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Specific $G\alpha_{11}\beta_3\gamma_5$ Protein Involvement in Endothelin Receptor-Induced Phosphatidylinositol Hydrolysis and Ca^{2+} Release in Rat Portal Vein Myocytes

NATHALIE MACREZ[1], JEAN-LUC MOREL[1], and JEAN MIRONNEAU

Laboratoire de Physiologie Cellulaire et Pharmacologie Moléculaire, Centre National de la Recherche Scientifique, Enseignement Supérieur Associé 5017, Université de Bordeaux II, 33076 Bordeaux Cedex, France

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ABSTRACT

In this study, we identified the receptor subtype activated by endothelin-1 (ET-1) and the subunit composition of the G protein coupling this receptor to increase in cytosolic Ca^{2+} concentration in rat portal vein myocytes. We used intranuclear antisense oligonucleotide injection to selectively inhibit the expression of G protein subunits. We show here that the endothelin receptor subtype A (ET_A)-mediated increase in cytosolic Ca^{2+} concentration was mainly dependent on Ca^{2+} release from the intracellular store. ET_A receptor-mediated Ca^{2+} release was selectively inhibited by antisense oligonucleotides that inhibited the expression of α_{11}, β_3, and γ_5 subunits, as checked by immunocytochemistry. Intracellular dialysis of a carboxyl terminal anti-β_{com} antibody and a peptide corresponding to the $G\beta\gamma$ binding region of the β-adrenergic receptor kinase-1 had no effect on the ET_A receptor-mediated Ca^{2+} release. In contrast, a synthetic peptide corresponding to the carboxyl terminus of the α_q/α_{11} subunit, heparin (an inhibitor of inositol 1,4,5-trisphosphate receptors), and U73122 (an inhibitor of phosphatidylinositol-phospholipase C) inhibited, in a concentration-dependent manner, the ET_A receptor-mediated Ca^{2+} responses. Accumulation of [^{3}H]inositol trisphosphate evoked by norepinephrine peaked at ~15 s, whereas that evoked by ET-1 progressively increased within 2 min. In myocytes injected with anti-α_q antisense oligonucleotides, both amplitude and time course of the norepinephrine-induced Ca^{2+} release became similar to those of the ET-1-induced Ca^{2+} response. We conclude that the ET_A receptor-mediated Ca^{2+} release is selectively transduced by the heterotrimeric G_{11} protein composed of α_{11}, β_3, and γ_5 subunits, and that a delayed stimulation of phospholipase C occurs via the α_{11} subunit.

Endothelin-1 (ET-1) has been implicated in a wide variety of physiological functions associated with the cardiovascular, endocrine, pulmonary, renal, and nervous systems. Two main receptor subtypes (ET_A and ET_B) have been cloned (Arai et al., 1990; Sakurai et al., 1990). Molecular characterization of ET receptors has revealed that they belong to the G protein-coupled receptor superfamily. The mechanisms of ET-1 action involve second messenger generation via different signal transduction processes. These include activation of phospholipase C (PLC) degrading phosphatidylinositol 4,5-bisphosphate with an increased production of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol, inhibition or activation of adenylyl cyclase, activation of phospholipase A_2 and D (Rubanyi and Polokoff, 1994; Webb and Meek, 1997), and activation of Ca^{2+}-permeable nonselective cation channel via a cyclic guanosine monophosphate signaling system (Minowa et al., 1997).

In smooth muscle cells, both ET_A and ET_B are expressed and may evoke an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) via different G proteins (Palacios et al., 1997; Saita et al., 1997). $InsP_3$ generation induced by either ET_A or ET_B receptors has been reported to be inhibited by pertussis toxin in various cell types, suggesting a coupling to PLC via $G_{i/o}$ proteins. However, in rat R22-D vascular myocytes, the ET receptors are coupled to PLC via a pertussis toxin-insensitive G protein (Douglas and Ohlstein, 1997). Members of the G_q family (G_q, G_{11}, G_{14}, and G_{15}) can activate PLC-β_1, -β_3, and -β_4 through their α subunit (Jhon et al., 1993), whereas $\beta\gamma$ dimers released from either pertussis toxin-sensitive or -insensitive G protein can activate the PLC-β_2, β_3, and β_1 (Camps et al., 1992; Ushio-Fukai et al., 1998).

The aim of the present study was two fold: 1) to identify the ET receptor subtype responsible for the increase in $[Ca^{2+}]_i$ in

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[1] Contributed equally to the work.

ABBREVIATIONS: ET-1, endothelin-1; AII, angiotensin II; βARK_1, β-adrenergic receptor kinase-1; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; InsP, inositol phosphate; $InsP_1$, inositol monophosphate; $InsP_2$, inositol bisphosphate; $InsP_3$, inositol trisphosphate; NE, norepinephrine; PLC, phospholipase C; ET_A, endothelin receptor subtype A; ET_B, endothelin receptor subtype B; U73122, 1-(6-((17β-3 methoxystra 1,3,5 (10)-trien-17-yl) amino) hexyl)-1H-pyrrole-2,5-dione; PTX, pertussis toxin; AM, indo-1-acetoxymethylester.

rat portal vein myocytes and 2) to determine the composition of the G protein that couples the ET-1 receptor subtype to PLC. Experiments with antisense oligonucleotides designed to block synthesis of G protein subunits revealed the G protein heterotrimer, composed of α_{11}, β_3, and γ_5 subunits, which coupled ET-1 receptors to elevation in $[Ca^{2+}]_i$. Identification of the G protein subunits involved in the activation of PLC was resolved by using $G\beta\gamma$ scavengers. In addition, the delayed accumulation of $[^3H]InsP_3$ induced by ET-1 was associated with the slow kinetics of the ET-1-evoked Ca^{2+} response.

Materials and Methods

Cell Preparation. Isolated myocytes from rat portal vein were obtained by enzymatic dispersion, as described previously (Macrez-Leprêtre et al., 1994). Cells were seeded at a density of about 10^3 cells/mm^2 on glass slides imprinted with squares for localization of injected cells, and maintained in short-term primary culture in M199 medium containing 2% fetal calf serum, 2 mM glutamine, 1 mM pyruvate, 200 U/ml penicillin, and 200 μg/ml streptomycin; they were kept in an incubator gassed with 95% air and 5% CO_2 at 37°C, and were used within 96 h.

Fluorescence Measurements. Cells were loaded by incubation in physiological solution containing either 1 μM Indo-1-acetoxymethylester (AM) or 1 μM Fura-2-acetoxymethylester for 30 min at room temperature. These cells were washed and allowed to cleave the dye to the active Indo-1 or Fura-2 for 60 min. Indo-1 or Fura-2 loading was usually uniform over the cytoplasm and compartmentalization of the dyes was never observed. Measurement of $[Ca^{2+}]_i$ in single cells and calibration curves for Indo-1 and Fura-2 were determined within cells after 1 or 4 days in primary culture, as reported previously (Macrez-Leprêtre et al., 1994; Morel et al., 1996). Briefly, $[Ca^{2+}]_i$ was calculated from mean ratios according to Grynkiewicz's formula (Grynkiewicz et al., 1985): $[Ca^{2+}]_i = K_d\,\beta\,(R - R_{min})/R_{max} - R)$, where R_{min} and R_{max} are the ratios obtained in the absence of Ca^{2+} and at saturating Ca^{2+}. K_d is the effective dissociation constant of the Ca^{2+}-sensitive dyes. β is defined as the ratio of the signals observed at excitation wavelength 380 nm (Fura-2) or at emission wavelength 480 nm (Indo-1) in the absence and presence of saturating Ca^{2+}, and takes into account corrections for the optics of the systems. Mean $K_d\beta$ values of 825 nM (Fura-2) and 1350 nM (Indo-1) were obtained with their respective set-up. Cells were made permeable to external Ca^{2+} using 20 μM ionomycin and R_{min} and R_{max} were obtained by either Ca^{2+} depletion (no added Ca^{2+}, 10 mM EGTA) or Ca^{2+} saturation (2 mM Ca^{2+}) of the physiological solution. At 1 day of culture, the mean R_{min} and R_{max} values for Fura-2 were 0.61 ± 0.02 and 6.10 ± 0.25 (n = 12), respectively. These values were not significantly different at 4 days of culture as R_{min} and R_{max} values were 0.60 ± 0.02 and 5.90 ± 0.20 (n = 12), respectively. Similarly, the mean R_{min} and R_{max} values for Indo-1 were calculated at 1 and 4 days of culture. The R_{min} and R_{max} values were 0.30 ± 0.01 and 0.32 ± 0.02 (n = 10) and 4.25 ± 0.21 and 4.18 ± 0.15 (n = 10), respectively at 1 and 4 days of culture. Indo-1- or Fura-2-loaded cells were mounted in a perfusion chamber and placed on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan). Most experiments were carried out in the presence of 1 μM oxodipine (a light-stable dihydropyridine derivative) to inhibit voltage-dependent Ca^{2+} channels. All measurements were made at 25 ± 1°C.

