Distinct Functions of Gq and G11 Proteins in Coupling α1-Adrenoreceptors to Ca\(^{2+}\) Release and Ca\(^{2+}\) Entry in Rat Portal Vein Myocytes

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In this study, we identified the subunit composition of G\(_i\) and G\(_{11}\) proteins coupling α\(_1\)-adrenoreceptors to increase in cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in rat portal vein myocytes maintained in short-term primary culture. We used intranuclear antisense oligonucleotide injection to inhibit selectively the expression of subunits of G protein. Increases in [Ca\(^{2+}\)]\(_i\), were measured in response to activation of α\(_i\)-adrenoreceptors, angiotensin AT\(_1\) receptors, and caffeine. Antisense oligonucleotides directed against the mRNAs coding for α\(_i\), α\(_{11}\), β\(_{1\alpha}\), γ\(_{9}\), and γ\(_{12}\) subunits selectively inhibited the increase in [Ca\(^{2+}\)]\(_i\) activated by α\(_i\)-adrenoreceptors. A corresponding reduction of the expression of these G protein subunits was immunochemically confirmed. In experiments performed in Ca\(^{2+}\)-free solution only cells injected with anti-α\(_i\) antisense oligonucleotides displayed a reduction of the α\(_i\)-adrenoreceptor-induced Ca\(^{2+}\) release. In contrast, in Ca\(^{2+}\)-containing solution, injection of anti-α\(_{11}\) antisense oligonucleotides suppressed the α\(_i\)-adrenoreceptor-induced stimulation of the store-operated Ca\(^{2+}\) influx. Agents that specifically bound G\(_i\) by subunits (anti-β\(_{1\alpha}\) com antibody and overexpression of a β-adrenergic receptor kinase carboxyl-terminal fragment) had no effect on the α\(_i\)-adrenoreceptor-induced signal transduction. Taken together, these results suggest that α\(_i\)-adrenoreceptors utilize two different G\(_i\) subunits to increase [Ca\(^{2+}\)]\(_i\). G\(_{11}\) may activate phosphatidylinositol 4,5-bisphosphate hydrolysis and induce release of Ca\(^{2+}\) from intracellular stores. G\(_{11}\) may enhance the Ca\(^{2+}\)-activated Ca\(^{2+}\) influx that replenishes intracellular Ca\(^{2+}\) stores.

In vascular smooth muscle, activation of α\(_i\)-adrenoreceptors stimulates phospholipase C-β which hydrolyzes phosphatidylinositol-4,5-bisphosphate to yield diacylglycerol and inositol 1,4,5-trisphosphate. In portal vein myocytes, the α\(_i\)-adrenoreceptors are coupled to phospholipase C-β through G proteins which have been identified to be G\(_i\) and/or G\(_{11}\), on the basis of intracellular applications of an anti-G\(_{11}\) antibody. Inositol 1,4,5-trisphosphate subsequently releases Ca\(^{2+}\) from the intracellular store. Diacylglycerol in concert with cellular Ca\(^{2+}\) activates protein kinase C which, in turn, stimulates Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels (1–2). Both norepinephrine-induced Ca\(^{2+}\) release and Ca\(^{2+}\) entry lead to a biphasic rise of the cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). Although the G protein subtypes are currently defined by their α subunits, of which 23 (including splice variants) are known, a functionally active heterotrimeric G protein includes an α, β, and γ subunit. Up to now, 5 different β and 11 different γ subunits have been identified (4). Thus, a great number of heterotrimers composed of specific α, β, and γ subunits may exist and be involved in signal transduction pathways. In many cases, the coupling between receptor and G protein may appear unselective since one receptor may activate more than one G protein and thus initiate more than one signal-transduction pathway. However, there are many examples showing that different receptors activate the same heterotrimeric G protein to regulate the same effector system (5–6). The question remains whether in portal vein myocytes α\(_i\)-adrenoreceptors recognize a single heterotrimeric G protein (G\(_i\) or G\(_{11}\)) to induce a rise of [Ca\(^{2+}\)]\(_i\), or whether different heterotrimers varying in the composition of α, β, and γ subunits are required for this coupling.

Antisense oligonucleotides can be used for selective and transient knockout of cellular proteins (7). So far, microinjection is the only method available that allows for controlled intranuclear application of antisense oligonucleotides. Studies with this method in GH\(_3\) cells have revealed that the M\(_4\) muscarinic receptor in GH\(_3\) cells couples to the G protein trimer consisting of α\(_{3\beta}\)γ\(_4\), the somatostatin receptor to the trimer α\(_{3}\)β\(_1\)γ\(_3\), and the galanin receptor to the trimers α\(_{4}\)β\(_2\)γ\(_2\) and α\(_{4}\)β\(_3\)γ\(_4\) to inhibit voltage-dependent Ca\(^{2+}\) channels (8–11). In RBL-2H3-hm1 cells, G proteins composed of G\(_{11}\)/α\(_{11}\)β\(_1\)γ\(_3\) are required for effective coupling between the stably expressed human muscarinic m\(_4\) receptor and cellular increase in [Ca\(^{2+}\)]\(_i\) (12).

