Cell Cycle-Dependent Coupling of the Vasopressin V$_{1a}$ Receptor to Different G Proteins

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Arginine vasopressin (AVP) regulates biological processes by binding to G protein-coupled receptors. In Swiss 3T3 fibroblasts, expressing the V$_{1a}$ subtype of vasopressin receptors, AVP mobilizes calcium from intracellular stores. In proliferating cells, the AVP-induced increase in intracellular calcium concentration ([Ca$^{2+}$])$_i$ was mediated by G proteins of the G$_i$ family, which are insensitive to pertussis toxin (PTX) pretreatment of the cells. In quiescent cells, the AVP-induced increase in [Ca$^{2+}$]$_i$ was partially PTX-sensitive, suggesting an involvement of G$_i$ proteins. We confirmed this by photoaffinity labeling of G proteins in Swiss 3T3 cell membranes activated by AVP. In Swiss 3T3 cells arrested in the G$_0$/G$_1$ phase of the cell cycle, the AVP-induced increase in [Ca$^{2+}$]$_i$ was also partially PTX-sensitive but was PTX-insensitive in cells arrested in other phases of the cell cycle. The blocking effect of PTX pretreatment in G$_i$/G$_i$ cells was mimicked by microinjection of antisense oligonucleotides suppressing the expression of the G$_{0i}$ subunits. These results were confirmed by microinjection of antibodies directed against the C terminus of G protein $\alpha$-subunits. The data presented indicate that in Swiss 3T3 fibroblasts synchronized in the G$_0$/G$_1$ phase of the cell cycle the V$_{1a}$ receptor couples to G$_{0i11}$ and G$_{i10}$ to activate the phospholipase C-\(\beta\), leading to release of intracellular calcium.

The biological effects of the neuropeptide hormone arginine vasopressin (AVP) are induced by binding of AVP to specific membrane receptors that are members of the GTP-binding protein (G protein)-coupled receptor (GPCR) superfamily. To date, three AVP receptor subtypes (V$_{1a}$, V$_{1b}$, and V$_2$) have been cloned and characterized according to their tissue distribution and functional properties. The V$_2$ receptor subtype is predominantly expressed in the kidney, mediating the antidiuretic effects of AVP. The activated V$_2$ receptor couples to the heterotrimeric G protein G$_{11}$ and activates adenylyl cyclases (1, 2). Two V$_1$-receptor subtypes have been cloned (3) and can be pharmacologically differentiated by their binding affinities to various AVP agonists and antagonists (4, 5). While the expression of the V$_{1b}$ receptor subtype is restricted mainly to the central nervous system (6), the V$_{1a}$ receptor is expressed in different neuronal and nonneuronal tissues. In nonneuronal tissues, the V$_{1a}$ receptor induces a wide range of physiological effects such as contraction of vascular smooth muscles, stimulation of hepatic glycogenolysis and cell proliferation (for a review, see Refs. 7 and 8).

By binding to GPCRs, many hormones and neurotransmitters activate various subtypes of the phospholipase C-\(\beta\) (PLC-\(\beta\)) (9). These enzymes catalyze the hydrolysis of phosphatidylinositol 4,5-biphosphate to the two second messenger molecules, diacylglycerol and inositol 1,4,5-trisphosphate. While diacylglycerol stimulates the activation of protein kinase C and Ca$^{2+}$ influx, inositol 1,4,5-trisphosphate binds to receptors in the endoplasmic reticulum leading to the release of Ca$^{2+}$ from the intracellular stores and increases the intracellular calcium concentration ([Ca$^{2+}$]$_i$). The activity of most PLC-\(\beta\) isoforms is regulated by G protein $\alpha$-subunits of the G$_{11}$ family ($\alpha_{i1}, \alpha_{i11}, \alpha_{i14}, \alpha_{i15/16}$). Thus, hydrolysis of phosphatidylinositol 4,5-biphosphate and the subsequent increase in [Ca$^{2+}$]$_i$ is mediated by receptors coupling to G proteins of the G$_i$ family that are not inhibited by pertussis toxin (PTX). However, in some cell types, e.g. cells of the hematopoietic origin, the receptor-mediated increase in [Ca$^{2+}$]$_i$ can be blocked by pretreatment of the cells with PTX (10, 11), suggesting the involvement of PTX-sensitive G proteins of the G$_i$ family in the activation of PLC-\(\beta\). Indeed the PLC-\(\beta_2\) and -\(\beta_4\) isoforms have been found to be activated by $\beta y$ dimers released from G$_i$ proteins (11–13). Nevertheless, PLC-\(\beta_1\), -\(\beta_3\), and -\(\beta_4\) are preferentially activated by the $\alpha$ subunits of the G$_{11}$ family (14–16).