Membrane Current and $[Ca^{2+}]_i$ Measurements. Voltage-clamp and membrane current recordings were made with a standard patch-clamp technique using an EPC-7 patch-clamp amplifier (List, Darmstadt-Eberstadt, Germany). Patch pipettes had resistances of 3–4 MΩ. When $[Ca^{2+}]_i$ measurements were carried out simultaneously, Indo-1 (50 μM) was added to the pipette solution in the whole-cell recording mode and $[Ca^{2+}]_i$ was estimated from the 405/480 nm fluorescence ratio using a calibration determined within cells as described previously (Morel et al., 1996). R_{min} was determined by loading cells with a pipette solution containing 10 mM EGTA. Mean R_{min} values were 0.25 ± 0.02 (n = 10) and 0.26 ± 0.03 (n = 10) at 1 and 4 days of culture, respectively. R_{max} was determined by hyperpolarizing the cells to −200 mV. This caused membrane breakdown and a large increase in Ca^{2+} permeability without resulting in a significant dye leakage. Mean R_{max} values were 4.19 ± 0.25 (n = 10) and 4.25 ± 0.30 (n = 10) at 1 and 4 days of culture, respectively. An intracellular value for $K_d\beta$ was determined according to Almers and Neher (1985). The 405/480-nm ratio was determined in cells loaded with a pipette solution containing 8 mM Ca^{2+} and 10 mM EGTA so that the free Ca^{2+} concentration was around 240 nM. A mean value for $K_d\beta$ of 1156 nM was obtained under these conditions. Patch-clamp experiments were performed at 30 ± 1°C.

Antibodies, Peptides, and Heparin. Anti-$G\beta_{com}$ antibody, $G\alpha_q$/α_{11} peptide, β-adrenergic receptor kinase-1 (βARK_1) peptide, and heparin were added to the pipette solution to allow dialysis of the cell after a breakthrough in whole-cell recording mode for at least 7 to 8 min, a time longer than that expected theoretically for diffusion of substances in solution (Pusch and Neher, 1988).

Microinjection of Oligonucleotides. Antisense or nonsense oligonucleotides were injected into the nucleus of myocytes by a manual injection system, as described previously (Macrez-Leprêtre et al., 1997a,b). The injection solution contained 10 μM oligonucleotides in water; approximately 10 fl were injected into the nucleus. The myocytes were cultured for 72 h (the time needed to obtain the highest inhibition of protein expression) in culture medium before $[Ca^{2+}]_i$ measurements. The sequences of the anti-α_q, anti-α_{11}, anti-α_{12}, anti-α_{13}, anti-α_{14}, anti-α_{15}, anti-α_{icom}, anti-α_{01}, anti-α_z, anti-β_1, anti-β_2, anti-β_3, anti-β_4, anti-β_5, anti-γ_1, anti-γ_2, anti-γ_3, anti-γ_4, anti-γ_5, anti-γ_7, and anti-γ_8 antisense oligonucleotides have been published previously (Gollasch et al., 1993; Macrez-Leprêtre et al., 1997a,b). Nonsense oligonucleotides had the same composition that the corresponding antisense oligonucleotides, but in the opposite sense.

Solutions. The normal physiological solution contained (in mM): NaCl 130, KCl 5.6, $MgCl_2$ 1, $CaCl_2$ 2, glucose 11, and HEPES 10, pH 7.4 with NaOH. The basic pipette solution contained (in mM): KCl 130 and HEPES 10, pH 7.3 with NaOH. Ca^{2+}-free solution was prepared by omitting $CaCl_2$ and by adding 0.5 mM EGTA. ET-1 and active compounds were applied to the recorded cell by pressure ejection for the period indicated on the records.

Immunocytochemistry. Three days after injection, venous myocytes were washed with PBS, fixed with 3% formaldehyde (v/v) for 30 min at room temperature, and permeabilized in PBS containing 3% fetal calf serum and 0.1 (w/v) saponin for 30 min. Cells were incubated with the same buffer containing 5% fetal calf serum, 0.1 (w/v) saponin, and the anti-G protein antibody at 1:100 dilution overnight at 4°C. Then, cells were washed in PBS containing 3% fetal calf serum and 0.1 (w/v) saponin (4 × 10 min) and incubated with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (diluted 1:200) in the same solution for 3 h at 20°C. Thereafter, cells were washed (4 × 10 min) in PBS and mounted in Vectashield (Biosys, Compiègne, France). Images of the stained cells were obtained with a confocal microscope (Bio-Rad MRC 1000, Bio-Rad Laboratories, Inc., Hemel Hempstead, UK). Only cells on the same glass slide were compared with each other by keeping acquisition parameters (gray values, exposure time, aperture, etc.) constant. Immunostaining fluorescence was estimated by gray level analysis using MPL software (Bio-Rad).

Measurement of $[^3H]$Inositol Phosphates. Experiments on intact cells were performed as described previously (Mouillac et al., 1989; Meneton et al., 1992). Cells (0.6×10^6) were incubated in 2 ml of culture medium containing 5 μCi/ml myo[2-^{3}H]inositol (20 Ci/mmol) for 3 days leading to steady-state labeling of cellular inositol lipids (Menniti et al., 1990; Mouillac et al., 1990). Then, cells were washed with M199 without serum and myo[2-^{3}H]inositol during 30 min. The M199 medium was removed and replaced by physiological solution with 10 mM LiCl. After a 10-min delay, ET-1 or norepineph-

rine (NE) was added. Inositol phosphate (InsP) formation was stopped by ice-cold perchloric acid (final concentration 5%). Acid-lysed cells were centrifuged at 2500 rpm, 4°C for 5 min. Supernatant contained InsPs and the pellet contained phosphatidylinositols and lipids. Supernatant was neutralized by HEPES 75 mM and KOH 1.5 M (pH = 6–8) and incubated 5 min on ice for the precipitation of potassium perchlorate. Neutralized supernatant was centrifuged at 2500 rpm, 4°C for 5 min. Supernatant was eluted through a polyprep chromatography column (Bio-Rad) containing 1.6 ml anion exchange resin (DOWEX AG1-X8, formate form, 200–400 mesh). On the basis of control experiments with labeled inositol phosphate standards, free inositol was eluded with 12 ml water, glycerophosphoinositol with 12 ml of 30 mM ammonium formate (pH = 5), inositol monophosphate ($InsP_1$) with 12 ml of 180 mM ammonium formate/0.1 M formic acid, inositol bisphosphate ($InsP_2$) with 12 ml of 400 mM ammonium formate/0.1 M formic acid, and $InsP_3$ with 12 ml of 700 mM ammonium formate/0.1 M formic acid. The perchloric acid-precipitated pellets that contained phosphoinositides were resuspended with 1 ml chloroform-methanol-10 M HCl (200:100:1, v/v/v). These suspensions were mixed with 350 μl HCl and 350 μl chloroform and centrifuged for 5 min at 2500 rpm to separate the phases. The lower hydrophobic phase was recovered and dried in counting vials to determine radioactivity in total phosphoinositides. Recoveries of inositol phosphates and phosphoinositides were about 75%.