In the present study, we used the method of intranuclear microinjection of antisense oligonucleotides directed against individual G protein subunits and determined the composition of G\(_i\) and G\(_{11}\) proteins mediating the α\(_i\)-adrenoreceptor-induced increase in [Ca\(^{2+}\)]\(_i\) in short-term primary cultured rat portal vein myocytes. We show that α\(_i\)-adrenoreceptors utilize G proteins composed of α\(_i\), α\(_{11}\), β\(_1\), β\(_3\), γ\(_2\), and γ\(_3\) subunits to

1 The abbreviations used are: [Ca\(^{2+}\)]\(_i\), cytoplasmic Ca\(^{2+}\) concentration; PBS, phosphate-buffered saline solution; nt, nucleotide(s); βARK, β-adrenergic receptor kinase.
increase [Ca\(^{2+}\)], and that the effector coupling is mediated by the \(\alpha\) subunits. Go\(_a\) subunit may activate release of Ca\(^{2+}\) from intracellular stores and Go\(_{11}\) subunit may modulate intracellular store-dependent Ca\(^{2+}\) entry.

**EXPERIMENTAL PROCEDURES**

**Microinjection of Oligonucleotides—**Isolated myocytes from rat portal vein were obtained by enzymatic dispersion, as described previously (1). Cells were seeded at a density of about 10\(^5\) cells per mm\(^2\) on glass slides imprinted with squares for localization of injected cells and maintained in short-term primary culture in medium M199 containing 2% fetal calf serum, 2% glutamine, 10\(\mu\)M ascorbic acid, 0.1\(\mu\)g/ml penicillin, and 20 \(\mu\)g/ml streptomycin; they were kept in an incubator gassed with 95% air, 5% CO\(_2\) at 37 \(^\circ\)C. The sequences of the oligonucleotides used in this study were determined by sequence comparison and multiple alignment using Mac Molly Tetra software (Soft Gene, Berlin, Germany). Oligonucleotides were from MWG-Biotech (Ebersberg, Germany) or synthesized in a DNA synthesizer (Milligen, model 8600); for synthesis of phosphorothioate oligonucleotides, the method described by Iyer et al. (13) was used. Injection of oligonucleotides was performed into the nucleus of myocytes by a manual injection system (Eppendorf, Hamburg, Germany). The injection solution contained 10 \(\mu\)M oligonucleotides in water; approximately 10 \(\mu\)l were injected into the nucleus of myocytes, as described for oligonucleotides. The signal was processed (Hamamatsu DVS 3000) by correcting the intracellular stores and G\(_q/11\) complex (Eppendorf, Hamburg, Germany) or synthesized in a DNA synthesizer (Milligen, model 8600); for synthesis of phosphorothioate oligonucleotides, the method described by Iyer et al. (13) was used. Injection of oligonucleotides was performed into the nucleus of myocytes by a manual injection system (Eppendorf, Hamburg, Germany). The injection solution contained 10 \(\mu\)M oligonucleotides in water; approximately 10 \(\mu\)l were injected into the nucleus of myocytes, as described for oligonucleotides. The signal was processed (Hamamatsu DVS 3000) by correcting

**Chemicals and Drugs—**M199 medium was from Flow Laboratories (Puteaux, France). Fetal calf serum was from Flobio (Courbevoie, France). Streptomycin, penicillin, glutamate, and pyruvate were from Life Technologies, Inc. (Paisley, UK). Fura-2, Fura-2/AM, and anti-\(\alpha\) (CN 371752) antibody were from Calbiochem (Meudon, France). Norepinephrine, ruvalcaine, and proparanol were from Sigma (St. Quentin Fallavier, France). Angiotensin II, CGP42112A (\(N\)-ac-nicotinyl-Tyr-Lys\((N\)-\(N\)-CBZ-Arg\)-His-Pro-Ile-\(OH\)) was from Neosystem Laboratories (Strasbourg, France). Caffeine was from Merck (Nogent sur Marne, France). Anti-\(\alpha\)1 (SC 384), anti-\(\beta\) (SC 378), and anti-\(\gamma\) (SC 375) were from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was from Immunotech (Marseille, France). Green fluorescent protein S65T expression construct was from Clontech (Palo Alto, CA). Oxidipine was a gift from Dr. Galiano (IQB, Madrid, Spain).