Both V$_1$ receptor subtypes, V$_{1a}$ and V$_{1b}$, have been shown to activate PLC-\(\beta\) leading to a transient increase in [Ca$^{2+}$]$_i$ (17, 18). In most of the cell lines studied, the AVP-induced increase in [Ca$^{2+}$]$_i$ involves coupling of the V$_{1a}$ receptor to the PTX-insensitive G proteins G$_{11}$ and G$_{11}$ and subsequent activation of PLC-\(\beta\) (19, 20).

Swiss 3T3 fibroblasts, which are derived from embryonic mouse cells, endogenously express several GPCRs for neuropeptides such as bradykinin, bombesin, or AVP and, therefore, have widely been used to study intracellular signal transduction pathways induced by neuropeptides. In addition, these cells provide a useful model to study the regulation of cell growth. Swiss 3T3 cells express the V$_{1a}$ receptor subtype (21). Using the technique of photoaffinity labeling of receptor-activated G$_i$ subunits with the GTP analog [$\alpha$-\(^{32}\)P]GTP azidoani-
lized (aaGTP), it has been shown that the AVP receptor in Swiss 3T3 cells activates Gq and G11. No activation of Gi by AVP was detected in this study (22). These results are in accordance with other studies showing that the AVP-activated induction of PLC-β through the V1a receptor in various cell systems is insensitive to pretreatment of the cells with PTX (for a review, see Ref. 23). However, AVP-induced stimulation of DNA synthesis has been reported to be partially sensitive to PTX (24), suggesting that the V1a receptor subtype principally is able to couple to PTX-sensitive G proteins in these cells. Remarkably, AVP-induced 3H-labeled inositol phosphate formation was also inhibited by 23% by PTX pretreatment of the Swiss 3T3 cells in that study.

Herein, we studied the functional coupling of the AVP receptors in Swiss 3T3 cells to G proteins leading to the release of calcium from intracellular stores. We will provide evidence that AVP-evoked calcium release is mediated solely by the V1a receptor subtype and that coupling of these receptors to Gq and G11 is dependent on the cell cycle.

