, 1993]. G_s has been implicated in endosome fusion [Colombo et al., 1994a; 1994b] and in basolateral to apical transcytosis [Barroso and Sztul, 1994]. Furthermore, it has been demonstrated by biochemical and morphological means that $G_i\alpha$ and $G\beta$ subunits are associated with various fractions of liver plasma mem-

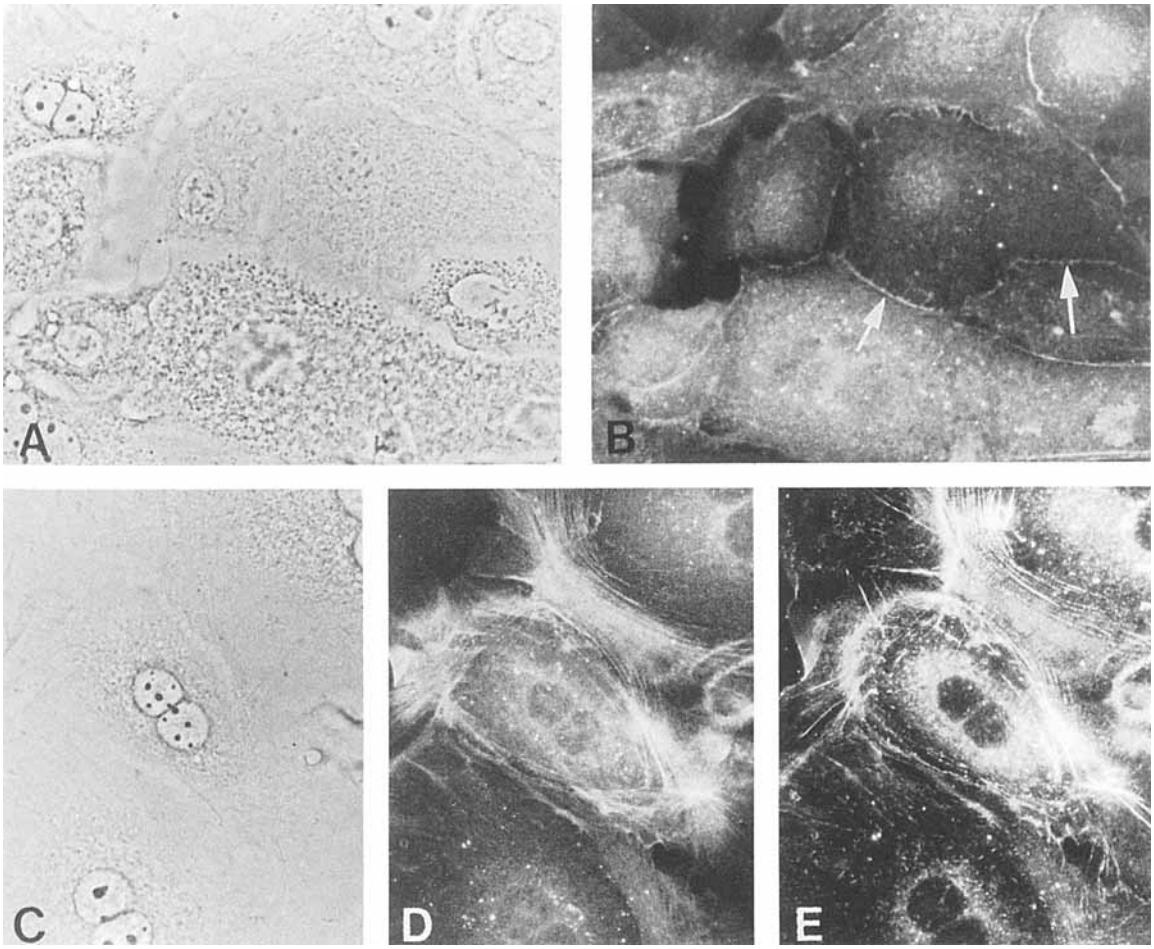

Fig. 3. Distribution of $G_i\alpha 2$ in primary-cultured hepatocytes. **A,C** phase contrast micrograph **B,E,** staining for the detection of $G_i\alpha 2$ and **D,** staining for the detection of actin. B: $G_i\alpha 2$ shows some localization at the cell-cell border of some hepatocytes (*arrows*). D: $G_i\alpha 2$ is associated with filamentous structures. E: Actin staining of the same field as shown in (D) indicating that the filamentous structures recognized by $G_i\alpha 2$ antibody correspond to actin stress fibers.