**RESULTS**

**Identification of Subunit Composition of the G Proteins Coupling \(\alpha\)1-Adrenoceptors to Increase in [Ca\(^{2+}\)] in Single Rat Portal Vein Myocytes—**We previously showed that in portal vein myocytes, activation of \(\alpha\)1-\(\beta\)1-adrenoceptors mediates both release of Ca\(^{2+}\) from intracellular stores and stimulation of voltage-dependent Ca\(^{2+}\) channels through a G\(_q/11\) protein that activates phospholipase C-\(\beta\) (1). In order to identify the heterotrimeric G proteins involved in the \(\alpha\)1-adrenoceptor-induced increase in [Ca\(^{2+}\)], we injected phosphorothioate-modified antisense oligonucleotides directed against \(\alpha\), \(\beta\), and \(\gamma\) subunits into the nucleus of vascular myocytes. By measuring the norepinephrine-induced increases in [Ca\(^{2+}\)] after injection of an antisense oligonucleotide directed against both \(\alpha\) and \(\alpha\)11 subunits (anti-\(\alpha\) \(\alpha\) \(\alpha\) \(\alpha\)11), the highest inhibition (76 ± 12\%, \(n = 7\)) was obtained 3 days after injection (data not shown). Therefore, all further measurements were performed 3 days after injection. We measured the increase in [Ca\(^{2+}\)], induced by successive applications of 10 \(\mu\)M norepinephrine (in the presence of 10 \(\mu\)M ruvalcaine and 1 \(\mu\)M propranolol) to the cells both with \(\alpha\) and \(\beta\)-adrenoceptors and with \(\alpha\) and \(\gamma\)-adrenoceptors (100 nM each). The responses of antisense oligonucleotide-injected cells were normalized to the area of the glass slide to sense or scrambled oligonucleotide-injected cells or non-injected cells outside this marked area. This procedure guaranteed that antisense oligonucleotide-injected cells...
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Fig. 1. Increase in [Ca2+]i evoked by norepinephrine (NE), caffeine (Caf), and angiotensin II (A II) in myocytes injected with 10 μM antisense oligonucleotides directed against the mRNAs of Goq, Gβ11, and Gα14 proteins. Ca2+ responses were obtained for successive applications of 10 μM norepinephrine, 10 mM caffeine, and 10 nM angiotensin II in non-injected myocytes (A) and in myocytes injected with anti-αq (B), anti-α11 (C), or anti-α14 (D) antisense oligonucleotides. Myocytes were used 3 days after nuclear microinjection of oligonucleotides. Norepinephrine, caffeine, and angiotensin II were ejected from a glass pipette close to the cell for the period indicated on the records. External solution contained 10 mM rauwolscine, 1 μM propranolol, and 1 μM CGP42112A.

were always compared with control cells that were otherwise grown, treated, and analyzed under identical conditions, i.e. culture, incubation, microinjection, and loading with fura-2AM. The increase in [Ca2+]i was measured for each cell, and mean values were calculated from all cells of each experiment. Myocytes injected with 10 μM antisense oligonucleotides directed against the mRNAs encoding for αq subunit (anti-αq) showed strongly reduced (75%) α1-adrenoreceptor-induced Ca2+ responses, as compared with non-injected cells (Figs. 1B and 2A). Myocytes injected with 10 μM antisense oligonucleotides directed against the α11 (anti-α11) subunit showed reduced (40%) α1-1-adrenoreceptor-induced Ca2+ responses as well (Figs. 1C and 2A). Interestingly, injection of both anti-αq and anti-α11 oligonucleotides (anti-αq+α11) did not induce a larger decrease of the α1-adrenoreceptor-induced Ca2+ response than that evoked by anti-αq oligonucleotides alone (Fig. 2A). Myocytes injected with 10 μM antisense oligonucleotides directed against αq1 and αq2 (anti-αqcom), α12 (anti-α12), and α14 (anti-α14) subunits were comparable with non-injected cells. None of them showed a significant reduction in [Ca2+]i responses evoked by activation of α1-adrenoreceptors (Figs. 1D and 2A).

Furthermore, we used sense αq1com and scrambled anti-αq11com oligonucleotides which do not efficiently anneal to the target sequence of Goq11 subunits. Ca2+ responses evoked by activation of α1-adrenoreceptors were not significantly affected by injection of these oligonucleotides (non-injected cells = 418 ± 53 nM, n = 12; sense αq11-complinjected cells = 379 ± 42 nM, n = 9; and scrambled anti-αq11com-injected cells = 390 ± 32 nM, n = 13).