The radioactivity contained in 3 ml of each eluate and phospholipid extract was counted with a Packard Tricarb 1500 counter (Downers Grove, IL) after the addition of 10 ml of scintillation liquid for 10 or 20 min after experiments. The radioactivity was corrected for variable quenching due to the different elution solutions by means of quench curves obtained with each solution, and was thereby converted into dpm.

Chemicals and Drugs. Collagenase was obtained from Worthington Biochemical Corp. (Freehold, NJ); pronase (type E), bovine serum albumin, NE, pertussis toxin, heparin (from porcine intestinal mucosa; molecular weight, 6000), and chondroitin sulfate were purchased from Sigma (St. Louis, MO). M199 medium was obtained from Flow Laboratories (Puteaux, France). Fetal calf serum was obtained from Flobio (Courbevoie, France). Streptomycin, penicillin, glutamine, and pyruvate were purchased from Gibco (Paisley, UK). Oxodipine was a gift from Dr. Galiano (Instituto de Investigacion y Desarrollo Quimico Biologica, Madrid, Spain). Caffeine was purchased from Merck (Nogent sur Marne, France). Indo-1 and Indo-1 AM were obtained from Calbiochem (Meudon, France). AII, ET-1, BQ123, and BQ788 were purchased from Neosystem Laboratories (Strasbourg, France). Carboxyl-terminal $G\alpha_{q/11}$ peptide (LQLNLKEYNLV) and peptide corresponding to the $G\beta\gamma$-binding protein domain of βARK_1 (WKKELRDAYREAQQLVQRVPKMKNKPRS) were synthesized by Genosys (Cambridge, UK). 1-(6-((17β-3 methoxystra 1,3,5 (10)-trien-17-yl) amino) hexyl)-1H-pyrrole-2,5-dione (U73122) and 1-(6-((17β-3 methoxystra 1,3,5 (10)-trien-17-yl) amino) hexyl)-1H-pyrrolipine-dione (U73343) were obtained from Biomol (Plymouth Meeting, PA). Anti-$G\alpha_q$ (SC393), anti-$G\alpha_{11}$ (SC394), anti-$G\beta_{com}$ (SC378), anti-β_3 (SC381) and anti-γ_5 (SC376) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Oligonucleotides were a gift from F. Kalkbrenner (University of Berlin, Germany). Myo[2-^{3}H]inositol (20 Ci/mmol) was purchased from NEN (Paris, France). Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was obtained from Immunotech (Marseille, France).

Results

Effects of ET-1 on $[Ca^{2+}]_i$ in Vascular Myocytes. Application of 10 nM ET-1 for 30 s resulted in a delayed increase in $[Ca^{2+}]_i$ (Fig. 1A). In 20% of the cells tested (n = 30), the ET-1-induced Ca^{2+} response seemed to possess two components (Fig. 1A) with a faster component followed by a more or less sustained increase in $[Ca^{2+}]_i$, which returned to basal $[Ca^{2+}]_i$ value within 2 to 3 min. The transient peak was obtained 35 ± 7 s (n = 9) after application of ET-1. Its mean amplitude was 135 ± 32 nM from a resting $[Ca^{2+}]_i$ of 43 ± 3 nM (n = 9) in Indo-1 AM-loaded myocytes. In 80% of the cells tested (n = 30), ET-1 initiated a slow and monophasic Ca^{2+} response (Fig. 1B) with a mean amplitude of 129 ± 25 nM (n = 21). Removal of external Ca^{2+} (in the presence of 0.5 mM EGTA) decreased the transient Ca^{2+} peak to 86 ± 12 nM (n = 30) and suppressed the sustained increase in $[Ca^{2+}]_i$ in all the types of Ca^{2+} responses (Fig. 1, A and B). External application of 1 μM oxodipine (to inhibit L-type Ca^{2+} channels) reduced the transient peak to 93 ± 15 nM (n = 30) and largely inhibited the sustained Ca^{2+} phase, but did not suppress it (Fig. 1, A and B). These results suggest that the peak increase in $[Ca^{2+}]_i$ is largely due to release of Ca^{2+} from the intracellular store. In the following experiments, ET-1-induced Ca^{2+} responses were measured either in the presence of 1 μM oxodipine for at least 5 min or in Ca^{2+}-free, 0.5 mM EGTA-containing solution for 30 s.

The concentration-response curve for ET-1 (Fig. 2A) showed that the maximal effect was obtained at 100 nM. The concentration producing half-maximal response was estimated to be 1.4 ± 0.5 nM with a Hill coefficient close to unity. As illustrated in Fig. 2B, the ET-1-induced peak Ca^{2+} response was concentration-dependently inhibited by BQ123 (an antagonist of ET_A receptors; Webb and Meek, 1997). The IC_{50} value for BQ123 was estimated to be 1.8 ± 0.3 nM, and complete inhibition of the ET-1-induced Ca^{2+} response was obtained at 100 nM. In contrast, BQ788 (an antagonist of ET_B receptors; Webb and Meek, 1997) showed little effect on the Ca^{2+} response evoked by 10 nM ET-1 (Fig. 2B). These results indicate that in portal vein myocytes the ET-1-induced increase in $[Ca^{2+}]_i$ is mainly mediated through activation of ET_A receptors.

Effects of Heparin and PLC Inhibitor. Heparin blocks $InsP_3$ receptors (Guillemette et al., 1989) and inhibits Ca^{2+} release via these receptors in smooth muscle cells (Somlyo

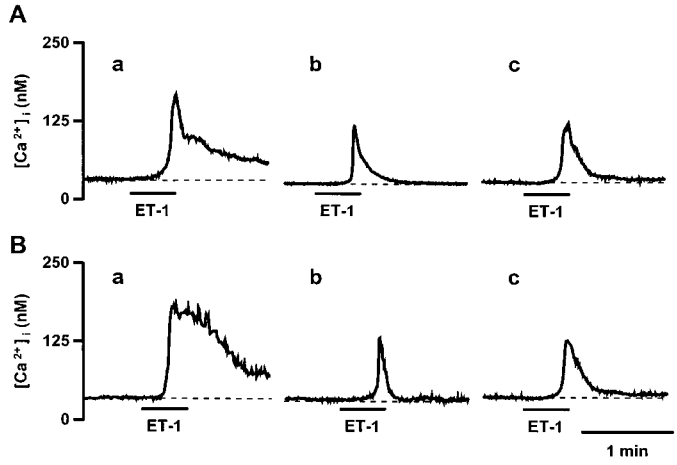

Fig. 1. Increase in $[Ca^{2+}]_i$ evoked by 10 nM ET-1 in rat portal vein myocytes. The cells were loaded with Indo-1 AM and not patch-clamped. A, biphasic Ca^{2+} response evoked by 30-s application of 10 nM ET-1 in physiological solution containing 2 mM $CaCl_2$ (a), becomes monophasic in Ca^{2+}-free (0.5 mM EGTA) solution for 30 s (b), and after application of 1 μM oxodipine for 5 min (c). B, monophasic Ca^{2+} response evoked by 30-s application of 10 nM ET-1 in 2 mM $CaCl_2$ solution (a), in Ca^{2+}-free solution (0.5 mM EGTA) solution for 30 s (b), and after application of 1 μM oxodipine for 5 min (c).

and Somlyo, 1994). The effect of ET-1 was studied in myocytes held at −50 mV with 1 mg/ml heparin in the pipette solution. The basal $[Ca^{2+}]_i$ level (61 ± 3 nM, n = 11) was not significantly different from that observed under control conditions (65 ± 5 nM, n = 11), but the ET-1-induced Ca^{2+} response was completely removed (Fig. 3A). This effect is specific to heparin as, when the glycosaminoglycan chondroitin sulfate (used at 1 mg/ml as a negative control; Worley et al., 1987) was added to the pipette solution instead of heparin, the Ca^{2+} response to ET-1 (102 ± 24 nM, n = 7) was similar to that obtained in control conditions (104 ± 25 nM, n = 7). U73122 has been shown to inhibit phosphatidylinositol-specific PLC in vascular myocytes without affecting the intracellular Ca^{2+} store (Macrez-Leprêtre et al., 1996). As shown in Fig. 3B, the ET-1-induced Ca^{2+} response was concentration-dependently inhibited by U73122 with an estimated IC_{50} value of 0.25 μM. Complete inhibition was obtained with 3 μM U73122. In contrast, 3 μM 73343 (the inactive analog of U73122) had no significant effects on the ET-1-induced Ca^{2+} response (Fig. 3B). These results suggest that ET-1 activates a PLC to generate $InsP_3$ and the subsequent activation of $InsP_3$-gated Ca^{2+} release channels in the sarcoplasmic reticulum.