branes and with endosomes [Ali et al., 1989]. These findings point to diverse roles of heterotrimeric G-proteins in cell function that go beyond their involvement in the plasma membrane signal transduction pathways. The data described above show that the localization of G-proteins in primary-cultured cells is different from that in tissue sections. In general, there is a greater abundance of the G-proteins in the intracellular compartment, as opposed to the plasma membrane in cultured cells.

In liver, hormone-, and neurotransmitter-activated AC activity are thought to be confined mainly to the sinusoidal plasma membranes [Wisher and Evans, 1975], while the enzymes of the inositol phosphate system are present in all plasma membrane domains as well as in endosomes [Shears et al., 1988]. Our results confirm the association of $G_i\alpha$ subunits with the inusoidal membranes [Ali et al., 1989] and also demonstrate that $G_s\alpha$ peptides are present in these areas. The variation in AC activity between the various preparations described herein correlates with the observed variation in G-protein distribution. Thus, where $G_s\alpha$ is largely in the cytoplasm, as in primary cultured hepatocytes, AC is not stimulated by ligands which act through G_s. However, forskolin and Mn stimulate the AC catalytic unit directly.

The results described in this paper suggest that the state of organization of the cells i.e., cell-cell interaction, cell-extracellular matrix interaction, and the relationship to organ elements, dictates the distribution of these proteins and determines their role in signal transduction.