In order to identify the β subunits involved in the α1-adrenoreceptor-induced Ca2+ response, we used antisense oligonucleotides directed against the mRNAs coding for β1, β2, β3, β5, and β6 subunits (Fig. 3A). Injection of 10 μM oligonucleotides directed against the β1 (anti-β1) and β5 (anti-β5) subunits significantly reduced the α1-adrenoreceptor-induced Ca2+ responses. Inhibition of the α1-adrenoreceptor-induced Ca2+ responses evoked by the oligonucleotides directed against the β1 and β3 subunits (80% and 40%, respectively) was quantitatively similar to those induced by the oligonucleotides directed against the αq and α11 subunits. No significant reduction of the α1-adrenoreceptor-induced Ca2+ response was seen in myocytes injected with oligonucleotides directed against β2 (anti-β2), β4 (anti-β4), and β6 (anti-β6) subunits. Injection of 10 μM antisense oligonucleotides against different γ subunits showed that anti-γ1, anti-γ5, anti-γ11, and anti-γ14 oligonucleotides had no significant effect on the α1-adrenoreceptor-induced Ca2+ responses (Fig. 3B). In contrast, injection of anti-γ14 and γ14 oligonucleotides resulted in significant reduction of the α1-adrenoreceptor-induced Ca2+ responses (73% and 40%, respectively). Amplification of cDNA fragments revealed that in portal vein smooth muscle five β (β1–β5) and six γ (γ1–γ6) subunits were expressed (data not shown). These results indicate that α1-adrenoreceptors utilize G proteins composed of αq, α11, β1, β5, γ2, and γ6 subunits to increase [Ca2+]i.

Specificity of the Antisense Oligonucleotides—In order to verify that injection of antisense oligonucleotides directed against specific G protein subunits suppressed involvement of these subunits in the α1-adrenoreceptor-activated transduction couplings, we performed two types of control experiments. First, we showed that injection of a specific antisense oligonucleotide inhibited only the immunofluorescence signal of the corresponding G protein subunit and did not affect the expression of other subunits. Cells were stained with either anti-αq or anti-α11, specific antibodies, and the immunofluorescence was quantified by using the MPL software of the confocal microscope (Fig. 4A). Cells injected with either of the two different antisense oligonucleotides and non-injected cells located on the same glass slide were compared with each other, so that the
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**Fig. 2.** Peak increase in [Ca^{2+}] evoked by norepinephrine (A), caffeine (B), and angiotensin II (C) in myocytes injected with 20 μM anti-α_1q antisense oligonucleotides and 10 μM anti-α_1b antisense oligonucleotides. Myocytes were used 3 days after injection. Norepinephrine (10 μM), caffeine (10 mM), and angiotensin II (10 nM) were ejected from a glass pipette close to the cell in the presence of 10 nM rauwolscine, 1 μM propranolol, and 1 μM CGP42112A. Data are given as mean ± S.E. with the number of experiments in parentheses, in non-injected cells (open bar), and in cells injected with antisense oligonucleotides (filled bar). * values significantly different from those obtained under control conditions (p < 0.05).

staining procedure 3 days after injection of oligonucleotides was identical for the different cells. In cells injected with anti-α_1q oligonucleotides the immunofluorescence signal for the G_{α_q} subunit was reduced by 78% (n = 9), whereas that for the G_{α_11} subunit was only slightly affected (10%, n = 8). Similarly, in cells injected with anti-α_11 antisense oligonucleotides, the immunofluorescence signal for the G_{α_11} subunit was reduced by 70% (n = 7), whereas that for the G_{α_q} subunit was only slightly affected (12%, n = 12). Then we tested the effects of injection of anti-β_3, anti-β_2, anti-γ_2, and anti-γ_3 antisense oligonucleotides on the expression of G_{α_q/α_11} subunits by staining with an anti-α_1q/α_11 antibody (Fig. 4B). Although the immunofluorescence signal appeared to be slightly reduced in cells injected with β and γ antisense oligonucleotides (between 15 and 20%, n = 21), only the cells injected with the α_1q/α_11 antisense oligonucleotides showed a considerable inhibition of the immunofluorescence signal (85%, n = 7). Finally, we verified that in cells stained with an anti-β_3 antibody, the immunofluorescence signal was inhibited in cells injected with anti-β_3 antisense oligonucleotides (77%, n = 13), whereas it was slightly affected in cells injected with either anti-β_3 or anti-γ_3 antisense oligonucleotides (22%, n = 12). In cells stained with an anti-γ_2 antibody, the immunofluorescence signal was inhibited in cells injected with anti-γ_2 antisense oligonucleotides (81%, n = 12), whereas it was slightly affected in cells injected with either anti-γ_1 or anti-γ_2 antisense oligonucleotides (22%, n = 12). In cells stained with an anti-γ_2 antibody, the immunofluorescence signal was inhibited in cells injected with anti-γ_2 antisense oligonucleotides (81%, n = 12), whereas it was slightly affected in cells injected with either anti-γ_1 or anti-γ_2 antisense oligonucleotides (22%, n = 12). In cells stained with an anti-γ_2 antibody, the immunofluorescence signal was inhibited in cells injected with either anti-γ_1 or anti-γ_2 antisense oligonucleotides (22%, n = 12). In cells stained with an anti-γ_2 antibody, the immunofluorescence signal was inhibited in cells injected with either anti-γ_1 or anti-γ_2 antisense oligonucleotides (22%, n = 12). In cells stained with an anti-γ_2 antibody, the immunofluorescence signal was inhibited in cells injected with either anti-γ_1 or anti-γ_2 antisense oligonucleotides (22%, n = 12). In cells stained with an anti-γ_2 antibody, the immunofluorescence signal was inhibited in cells injected with either anti-γ_1 or anti-γ_2 antisense oligonucleotides (22%, n = 12). In cells stained with an anti-γ_2 antibody, the immunofluorescence signal was inhibited in cells injected with either anti-γ_1 or anti-γ_2 antisense oligonucleotides (22%, n = 12). In cells stained with an anti-γ_2 antibody, the immunofluorescence signal was inhibited in cells injected with either anti-γ_1 or anti-γ_2 antisense oligonucleotides (22%, n = 12). In cells stained with an anti-γ_2 antibody, the immunofluorescence signal was inhibited in cells injected with either anti-γ_1 or anti-γ_2 antisense oligonucleotides (22%, n = 12). In cells stained with an anti-γ_2 antibody, the immunofluorescence signal was inhibited in cells injected with either anti-γ_1 or anti-γ_2 antisense oligonucleotides (22%, n = 12).