Subunit Composition of the G Protein. Previous identification of the α subunits of G proteins in rat portal vein membranes by immunoblot analysis has revealed the presence of pertussis toxin (PTX)-sensitive and PTX-insensitive G proteins (Macrez-Leprêtre et al., 1995). When cells were incubated in the presence of 0.5 μg/ml PTX for 20 h, the ET-1-induced Ca^{2+} response was not significantly affected (control: 101 ± 16 nM, n = 11; PTX-pretreated: 99 ± 20 nM, n = 11). In contrast, intracellular application of the carboxyl-terminal $G\alpha_{q/11}$ peptide inhibited in a concentration-dependent manner the ET-1-evoked increase in $[Ca^{2+}]_i$ (Fig. 4) with complete inhibition obtained at 0.5 μg/ml. To identify the heterotrimeric G protein that transduced the ET_A receptor signal, 10 μM phosphorothioate-modified antisense oligonucleotides directed against the α, β, and γ subunits of G proteins were injected into the nucleus of vascular myocytes (Macrez-Leprêtre et al., 1997a,b). Figure 5, A and B, illustrates the time course of the efficiency of anti-α_q and anti-α_{11} antisense oligonucleotides in inhibiting the NE- and ET-1-induced increases in $[Ca^{2+}]_i$, respectively. In both cases, the highest inhibition (75–80%) was obtained 3 days after injection. A slight recovery was observed 4 days after injection. A similar delay after injection (48–72 h) was needed to obtain the highest inhibition of Ca^{2+} responses by various anti-β and anti-γ antisense oligonucleotides. Figure 5C illustrates the efficiency and specificity of anti-β_1, anti-β_3, anti-γ_3, and anti-γ_5 antisense oligonucleotides in inhibiting ET-1 and AII-induced increase in $[Ca^{2+}]_i$. Therefore, in the following experiments, the increases in $[Ca^{2+}]_i$ were measured 3 days after oligonucleotide injection, in response to successive applications of 10 nM ET-1, 10 nM AII, and 10 mM caffeine on the same cells (Fig. 6). For each experiment, the Ca^{2+} responses of antisense or nonsense oligonucleotide-injected cells located within a marked area on the glass slide were compared with those of noninjected cells outside this marked area. This procedure ensures that oligonucleotide-injected cells were always compared with control cells that were grown, treated (i.e., incubation and loading with Fura-2 AM), and analyzed under identical conditions (Macrez-Leprêtre et al., 1997a,b). The increases in $[Ca^{2+}]_i$ were measured for

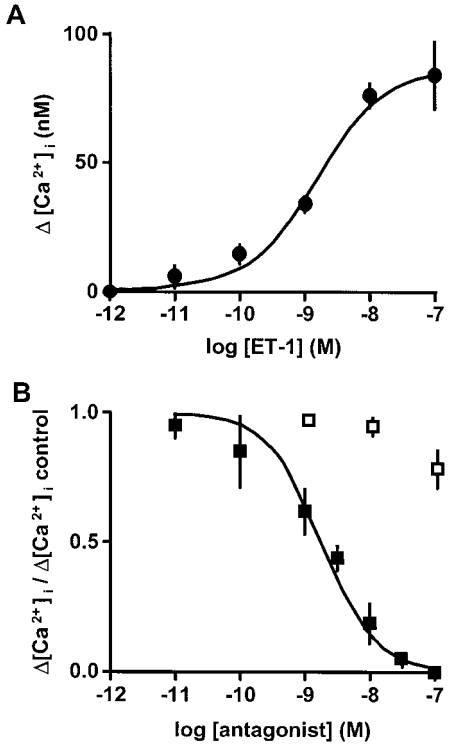

Fig. 2. Effects of ET-1 and endothelin receptor antagonists on $[Ca^{2+}]_i$. A, concentration-response curve to ET-1 obtained by fitting the experimental points. The Hill coefficient is equal to unity. B, inhibitions of the ET-1-stimulated $[Ca^{2+}]_i$ changes by BQ123 (■) and BQ788 (□). $[Ca^{2+}]_i$ values are expressed as a percentage of the response obtained with 10 nM ET-1. Data are means ± S.E.M. for five to eleven cells. Myocytes were loaded with Indo-1 AM and not patch-clamped. External solution contained 1 μM oxodipine.

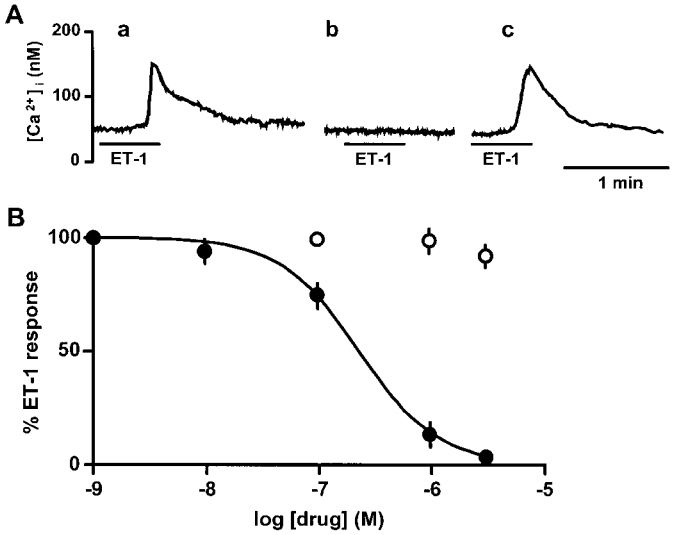

Fig. 3. Effects of heparin and PLC inhibitor on ET-1-induced Ca^{2+} release. A, Ca^{2+} responses evoked by 10 nM ET-1 in control conditions (a) and in cells dialyzed with a pipette solution containing 1 mg/ml heparin (b) or chondroitin sulfate for 7 to 8 min (c). Myocytes were loaded with Indo-1 through the patch pipette and held at −50 mV. B, inhibition of the ET-1-induced Ca^{2+} release by U73122 (●) and U73123 (○). $[Ca^{2+}]_i$ values are expressed as a percentage of the response obtained with 10 nM ET-1. Data are means ± S.E.M. for seven cells. Myocytes were loaded with Indo-1 AM and not patch-clamped. External solution contained 1 μM oxodipine.

each cell, and mean values were calculated from all cells of each experiment. Myocytes injected with anti-α_{11}, anti-β_3, and anti-γ_5 antisense oligonucleotides showed a strong and similar inhibition (65–80%) of the ET-1-induced Ca^{2+} responses when compared with noninjected cells or cells injected with α_{11}, β_3, and γ_5 nonsense oligonucleotides (Fig. 7). These results suggest that the heterotrimeric G protein composed of α_{11}, β_3, and γ_5 subunits controls the Ca^{2+} response evoked by ET-1 application.