**EXPERIMENTAL PROCEDURES**

**Materials—**[Arg]vasopressin, [1-β-mercapto-β-cyclopenanthylene propionic acid], O-Me-Tyr(3)-Arg9-vasopressin, aphidicolin, and nocodazole were obtained from Sigma. Fura-2/AM and PTX were purchased from Calbiochem. 125I-Phenylacetyl-d-Tyr(Me)-Phe-Asn-Arg-Pro-Arg-Tyr-NH2 was from PerkinElmer Life Sciences. Phosphorothioate oligonucleotides were purchased from Eurogentec (Seraing, Belgium). The generation and the specificity of the polyclonal antibodies directed against Gαq (AS266 and AS86, C-terminal antibodies), Gαq1 (AS370), and Gαs (AS404) has been described (25). Additional affinity-purified polyclonal antibodies against Gαq1 (SC-392) and Gαs (SC392) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Cell Culture and Membrane Preparation—**Swiss 3T3 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere containing 10% CO2 and 90% air at 37 °C. Pretreatment with PTX was performed by adding 100 ng/ml to the culture medium for 24 h prior to experiments. For measurement of intracellular calcium concentration ([Ca2+]i), and for photoaffinity labeling of G proteins, cells were cultured until they reached confluence. Before processing for experiments, cells were washed twice with PBS and incubated for 1 h either in fresh DMEM containing 10% FCS (proliferating cells) or DMEM without FCS (quiescent cells). Synchronization of cell growth in individual phases of the cell cycle was performed with exponentially growing Swiss 3T3 using the following protocols. For G1/G0 phase, cells were washed with PBS and cultured in DMEM without FCS for 12 h, cultured another 16 h in fresh medium containing 10% FCS (G2/G1 cells) and were detached with the culture flask with Hank’s balanced salt solution buffer (118 mM NaCl, 4.6 mM KCl, 1 mM MgCl2, 10 mM glucose, 5 mM EGTA, 20 mM HEPES, pH 7.2). For G1/S phase, aphidicolin (5 μM/ml) was added to freshly changed DMEM containing 10% FCS (proliferating cells) or DMEM without FCS to arrest cells in G1 (0 h). After 16 h, nocodazole (500 ng/ml) was added to freshly changed DMEM containing 10% FCS and cells were detached 20 h later with Hanks’ balanced salt solution buffer. For G2/M phase, nocodazole (500 ng/ml) was added to freshly changed DMEM containing 1% FCS (proliferating cells) and synchronized cells were detached after 16 h with 0.5% trypsin. For membrane preparation, Swiss 3T3 cells were washed twice with PBS; scraped off the tissue culture flask; resuspended in ice-cold buffer consisting of 100 mM NaCl, 0.5 mM EDTA, 50 mM KH2PO4, and homogenized by nitrogen cavitation. Membranes were then sedimented at 100,000 × g for 1 h and resuspended in 10 mM triethanolamine (pH 7.4). Protein concentration was determined with a BCA protein assay kit (Pierce), and membranes were stored at -70 °C until they were used.

**Flow Cytometric Analysis of Cell Cycle—**For analysis of cell cycle progression, samples of differently cultured cells were treated with ice-cold ethanol. Cell nuclei were sedimented by centrifugation, stained with propidium iodide in Ca2+- and Mg2+-free PBS supplemented with 0.1% (w/v) Triton X-100 (20 μg/ml) and resuspended in DiO-EdU-labeling buffer containing 0.05% Triton X-100. DNA fragmentation using a fluorescein-activated cell sorter (FACScan, Heidelberg, FRG). Cells with an unreplicated complement of DNA (2n) were assigned to G0/G1 phase, cells with a fully replicated complement of DNA (4n) were assigned to the G2/M phase, and those cells containing an intermediate amount of DNA were assigned to the G1/S phase.
excitation of 340 and 380 nm and an emission wavelength of 510 nm. Before cells were stimulated with 100 nM AVP, extracellular Ca\(^{2+}\) was depleted by the gradual addition of 20-μM aliquots of Tris/EGTA (20 mM) until a stable base line was reached. For measurement of [Ca\(^{2+}\)]\(i\), in single cells, cells were grown on glass coverslips and loaded with 5 μM fura 2-AM in HBSS for 30 min. The cells were washed twice in incubation buffer, mounted on an inverted microscope (Zeiss Axiovert 100), and washed with incubation buffer supplemented with 1 mM EGTA instead of CaCl\(_2\). Determination of [Ca\(^{2+}\)]\(i\), in individual cells was performed with an imaging system (T.I.L.L. Photonics) as described (27). [Ca\(^{2+}\)]\(i\) values were calculated using a formula provided by Grynkiewicz et al. (28). The Δ[Ca\(^{2+}\)] was calculated as the difference of maximum [Ca\(^{2+}\)]\(i\), after stimulation with AVP and basal [Ca\(^{2+}\)]\(i\), prior to stimulation. The significance of the results was determined by a U test, and errors are given as S.E.