Second, we compared the effects of norepinephrine to those of angiotensin II (activating AT_1 receptors, 22) and caffeine (releasing Ca^{2+} from the intracellular stores) in each cell studied (Fig. 1). We recently showed that activation of angiotensin AT_1 receptors releases intracellularly stored Ca^{2+} without involving inositol 1,4,5-trisphosphate but through a Ca^{2+} release mechanism activated by Ca^{2+} influx through L-type Ca^{2+} channels (22–23). In the same cells injected with anti-α_1q/α_11, anti-α_1b, and anti-α_11 antisense oligonucleotides, angiotensin II (in the presence of 1 μM CGP42112A) and caffeine evoked large Ca^{2+} responses, whereas α_1-adrenoreceptor-induced Ca^{2+} responses were inhibited (Fig. 2, A–C). We noted unspecific effects of phosphorothioate-modified antisense oligonucleotides only when oligonucleotides were injected at concentrations of 50 μM, i.e. 5 times higher than the concentration used in these experiments (n = 15). Taken together, these data indicate that suppression of α_1-adrenoreceptor-activated effects by antisense oligonucleotides does not interfere with other signaling pathways (e.g. that of angiotensin II) and with the intracellular Ca^{2+} stores of vascular myocytes.

**Different G Proteins Are Involved in α_1-Adrenoreceptor-induced Ca^{2+} Release and Ca^{2+} Entry—** We previously showed that norepinephrine activates Ca^{2+} entry even if the intracel-
lular Ca\(^{2+}\) store is not completely emptied (3), possibly by involving a mechanism independent of Ca\(^{2+}\) store depletion. Therefore, experiments were performed in external Ca\(^{2+}\)-free solution (containing 0.5 mM EGTA) on myocytes injected with anti-a\(_1\)- or anti-a\(_{11}\) antisense oligonucleotides. As illustrated in Fig. 5, the a\(_1\)-adrenoreceptor-induced Ca\(^{2+}\) release was inhibited in cells injected with anti-a\(_1\) oligonucleotides but was not affected in cells injected with anti-a\(_{11}\) oligonucleotides. These results suggest different tasks for G\(_{q}\) and G\(_{11}\) proteins, i.e. induction of Ca\(^{2+}\) release from intracellular stores and induction of Ca\(^{2+}\) influx from extracellular medium, respectively.

The sarcoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor thapsigargin is commonly used to study the Ca\(^{2+}\) entry pathway activated after Ca\(^{2+}\) store depletion (24). Application of 1 \(\mu\)M thapsigargin depleted the intracellular Ca\(^{2+}\) stores (evidenced by the lack of caffeine-induced Ca\(^{2+}\) response) and increased the [Ca\(^{2+}\)] level to 118 \(\pm\) 17 nM (n = 29; Fig. 6A). In the continuous presence of 10 \(\mu\)M oxodipine (a light-resistant dihydropyridine) to block voltage-dependent Ca\(^{2+}\) channels, activation of a\(_1\)-adrenoreceptors during the thapsigargin-induced Ca\(^{2+}\) plateau evoked a further rise in [Ca\(^{2+}\)], reaching 161 \(\pm\) 15 nM (n = 5; Fig. 6A). This a\(_1\)-adrenoreceptor-induced Ca\(^{2+}\) response was never observed in Ca\(^{2+}\)-free, 0.5 mM EGTA-containing solution (n = 15). In cells injected with anti-a\(_{11}\) oligonucleotides, the a\(_1\)-adrenoreceptor-induced rise in [Ca\(^{2+}\)], was not observed (n = 7; Fig. 6B), although thapsigargin produced a progressive increase in [Ca\(^{2+}\)], reaching 123 \(\pm\) 13 nM (n = 7). In contrast, in cells injected with anti-a\(_{11}\) oligonucleotides, the amplitude of the a\(_1\)-adrenoreceptor-induced increase in [Ca\(^{2+}\)], (52 \(\pm\) 12 mM, n = 6) was comparable with that obtained in non-injected control cells (48 \(\pm\) 14 nM, n = 11; Fig. 6B).