To verify that injection of antisense oligonucleotides directed against a G protein subunit specifically suppresses the expression of this subunit thus revealing its involvement in the ET-1-activated transduction coupling, we performed two types of control experiments. First, the cells were stained with rabbit anti-α_q, anti-α_{11}, anti-β_3, or anti-γ_5 antibodies and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. The immunofluorescence was quantified in gray level arbitrary units by using the MPL software of the confocal microscope (Fig. 8). The immunofluorescence signal for the $G\beta_3$ subunit was reduced by 70% in cells injected with anti-β_3 antisense oligonucleotides (n = 9; Fig. 8A), whereas it was not affected in cells injected with anti-β_1 (not shown) and anti-γ_5 antisense oligonucleotides (n = 12; Fig. 8A). Similarly, the immunofluorescence signal for the $G\gamma_5$ subunit was reduced by 75% in cells injected with anti-γ_5 antisense antibody (n = 10), whereas it was not affected in cells injected with anti-γ_3 (not shown) and anti-β_3 antisense oligonucleotides (n = 9; Fig. 8A). We had previously shown that in cells stained with an anti-α_q antibody, the immunofluorescence signal was inhibited in cells injected with anti-α_q antisense oligonucleotides, whereas it was not affected in cells injected with anti-α_{11} antisense oligonucleotides (Macrez-Leprêtre et al., 1997b). In cells stained with an anti-α_{11} antibody (Fig. 8B), the immunofluorescence signal was inhibited by anti-α_{11} antisense oligonucleotides (n = 11) but remained unchanged in cells injected with anti-α_q, anti-β_3, or anti-γ_5 antisense oligonucleotides. Second, we compared the effects of ET-1 with those of AII and caffeine in each cell studied. We have recently shown that angiotensin AT_1 receptors bind to a $G\alpha_{13}\beta_1\gamma_3$ protein, leading to increases in $[Ca^{2+}]_i$ (Macrez-Leprêtre et al., 1997a). In cells injected with anti-α_{11}, anti-β_3, and anti-γ_5 antisense oligonucleotides, increases in $[Ca^{2+}]_i$ evoked by AII and caffeine were unchanged when compared with control measurements (n = 29; Figs. 5 and 6). We noted unspecific effects of antisense oligonucleotides only at concentrations higher than 50 μM, i.e., 5 times higher than the concentration used in these experiments (n = 15). Taken together, these results indicate that suppression of ET-1-mediated effects by antisense oligonucleotides does not interfere with another signaling pathway (i.e., that of AII) and with the intracellular Ca^{2+} store.

$G\alpha_{11}$-Dependent ET-1-Induced Increase in $[Ca^{2+}]_i$. To determine which G protein subunit (α_{11} or $\beta\gamma$) was involved in effector activation, an anti-β_{com} antibody raised to the carboxyl terminus of the β subunit was dialyzed into the cell for 7 to 8 min, as reported previously (Macrez et al., 1997). Intracellular applications of 10 to 20 μg/ml anti-β_{com} antibody had no effect on the ET-1-induced increase in $[Ca^{2+}]_i$

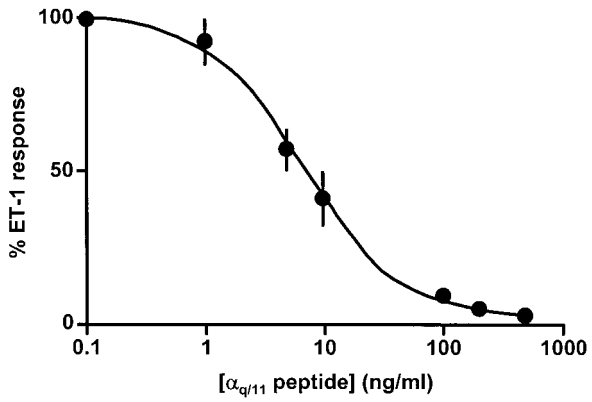

Fig. 4. Effects of a carboxyl-terminal $G\alpha_{q/11}$ peptide on the ET-1-induced Ca^{2+} release. Inhibition curve obtained with increasing concentrations of $G\alpha_{q/11}$ peptide. $[Ca^{2+}]_i$ values are expressed as a percentage of the response obtained with 10 nM ET-1. Data are means ± S.E.M. for five to nine cells. Myocytes were loaded with Indo-1 and held at −50 mV. External solution contained 1 μM oxodipine.

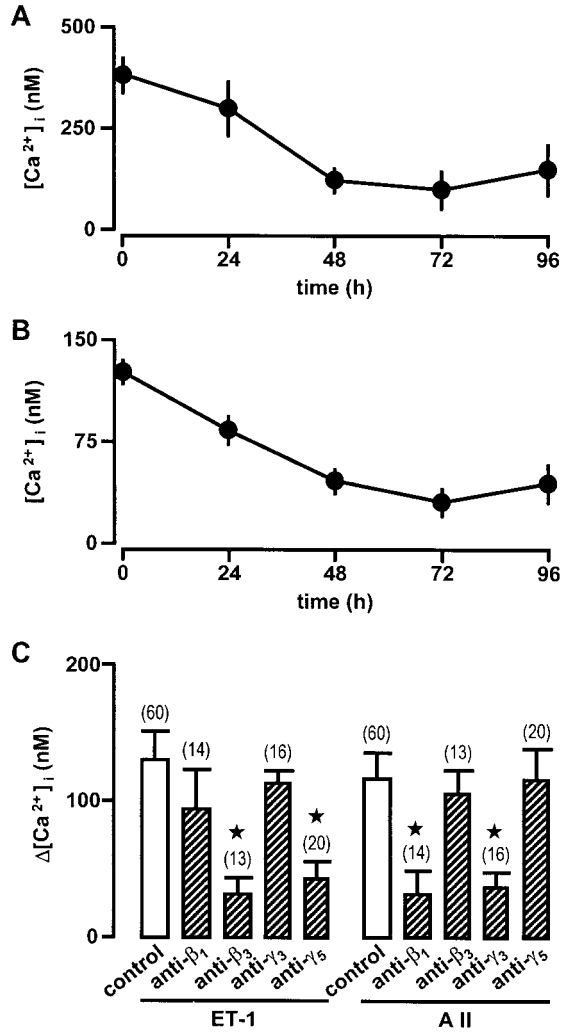

Fig. 5. Time course of inhibition of mediator-induced Ca^{2+} responses in cells injected with antisense oligonucleotides. A, mean amplitudes of Ca^{2+} responses evoked by 10 μM NE in cells injected with 10 μM anti-α_q antisense oligonucleotides in function of time after injection. B, mean amplitudes of Ca^{2+} responses evoked by 10 nM ET-1 in cells injected with 10 μM anti-α_{11} antisense oligonucleotides in function of time after injection. C, Ca^{2+} responses evoked by 10 nM ET-1 and 10 nM AII in the same cells 72 h after injection with 10 μM of the indicated antisense oligonucleotides. Controls correspond to Ca^{2+} responses in noninjected cells. Data are means ± S.E.M. for five to eight cells in (A) and (B) and for the number of cells tested indicated in parentheses in (C). Myocytes were loaded with Fura-2 AM and not patch-clamped.

(Fig. 9A) but removed the Ca^{2+} responses evoked by 10 nM AII (Fig. 9B). In a second set of experiments, a peptide corresponding to a fragment of βARK_1 was dialyzed into the cells for 7 to 8 min. Carboxyl-terminal fragments of βARK_1 have been used to bind $G\beta\gamma$ subunits and to block activation of effectors (Nair et al., 1995; Stehno-Bittel et al., 1995). Intracellular application of the peptide corresponding to the $G\beta\gamma$ binding domain of βARK_1 had no significant effects on the ET-1-induced increase in $[Ca^{2+}]_i$ (Fig. 9A), but removed the AII-induced increase in $[Ca^{2+}]_i$ (Fig. 9B). This is in agreement with previous data showing that angiotensin AT_1 receptor uses the $\beta\gamma$ dimers of G_{13} to transduce the signal leading to activation of Ca^{2+} channels and increase in $[Ca^{2+}]_i$ (Macrez et al., 1997). Taken together, these results suggest that the ET_A receptors transduce their signal to PLC through $G\alpha_{11}$, and not through $G\beta\gamma$ subunits.