Radioligand Binding Assay—Binding assays on Swiss 3T3 membranes were performed in 250 μl of incubation medium (1 mM mg/ml bovine serum albumin, 0.5 μg/ml bacitracin, 0.05 μg/ml soy bean trypsin inhibitor, pH 7.4) containing 1–300 pM of the linear AVP antagonist 11β[1-phenylacetly-l-tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH2 (PerkinElmer Life Sciences). The reaction was performed in duplicate and started by the addition of membranes (5–15 μg/assay), and the mixture was incubated 1 h at 20 °C. The reaction was stopped by adding 4 ml of ice-cold buffer (10 mM Tris-HCl, pH 7.4, 5 mM MgCl\(_2\), 0.5 mM EDTA) allowed by filtration through GF/C glass microfiber filters (Whatmann, Maidstone, UK) presoaked in 0.3% polyethyleneimine (Fluka, Buchs, Switzerland). Filters were washed four times with 4 ml of ice-cold buffer, and radioactivity was counted in a γ-counter (COBRA 5002, Packard, Meriden, MA). Nonspecific binding was determined in the presence of 3 μM unlabeled AVP. Binding data were analyzed by nonlinear regression using Prism software (GraphPad Software, San Diego, CA). The significance of the results was determined using a U test, and errors are given as S.D.

Immunoblot Analysis of G Proteins—Aliquots of membrane preparations of G\(_{α}/G_{β}\), and G\(_{α}\)-subunits in membranes from proliferating and quiescent Swiss 3T3 cells. Membranes (200 μg of protein/tube) were photolabeled in the presence (−) or presence (+) of 100 nM AVP with [α-\(^{32}\)P]GTP 

**Fig. 1.** Stimulation of photoaffinity labeling of G protein α-subunits in membranes from proliferating and quiescent Swiss 3T3 cells. Membranes (200 μg of protein/tube) were photolabeled in the presence (−) or presence (+) of 100 nM AVP with [α-\(^{32}\)P]GTP aziadonilide. Membranes were solubilized and incubated with α\(_{α1}\) and α\(_{α-com}\) antisera. Immunoprecipitation was performed as described. Precipitated proteins were subjected to SDS-PAGE. Shown is an autoradiogram of an SDS gel with the position of the molecular mass marker for 43 kDa given on the left.

In proliferating cells, the AVP-induced increase in [Ca\(^{2+}\)]\(i\) was more prominent in the absence and presence of AVP. In addition, an antibody directed against all three subtypes of the G\(_{α}\) proteins (AS266) precipitated more α-GTP-labeled α\(_{α}-subunit in AVP-stimulated membranes than in control membranes (Fig. 1, compare lane 7 with lane 8, 557 and 547 arbitrary units, respectively). The significance of the results was determined using a U test, and errors are given as S.D.

**RESULTS**

In a previous publication, the G protein coupling of the AVP receptor in Swiss 3T3 cells was studied using the technique of photoaffinity labeling of receptor-activated G proteins with αaGTP and subsequent immunoprecipitation of activated G protein α-subunits with subtype-specific antibodies (22). In these experiments, membranes prepared from Swiss 3T3 cells grown under regular cell culture conditions were used, i.e. with change of the medium every second day, and cells were split before confluence was reached. The addition of AVP to the Swiss 3T3 membranes induced activation of both G\(_{αq}\) and G\(_{α11}\), but no activation of G\(_{α}\) was detected (22).