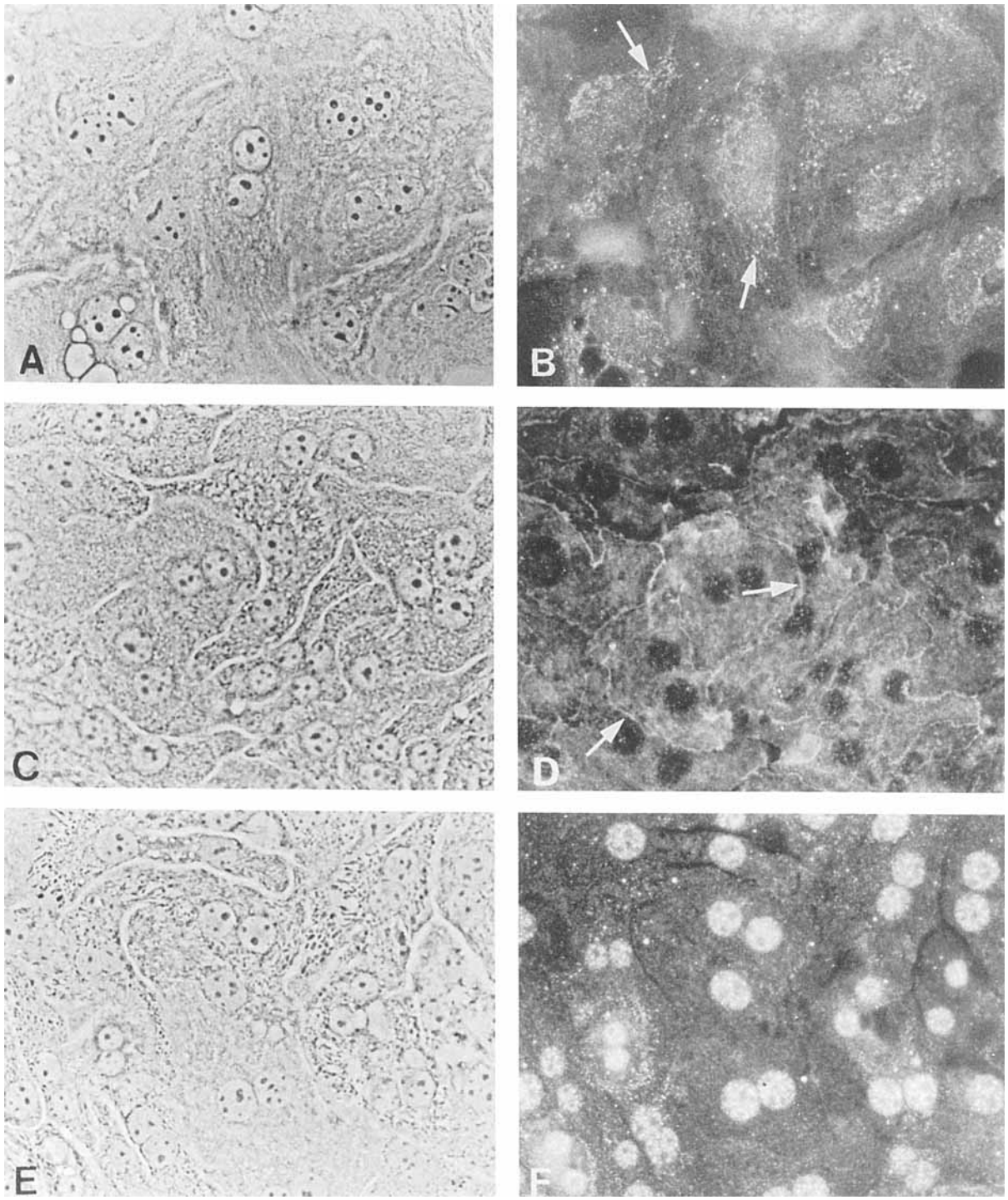

Fig. 4. Distribution of G proteins in primary-cultured hepatocytes. **A,C,E,** are phase contrast micrograph corresponding respectively to the same field as seen in **B,** $G_i\alpha 3$ **D,** $G\beta$, **F,** $G_s\alpha$. B: $G_i\alpha 3$ is almost entirely cytoplasmic and appeared to be associated with the Golgi apparatus (*arrows*). D: $G\beta$ is partially localized at the plasma membrane (*arrows*). F: $G_s\alpha$ is localized in the cytoplasm and the nucleus.

TABLE I. Adenylyl Cyclase Activity in Liver Membranes and Hepatocytes*

	Liver	Hepatocytes		
		Cultured	Fresh	
	Membrane	Homogenate	Homogenate	Membrane
Basal (pmol/mg)	25.9 ± 1.5	1.0 ± 0.2	2.3 ± 1.3	1.6 ± 0.6
		fold stimulation by:		
0.1 mM GTP	2.2 ± 0.2	2.0 ± 0.8	1.0 ± 0.2	1.5 ± 0.1
0.1 mM Gpp(NH)p	4.1 ± 0.4	2.6 ± 0.4	2.3 ± 0.5	3.5 ± 0.3
0.01 mM GTPγS	14.9 ± 1.5	3.7 ± 1.3	2.5 ± 1.5	16.4 ± 5.1
10 μM ISO + GTP	3.3 ± 0.2	nd	—	2.9 ± 0.5
1 μM glucagon	47.0 ± 2.2	1.6 ± 0.2	13.9 ± 1.6	52.7 ± 2.0
10 mM NaF	5.1 ± 0.3	2.0 ± 0.6	1.0 ± 0.5	8.7 ± 1.8
NaF + Al^{3+}	41.6 ± 3.3	4.5 ± 0.6	13.0 ± 7.2	63.5 ± 11.9
5 mM Mn^{2+}	4.5 ± 1.1	2.4 ± 1.0	2.9 ± 0.8	8.6 ± 3.2
0.1 mM Forskolin	18.7 ± 1.5	3.5 ± 0.8	4.2 ± 1.3	15.8 ± 3.6
Forskolin + Mn^{2+}	59.3 ± 6.1	16.7 ± 3.7	25.7	112 ± 21.2

*Adenylyl cyclase activity was measured as described in Materials and Methods. Basal values are expressed as pmol cAMP formed/min/mg protein. The other values are expressed as fold stimulation of basal values. Data are means ± SE for three to five observations with different preparations; nd = not detected; — = not assayed. Additional data relating to membranes isolated from cultured hepatocytes are given in the text.

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