We have previously shown that short (1-s) applications of NE induce increases in $[Ca^{2+}]_i$ that are strongly inhibited (~75%) in myocytes injected with anti-α_q antisense oligonucleotides (Macrez-Leprêtre et al., 1997b). The effects of durable (30-s) applications of 10 μM NE and 10 nM ET-1 were compared in Ca^{2+}-free solution in control myocytes and in myocytes injected with anti-α_q or anti-α_{11} antisense oligonucleotides. The Ca^{2+} responses evoked by NE and ET-1, which showed different amplitude and time course in control conditions (Fig. 10A), became similar after injection with anti-α_q antisense oligonucleotides (Fig. 10B). Interestingly, the delay between application of NE and the peak Ca^{2+} response increased from 0.9 ± 0.1 s (n = 12) in control conditions to 30 ± 5 s (n = 10) in cells injected with anti-α_q antisense oligonucleotides (Fig. 10B). In contrast, in cells injected with anti-α_{11} antisense oligonucleotides (Fig. 10C), NE evoked fast and transient Ca^{2+} responses as reported previously (Macrez-Leprêtre et al., 1997b), whereas ET-1 became ineffective. These results show that activation of $G\alpha_{11}$ by either NE or ET-1 receptors evokes a delayed and small Ca^{2+} response when compared with that observed when $G\alpha_q$ is activated.

Time Course of Inositol Phosphate Accumulation Induced by ET-1 and NE. Because the time courses of the Ca^{2+} responses evoked by ET-1 and NE in control conditions were largely different, we examined the time course of inositol phosphate accumulation induced by the two mediators in intact portal vein myocytes. With 10 mM LiCl, the unstimulated accumulations of total InsP and $InsP_3$ were 1136 ± 101 and 185 ± 15 dpm/10^6 cells (n = 3), respectively. After application of ET-1 (10 nM) or NE (10 μM) for 2 min, the total InsP-associated radioactivity increased against time

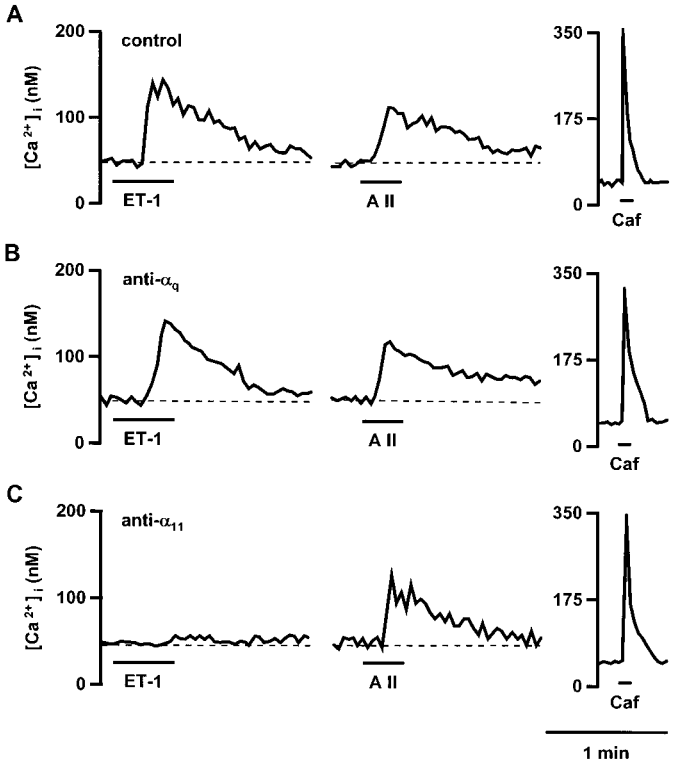

Fig. 6. Increases in $[Ca^{2+}]_i$ evoked by ET-1, AII and caffeine (Caf) in myocytes injected with 10 μM anti-sense oligonucleotides directed against the mRNAs of $G\alpha_q$ and $G\alpha_{11}$ proteins. Ca^{2+} responses were obtained in the same cells with successive applications of 10 nM ET-1, 10 nM AII, and 10 mM caffeine, separated by a 3-min interval, in noninjected control cells (A) and in cells injected with 10 μM anti-α_q (B), or anti-α_{11} (C) antisense oligonucleotides. Cells were used 3 days after nuclear injection of antisense oligonucleotides, were loaded with Fura-2 AM, and were not patch-clamped.

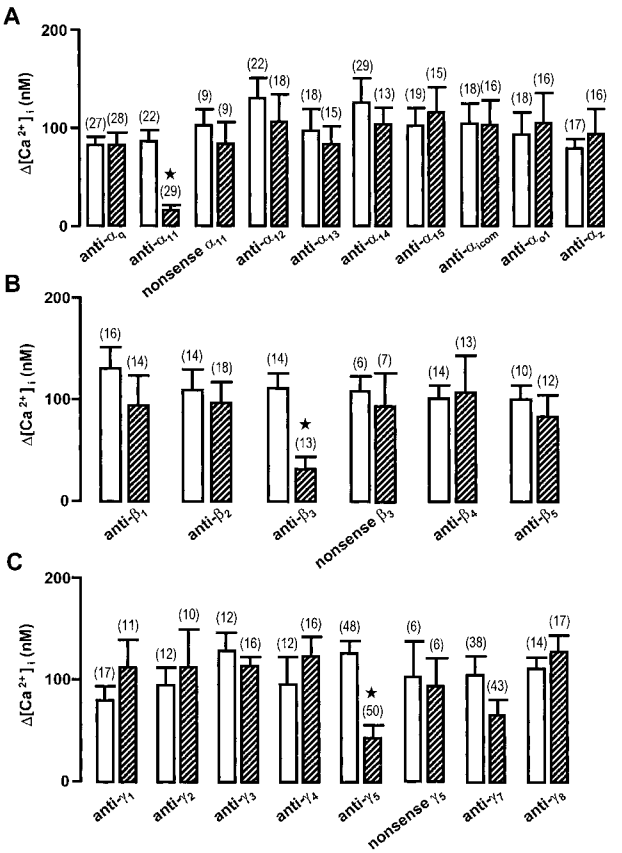

Fig. 7. ET-1-induced Ca^{2+} release in myocytes injected with 10 μM antisense oligonucleotides directed against the mRNAs of α, β, and γ subunits of G proteins. A, effects of anti-α_q, anti-α_{11}, anti-α_{12}, anti-α_{13}, anti-α_{14}, anti-α_{15}, anti-α_{icom}, anti-α_{01}, anti-α_z, and of α_{11} nonsense oligonucleotides on Ca^{2+} release evoked by 10 nM ET-1. B, effects of anti-β_1, anti-β_2, anti-β_3, anti-β_4, anti-β_5, and of β_3 nonsense oligonucleotides on ET-1-induced Ca^{2+} release. C, effects of anti-γ_1, anti-γ_2, anti-γ_3, anti-γ_4, anti-γ_5, anti-γ_7, anti-γ_8, and of γ_5 nonsense oligonucleotides on ET-1-induced Ca^{2+} release. Columns show means ± S.E.M. in noninjected cells (open columns) and in oligonucleotide-injected cells (hatched columns). Numbers in parentheses indicate the number of cells tested. ⋆, values significantly different from those obtained in noninjected cells ($P < .05$). Cells were loaded with Fura-2 AM and not patch-clamped. External solution contained 1 μM oxodipine.

and reached 2287 ± 130 dpm/10^6 cells and 2305 ± 145 dpm/10^6 cells, respectively (n = 3; Fig. 11A). These increases in InsP radioactivity were associated with a decrease in phosphoinositide radioactivity from 53316 ± 1850 dpm/10^6 cells in control to 43742 ± 1570 dpm/10^6 cells in the presence of the mediators for 2 min (n = 4). When focusing on the $InsP_3$ production (Fig. 11B), NE produced a rapid and transient stimulation of $InsP_3$ accumulation (~175% at 15 s) followed by a maintained stimulation (~50% at 2 min). In contrast, stimulation of $InsP_3$ accumulation induced by ET-1 increased progressively, reaching ~40% at 15 s and ~130% at 2 min (Fig. 11B). The sequential generation of inositol phosphates in the order of $InsP_3$, $InsP_2$, and $InsP_1$ (not shown) provides evidence that both ET-1 and NE mediate the stimulation of a PLC activity that degrades phosphatidylinositol bisphosphate. The profile of $InsP_3$ accumulation induced by ET-1 and NE appears to support the different time courses of the Ca^{2+} responses evoked by the two mediators.