Depletion of caffeine-sensitive intracellular Ca\(^{2+}\) stores induced a Ca\(^{2+}\) response similar to that evoked by activation of a\(_1\)-adrenoreceptors. Fig. 7 displays representative traces of...
Fig. 7. Effects of norepinephrine in the continuous presence of caffeine in cells injected with anti-α1 and anti-α11 antisense oligonucleotides. A, Ca2+-responses evoked by external application of 10 μM caffeine (50 s) in 2 mM Ca2+-containing solution (a) and in Ca2+-free solution with 0.5 mM EGTA for 10 s (b). B, during the caffeine-induced sustained Ca2+ response, norepinephrine (10 μM) was ejected in Ca2+-containing solution (a, c, and d) or in Ca2+-free solution with 0.5 mM EGTA (b), in non-injected cells (a and b), or in cells injected with 10 μM anti-α1 (c) or anti-α11 (d) oligonucleotides. All the experiments were performed in the presence of 10 μM oxidine, 10 μM rauwolscine, and 1 μM propranolol.

These experiments. In the continuous presence of 10 μM oxidine, application of 10 μM caffeine for 50 s in the external solution (Fig. 7Aa) produced a large transient increase in [Ca2+]i (375 ± 20 nM, n = 16) and a sustained plateau of 70 ± 9 nM (n = 16). The rapid initial increase in [Ca2+]i was reduced in Ca2+-free solution (298 ± 25 nM, n = 10), and the subsequent sustained phase was absent (Fig. 7Ab). This indicates that in venous myocytes caffeine is able to induce a transient increase in [Ca2+]i, due to Ca2+ release and a sustained phase representing Ca2+ entry into the cell from the extracellular space. As illustrated in Fig. 2B, the caffeine-induced Ca2+ responses were not affected by inhibition of the expression of any Go subunits, including Go5 and Go11 subunits. Activation of α1-adrenoreceptors (in Ca2+-containing solution) during the second sustained phase of the caffeine-evoked Ca2+ response resulted in a 2-fold increase in [Ca2+]i, which reached 134 ± 16 nM (n = 23; Fig. 7Ba). The α11-adrenoreceptor-induced enhancement of [Ca2+]i, during the second phase of the caffeine-induced Ca2+ response (64 ± 6 nM, n = 23) was not observed at all when norepinephrine was applied without Ca2+ and in the presence of 0.5 mM EGTA (n = 12; Fig. 7Bb), indicating that it corresponded to a Ca2+ entry from the extracellular medium. In myocytes injected with the anti-α11 antisense oligonucleotides, the α11-adrenoreceptor-induced Ca2+ entry in the continuous presence of caffeine (55 ± 9 nM, n = 6) was similar to that obtained in non-injected cells (61 ± 8 nM, n = 6; Fig. 7Bc). In contrast, in cells injected with anti-α11 antisense oligonucleotides, no α11-adrenoreceptor-induced Ca2+ entry in the continuous presence of caffeine was observed (n = 8; Fig. 7Bd). These results further support the idea that Go11 subunit is involved in the modulation of store-operated Ca2+ entry by α1-adrenoreceptors.

Effector Coupling Is Dependent on α1 and α11 Subunits—The anti-α1/α11 antibody and antisense oligonucleotide block of the α11-adrenoreceptor-induced Ca2+ response cannot distinguish whether α or βγ subunits are transducing the signal that activate Ca2+ release from the intracellular stores or Ca2+ entry. To determine which G protein subunits were involved in the α1-adrenoreceptor-mediated effects, an anti-βcom antibody was dialyzed into the cell by the patch pipette for 3 min. Anti-βcom antibody (10 μg/ml in pipette solution) had no effect on the α1-adrenoreceptor-induced Ca2+ response (Fig. 8A) since neither the transient peak (control = 305 ± 30 nM; in the presence of anti-βcom antibody = 295 ± 35 nM; n = 10) nor the sustained plateau (control = 55 ± 4 nM; in the presence of anti-βcom antibody = 53 ± 6 nM, n = 10) were significantly affected. In the same cells, the anti-βcom antibody inhibited the sustained angiotensin II-induced Ca2+ response in a concentration-dependent manner, with a maximal inhibition obtained at an antibody concentration of 10 μg/ml (n = 10).2 In a second set of experiments, we overexpressed a carboxyl-terminal fragment of βARK1 by intranuclear microinjection of expression plasmids containing cDNA inserts coding for βARK1. βARK1 has been used to bind βγ subunits and block activation of effectors (25–26). Overexpression of βARK1 had no effect on the α11-adrenoreceptor-induced Ca2+ response (Fig. 8B) since neither the transient peak (control = 290 ± 25 nM; in the presence of βARK1 = 285 ± 20 nM; n = 12) nor the sustained plateau (control = 65 ± 5 nM; in the presence of βARK1 = 60 ± 8 nM; n = 12) were significantly affected. In contrast, the angiotensin II-induced Ca2+ response was inhibited when βARK1 was overexpressed in the same cells (n = 12).2 Taken together, our results indicate that application of anti-βcom antibody and βARK1, both able to bind free βγ subunits, had no effects on both Ca2+ release and Ca2+ entry induced by activation of α1-adrenoreceptors.