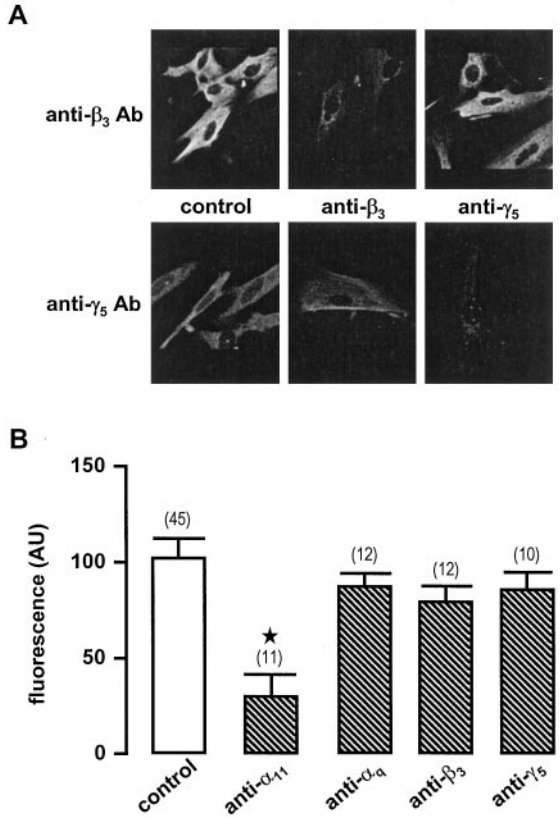

Fig. 8. Specific inhibition of $G\beta_3$, $G\gamma_5$, and $G\alpha_{11}$ protein expression in cells injected with anti-α, anti-β, and anti-γ antisense oligonucleotides. A: Top, myocytes stained 3 days after injection with rabbit anti-β_3 antibody. Visualization was obtained by staining with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200). Cells injected with anti-β_3 or anti-γ_5 antisense oligonucleotides were compared with control (noninjected cells) on the same glass slide. Bottom, myocytes stained with rabbit anti-γ_5 antibody. Cells injected with anti-γ_5 or anti-β_3 antisense oligonucleotides were compared with control (noninjected cells). B, diagram depicting $G\alpha_{11}$ expression in cells stained with anti-α_{11}, anti-α_q, anti-β_3, and anti-γ_5 antibody. Cells injected with anti-α_{11} and anti-α_q antisense oligonucleotides were compared with control (noninjected cells). Open column (noninjected cells) and hatched columns (antisense oligonucleotide-injected cells) show means ± S.E.M. with the number of cells tested in parentheses. Immunofluorescence was expressed in arbitrary units (AU). ⋆, values significantly different from those obtained under control conditions ($P < .05$).

Discussion

Our results show that, in rat portal vein myocytes, the G protein heterotrimer $G\alpha_{11}\beta_3\gamma_5$ is coupled to ET_A receptors and activates a transduction pathway leading to Ca^{2+} release from the intracellular store. This conclusion is based on experiments using oligonucleotides to block expression of G protein subunits, and a synthetic peptide corresponding to the carboxyl terminus of $G\alpha_{q/11}$ subunits to disrupt the ET_A receptor-evoked activation of G proteins. Use of antibodies raised against the carboxyl-terminus of Gβ subunits and of a synthetic peptide corresponding to the carboxyl terminus of βARK_1 fragments has allowed us to identify the G protein subunits interacting with the effector.

The ET-1-induced increase in $[Ca^{2+}]_i$ displayed a transient and a more or less sustained Ca^{2+} phase. Although reduced in amplitude, the transient Ca^{2+} phase persists after the removal of external Ca^{2+} or in the presence of oxodipine (an L-type Ca^{2+} channel antagonist), and is considered to be the result of a Ca^{2+} release from the intracellular store. The sustained Ca^{2+} phase depends on transmembrane Ca^{2+} influx, as it is removed in Ca^{2+}-free solution and reduced in the presence of oxodipine. These results are in good agreement with previous data obtained in other smooth muscles (Minowa et al., 1997; Saita et al., 1997). Although both ET_A and ET_B receptors have been identified in various smooth muscles including portal vein (Saita et al., 1997; Mickley et al., 1997; Wang et al., 1997), our data are consistent with the idea that only the ET_A receptor subtype is involved in intra-

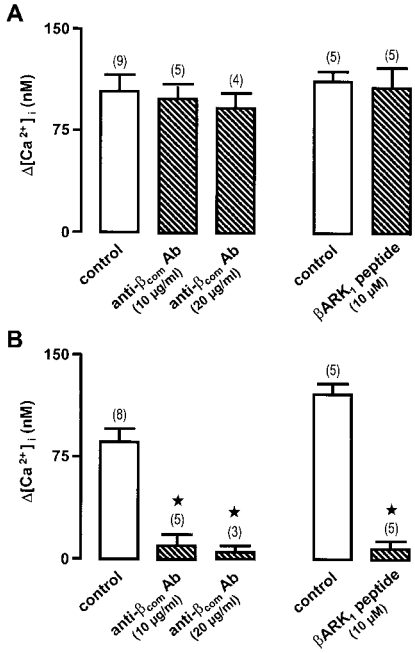

Fig. 9. Effects of anti-β_{com} antibody and βARK_1 peptide on ET-1- and AII-induced Ca^{2+} responses. A, Ca^{2+} responses evoked by 10 nM ET-1 in cells held at −50 mV in which antibody or peptide were dialyzed intracellularly for 7 to 8 min with Indo-1 (hatched columns) compared to control cells (open columns). Data show means ± S.E.M. with the number of experiments in parentheses. External solution contained 1 μM oxodipine. B, Ca^{2+} responses evoked by 10 nM AII in cells held at −50 mV in which antibodies or peptide were dialyzed intracellularly for 7 to 8 min (hatched columns) compared to control cells (open columns). Data show means ± S.E.M. with the number of experiments in parentheses. ⋆, Values significantly different from those obtained under control conditions ($P < .05$).

cellular Ca^{2+} release; BQ123 (an antagonist of ET_A receptors) inhibits, in a concentration-dependent manner, the ET-1-induced Ca^{2+} release, whereas BQ788 (an antagonist of ET_B receptors) is ineffective.

Antisense oligonucleotides have been used previously in these myocytes to identify the G protein heterotrimers selectively coupling α_1-adrenoceptors and angiotensin AT_1 receptors to increase in $[Ca^{2+}]_i$ (Macrez-Leprêtre et al., 1997a,b). The present results indicate that anti-α_{11} oligonucleotide injection into the nucleus of myocytes selectively inhibited the ET_A-induced Ca^{2+} release by ~75%, a value similar to that obtained with injection of anti-β_3 or anti-γ_5 antisense oligonucleotides. These inhibitions are correlated with reductions in the expression of the corresponding G protein subunits immunostained by anti-α_{11}, anti-β_3, and anti-γ_5 antibodies. The fact that the ET_A-induced Ca^{2+} release was selectively inhibited after blockade of each subunit expression (α_{11}, β_3, or γ_5) supports the concept that activation of G protein by ET-1 requires the presence of all three α_{11}, β_3, and γ_5 subunits. In addition, our results indicate that the $\beta\gamma$ subunits ($\beta_3\gamma_5$) associated with the α_{11} subunit in the ET-1-induced Ca^{2+} release differ from those associated with both α_q and α_{13} subunits in the transduction pathways activated by NE and AII (Macrez-Leprêtre et al., 1997a,b).

Antisense oligonucleotide blockade alone cannot distinguish which parts of the G protein are required for interaction with the ET_A receptor and the effector. Intracellular application of the carboxyl-terminal $\alpha_{q/11}$ peptide inhibits, in a concentration-dependent manner, the ET_A receptor-induced Ca^{2+} release, indicating that the receptor binds to the extreme carboxyl terminus of the α_{11} subunit to promote dissociation of the heterotrimeric G protein. The observation that ET_A receptor-induced Ca^{2+} release is not affected by an anti-β_{com} antibody or by a synthetic peptide derived from a βARK_1 fragment that selectively binds to the $G\beta\gamma$ subunits (Nair et al., 1995; Stehno-Bittel et al., 1995) supports the idea that the stimulation of the effector occurs via the α subunit of the G_{11} protein. In addition, ET-1 triggers a sequential generation of inositol phosphates in the order of $InsP_3$, $InsP_2$, and $InsP_1$, indicating that ET_A receptor activation leads to the stimulation of a PLC-degrading phosphatidylinositol bisphosphate. Although the PLC-β isoforms mediating Ca^{2+} release from the intracellular store have not been identified in rat portal vein myocytes, one may speculate that the $G\alpha_{11}$ subunit predominantly activated a PLC-β to hydrolyze phosphatidylinositol bisphosphate. The slow kinetics of ET_A-induced Ca^{2+} release reaching a peak within 30 to 40 s can be associated with the delayed accumulation of $InsP_3$. This is in contrast with the fast activation of PLC by $G\alpha_q$ subunit in response to activation of α_1-adrenoceptors, which produces a fast increase in $InsP_3$ (≤15 s) associated with a fast and large Ca^{2+} response. Although $G\alpha_q$ and $G\alpha_{11}$ have been reported to be similarly efficient in vitro and in overexpression experiments, with regard to stimulation of various PLC-β isoforms (Wu et al., 1993), our results indicate

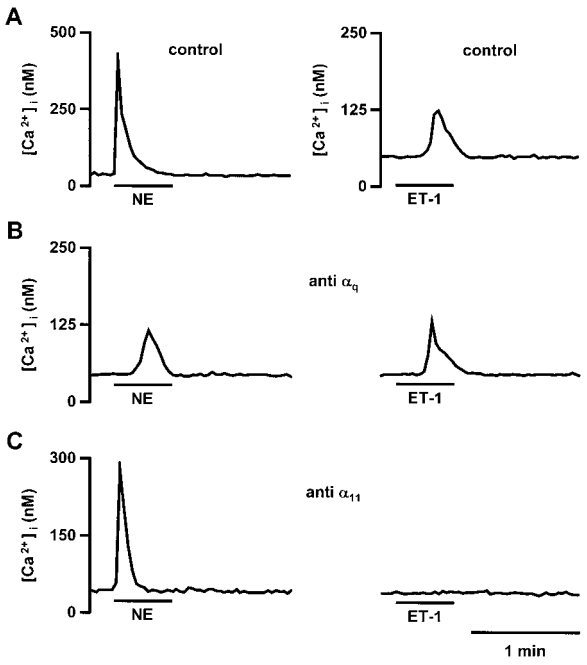

Fig. 10. Increase in $[Ca^{2+}]_i$ evoked by NE and ET-1 in myocytes injected with anti-α_q or anti-α_{11} antisense oligonucleotides. A, in Ca^{2+}-free (0.5 mM EGTA)-containing solution, Ca^{2+} releases obtained in the same noninjected cells (after 3 days of primary culture) in response to 10 μM NE or 10 nM ET-1. B, Ca^{2+} releases evoked by NE and ET-1 in the same cells, injected with 10 μM anti-α_q antisense oligonucleotides. C, Ca^{2+} releases evoked by NE and ET-1 in the same cells, injected with 10 μM anti-α_{11} antisense oligonucleotides. Myocytes were loaded with Fura-2 AM and not patch-clamped. Similar results were obtained in nine to fourteen cells.

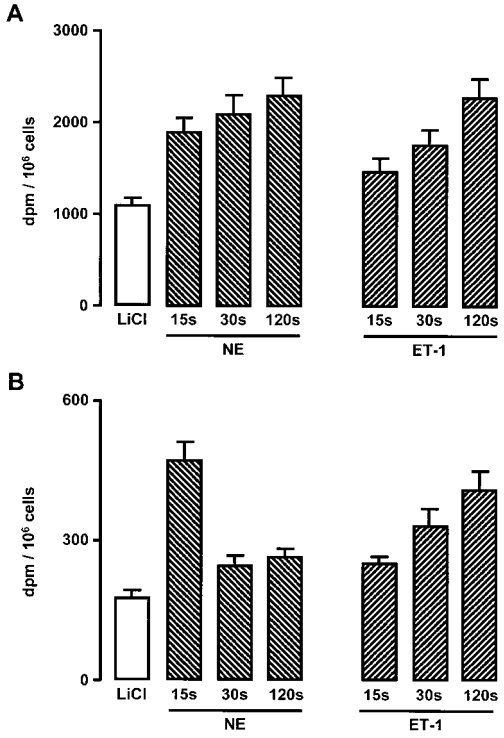

Fig. 11. Time course of NE and ET-1-induced accumulation of inositol phosphates in rat portal vein myocytes preincubated for 3 days with [^{3}H]inositol. The myocytes were incubated for 10 min with 10 mM LiCl before addition of 10 μM NE or 10 nM ET-1. Stimulations were stopped at times indicated, and individual [^{3}H]inositol phosphates were separated. A, total InsPs accumulation induced by NE and ET-1. Results are expressed in dpm/10^6 cells and are the means ± S.E.M. of three independent experiments, each done in duplicate. B, $InsP_3$ accumulation induced by NE and ET-1. In the presence of LiCl (basal), the mean total [^{3}H] radioactivity was 98280 ± 3350 dpm/10^6 cells (n = 3). The percentage of radioactivity present in free inositol, total phosphoinositide, and total inositol phosphate pools was 44.59 ± 1.55%, 54.25 ± 1.52% and 1.16 ± 0.11%, respectively (n = 3).

that the $G\alpha_{11}$-activated Ca^{2+} responses evoked by ET-1 are smaller and slower than the $G\alpha_q$-activated Ca^{2+} responses evoked by NE in the same cells. However, the $G\alpha_{11}$-mediated Ca^{2+} responses have similar amplitude and time course when activated by ET-1 or NE (after inhibition of the $G\alpha_q$ subunit). Several explanations may arise: 1) G_{11} protein may be distributed homogeneously in the plasma membrane but in a limited amount, so that its activation leads to a delayed stimulation of PLC-β. In contrast, G_q proteins have been reported to be clustered in membrane domains associated with cytoskeletal proteins including F-actin (Ibarrondo et al., 1995; Cornea et al., 1998). If ET-1 receptors are localized outside these G_q-rich membrane domains, this may also explain why ET-1-induced Ca^{2+} release is specifically transduced through G_{11} and not through G_q; 2) $G\alpha_{11}$ subunits may regulate the activity of intermediate effector proteins involved in the transduction of the ET_A-induced signal; 3) It has been recently shown that the γ_5 subunit is preferentially linked with membrane components rather than with F-actin, in contrast to γ_3 and γ_{12} subunits, which show a specific association with F-actin (Ueda et al., 1997). It is, however, unlikely that the $G\gamma_5$ subunit is responsible for the delayed effect of $G\alpha_{11}$ subunit on PLC activation and Ca^{2+} release because different Gγ subunits (γ_2 or γ_3) are dissociated from the NE-activated G_{11} protein; and 4) Finally, it is possible that the rate of dissociation of the activated $G\alpha_{11}\beta_3\gamma_5$ protein may be much slower than that of the activated $G\alpha_q\beta_1\gamma_3$ protein or that a more effective α_{11}-selective guanine nucleotide-regulatory protein (RGS-type) leads to a very fast reassociation of α_{11} subunits with $\beta_3\gamma_5$ subunits. In vitro experiments are needed to evaluate these different possibilities.

In conclusion, we show that, in portal vein myocytes, the ET_A receptor binds to a specific $G\alpha_{11}\beta_3\gamma_5$ heterotrimer, leading to a slow accumulation of $InsP_3$ and the subsequent delayed Ca^{2+} release from the intracellular store.

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Send reprint requests to: Dr J. Mironneau, Laboratoire de Physiologie Cellulaire et Pharmacologie Moléculaire, CNRS ESA 5017, Université de Bordeaux II, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France. Email: jean.mironneau@esa5017.u-bordeaux2.fr