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DISCUSSION

G Protein Subunits Mediating α₁-Adrenergoreceptor-induced [Ca²⁺], Increase—Here we show that the α₁-adrenergoreceptor-induced increase in [Ca²⁺]i in rat portal vein myocytes involves both Gz and G₁₁ proteins. Using nuclear injection of antisense oligonucleotides corresponding to the mRNA sequences coding for G protein α₁, β₁, and γ₁ subunits, we identified G protein heterotrimers composed of α₁q, α₁1, β₁, β₁q, γ₁, and γ₁q involved in the coupling of α₁-adrenergoreceptors to Ca²⁺ release and intracellular store-dependent Ca²⁺ entry.

We proved the specificity of the injected oligonucleotides by studying the increase in [Ca²⁺], induced by application of two hormonal stimuli (norepinephrine and angiotensin II) and caffeine. All three substances release Ca²⁺ from the same intracellular store via different pathways. Caffeine is known to release Ca²⁺ by acting on the ryanodine-sensitive Ca²⁺ channels of the sarcoplasmic reticulum. In portal vein myocytes, we recently showed that activation of angiotensin AT₁ receptors evoked an increase in [Ca²⁺], which depended on both activation of L-type Ca²⁺ channels and opening of ryanodine-sensitive Ca²⁺ channels of the sarcoplasmic reticulum, without involving inositol 1,4,5-trisphosphate generation (22); this effect is mediated by G proteins different from Gα₁₁ protein, as shown by the absence of effect of intracellular application of anti-α₁/γ₁1 antibodies (27). Therefore, we used caffeine as a control for the availability of the Ca²⁺ stores in control and oligonucleotide-injected cells, whereas the angiotensin AT₁ receptor-induced response was used as a control for the specificity of antisense oligonucleotide effects on G protein subunits.

Antisense oligonucleotides against Gα subunits showed no effect on the caffeine-induced Ca²⁺ responses excluding nonantisense effects on intracellular Ca²⁺ stores (see Fig. 2). Such non-antisense but sequence-specific effects have been described for oligonucleotides directed against c-myc and p53 (28–29). To have a second control for the specificity of the antisense oligonucleotides, we compared injected cells to non-injected control cells located on the same glass slide in each experiment. We show that the value of the increase in [Ca²⁺], induced by norepinephrine is not significantly different comparing noninjected cells to injected cells with α₁γ/1 sense or scrambled antisense oligonucleotides and antisense oligonucleotides directed against G protein subunits which are not involved in the α₁-adrenergoreceptor-induced increase in [Ca²⁺], (see Figs. 1 and 2). Furthermore, injection of antisense oligonucleotides directed against Gα subunits involved in α₁-adrenergoreceptor-mediated effects did not change the increase in [Ca²⁺], achieved by angiotensin II or caffeine (see Fig. 2, B–C). To demonstrate the extent of the antisense knockout effects, we studied protein depletion by immunocytochemistry of Gα subunits. The results of these experiments revealed that the time course by which anti-α₁q or anti-α₁1 antisense oligonucleotides were effective in suppressing functional receptor-mediated effects paralleled suppression of the Gαq or Gα₁₁ protein level which decreased maximally within 3 days after injection. Similar results were recently obtained using γ₁q, γ₁, and α₁/α₁qq antisense oligonucleotides in different cells (8, 12, 30). In addition, we demonstrated that injection of antisense oligonucleotides directed against a given G protein subunit did not modify significantly the expression of other G protein subunits (see Fig. 4). Therefore, the fact that anti-β₁, β₁, γ₁, and γ₁γ₁ oligonucleotides inhibit the α₁-adrenergoreceptor-induced [Ca²⁺], increase cannot be related to an inhibition of the Gα subunit expression and means that these β and γ subunits are necessary for activation of the Gz and G₁₁ proteins by α₁-adrenergoreceptors. Since anti-α₁q, -β₁q, and -γ₁γ₁ oligonucleotides largely inhibited the α₁-adrenergoreceptor-induced increase in [Ca²⁺], whereas anti-α₁1, β₁, and -γ₁γ₁ oligonucleotides produced a limited inhibition, one may speculate that the composition of G protein heterotrimers required for the two phases of the α₁-adrenergoreceptor-induced Ca²⁺ response is α₁/β₁/γ₁ and α₁1/β₁/γ₁.

Distinct Functions of Gα and G₁₁ Proteins—The experiments presented in this work suggest that different heterotrimeric G proteins mediate Ca²⁺ release from intracellular stores and Ca²⁺ entry in response to stimulation of α₁-adrenergoreceptors. Evidence supporting this proposal are the following.

1) Cells injected with a mixture of anti-α₁q and anti-α₁1 oligonucleotides (anti-α₁q,11) showed no further reduction of α₁-adrenergoreceptor-induced Ca²⁺ response compared with cells injected with either anti-α₁q or anti-α₁1/α₁q oligonucleotides. 2) In Ca²⁺-free solution, anti-α₁q oligonucleotides strongly reduced the α₁-adrenergoreceptor-induced Ca²⁺ release, whereas anti-α₁1 oligonucleotides were without effect, suggesting that Gα₁1 protein-mediated modulation of oxodipine-resistant Ca²⁺ entry required a preceding Ca²⁺ release mediated by Gαq protein. 3) The oxodipine-resistant Ca²⁺ entry evoked by activation of α₁-adrenergoreceptors in the presence of thapsigargin or caffeine was selectively suppressed by anti-α₁1 oligonucleotides. Therefore, we propose that the Gα₁1 subunit may enhance the α₁-adrenergoreceptor-induced Ca²⁺ entry activated by a previous release of Ca²⁺ from intracellular stores. Several types of Ca²⁺ entry mechanisms have been described in various cellular systems (31). In smooth muscle cells, activation of Ca²⁺ entry by application of mediators (histamine, endothelin, vasopressin) has been reported (32–34). We show that caffeine also induces intracellular store-operated Ca²⁺ entry in rat portal vein myocytes (see Fig. 6), and we used caffeine and thapsigargin pretreatment to study the modulation of the store-operated Ca²⁺ entry by activation of α₁-adrenergoreceptors. This dihydropyridine-resistant Ca²⁺ entry may be mediated by cation channels. Interestingly, a nonselective cation channel, the Drosophila trp channel, has been shown to be stimulated in a membrane-confined way by Gα₁₁ protein (35), and a similar nonspecific cation channel permeable for Ca²⁺ ions has been previously identified in portal vein myocytes (36). Furthermore, experiments performed in an epithelial cell line have shown that overexpression of Gαq protein increases Ca²⁺ release-activated Ca²⁺ influx (37). Thus, the ubiquitously expressed Gαq family may have a general role in modulating Ca²⁺ entry through Ca²⁺-permeable nonselective cation channels which may be controlled by both the filling state of Ca²⁺ stores (38) and, as shown here, more directly by G protein. Recently, the composition of G proteins coupling the stably expressed human muscarinic m₁ receptor in the rat basophilic leukemia cell line (RBL-2H3-hm1) to increase in [Ca²⁺], has been determined by using the same method and the same antisense oligonucleotides (12). In these cells, the authors have proposed that a complex of G protein subunits, i.e. Gα₁m₁γ₁/β₁q/γ₁qγ₁, is activated by m₁ receptors. As [Ca²⁺], measurements were performed in Ca²⁺-containing solution, a differential coupling of Gα₁ and Gα₁₁ subunits to Ca²⁺ release and Ca²⁺ entry, respectively, could not have been detected. Finally, transient expression of a carboxyl-terminal fragment of bARKⅤI that scavenged Gβγ subunits after their dissociation from the receptor-activated heterotrimer had no effect on α₁-adrenergoreceptor-induced Ca²⁺ responses suggesting that Gβγ subunits did not display direct interactions with the effectors, i.e. phospholipase C and Ca²⁺-, permeable nonselective cation channels. This conclusion is supported by the results showing that intracellular application of anti-β₁q antibody did not modify significantly the α₁-adrenergoreceptor-mediated Ca²⁺ release and Ca²⁺ entry. The possibility that Gβγ subunits that are dissociated from both Gαq and Gα₁₁ subunits after activation of α₁-adrenergoreceptors may activate
other cellular effectors remains to be investigated.

In conclusion, we show that in rat venous myocytes, the Gq proteins may couple by their α subunits endogenous Gq-adrenoceptors to phospholipase C, whereas the Gα11 proteins, activated at the same time by the same receptors, may couple to Ca2+ entry. These results point out distinct functions of Gq and G11 in receptor-activated [Ca2+]i increase.

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G Protein Coupling of α1-Adrenoceptors