We used the same technique, but compared membranes from cells grown under various cell culture conditions. The cells were grown in the same medium until they reached confluence for several days, and then the conditioned medium was replaced for 6 h by either fresh culture medium (DMEM) containing 10% FCS (“proliferating cells”) or DMEM devoid of FCS (“quiescent cells”). In membranes prepared from proliferating cells as well as in membranes prepared from quiescent cells, we confirmed the AVP-induced activation of G\(_{αq}\) and G\(_{α11}\) (Fig. 1A, lanes 1 and 2, 2470 and 222 arbitrary units, respectively, and lanes 5 and 6, 4846 and 1760 arbitrary units, respectively). In membranes prepared from quiescent cells, however, incorporation of αaGTP into G\(_{αq}\) and G\(_{α11}\) was more prominent in the absence and presence of AVP. In addition, an antibody directed against all three subtypes of the G\(_{α}\) proteins (AS266) precipitated more α-GTP-labeled α\(_{α}-subunit in AVP-stimulated membranes than in control membranes (Fig. 1, compare lane 7 with lane 8, 643 and 222 arbitrary units, respectively), indicating the coupling of AVP receptors to G\(_{α}\) proteins. No activation of G\(_{β}\) proteins by AVP could be detected in proliferating cells (see in Fig. 1, lanes 3 and 4, 557 and 547 arbitrary units, respectively). The AVP-induced activation of G\(_{α}\) proteins in membranes prepared from quiescent Swiss 3T3 cells leads to a functional coupling to an effector system, the AVP-induced increase in [Ca\(^{2+}\)]\(i\) was measured in Swiss 3T3 cell suspensions. The AVP-induced increase of [Ca\(^{2+}\)]\(i\) was completely abolished by preincubation of the cells with a linear peptide antagonist specific for the V\(_{1a}\) receptor [1-β-mercapto-β-cyclopentamethlylene propionic acid, O-Me-Tyr-Arg]\(_{6}\)-vasopressin (7, 31), indicating that the activation of PLC-β and subsequent increase in [Ca\(^{2+}\)]\(i\) in Swiss 3T3 cells is mediated solely by the V\(_{1a}\) receptor subtype (data not shown). Fig. 2 shows the mean values of the amplitude of the AVP-induced increase in [Ca\(^{2+}\)]\(i\) in Swiss 3T3 cells cultured under the described cell culture conditions and pretreated with or without PTX (100 ng/ml for 24 h). In quiescent cells, AVP induced a significant (p < 0.05) higher Ca\(^{2+}\) amplitude than in proliferating cells (Fig. 2, open bars). The basal calcium concentrations were not significantly different under both conditions (data not shown). In proliferating cells, the AVP-induced increase in [Ca\(^{2+}\)]\(i\), was not inhibited by preincubation of the cells with PTX (see Fig. 2). In contrast,
pretreatment of quiescent cells with PTX inhibited the AVP-induced increase in [Ca\(^{2+}\)], by 50% (see Fig. 2).

To confirm that the dual coupling of the V\(_{1a}\) receptor to G proteins of the G\(_{q/11}\) and the G\(_i\) subfamilies is a characteristic of the quiescent state of the cells, we analyzed the AVP-induced increase in [Ca\(^{2+}\)], in various phases of the cell cycle. To clearly define the quiescent state of cell growth as G\(_0/G1\) phase of the cell cycle in (in the following referred to as G\(_0/G1\) cells) we used defined protocols for synchronization of proliferating, subconfluent cells. For arresting the cell growth in the G\(_0/G1\) phase, subconfluent cells were serum-starved for 8 h, and then the serum-free medium was replaced by serum-containing medium. Synchrony of cell growth in the G\(_1/S\) phase of the cell cycle was reached by preincubation of the cells with aphidicolin and in G\(_2/M\) phase by using nocodazole. The phases of the cell cycle were analyzed by staining the DNA with propidium iodide and measurement of the DNA content by flow cytometric analysis (Fig. 3A). Control cells cultured in medium containing FCS, which was changed every second day, were found to proliferate desynchronously. Sixty-two percent of the cells were in the G\(_0/G1\) phase, 26% in the S phase, and 12% in the G\(_2/M\) phase (see Fig. 3A, a). From the cells that were serum-starved (DMEM without FCS for 12 h and subsequent incubation in medium with 10% FCS for 16 h), 92% were found in the G\(_0/G1\) phase, 1% in the S phase, and 4% in the G\(_2/M\) phase (see Fig. 3A, b). From the cells treated with aphidicolin (4 \(\mu\)g/ml for 12 h), 75% were found in the G\(_0/G1\) phase, 24% in the S phase, and 0.3% in the G\(_2/M\) phase (see Fig. 3A, c). From the cells arrested with nocodazole (500 ng/ml for 12 h), 6% were found in the G\(_0/G1\) phase, 5% in the S phase, and 88% in the G\(_2/M\) phase (see Fig. 3A, d). Fig. 3B shows the AVP-induced increase in [Ca\(^{2+}\)], (mean results of 7–10 experiments) in cell suspensions from Swiss 3T3 cells not arrested or arrested in the various phases of the cell cycle as indicated in Fig. 3A. As previously observed in confluent cells, in cells preincubated in serum-free DMEM (G\(_0/G1\) cells) the AVP-induced increase in [Ca\(^{2+}\)], was significantly \((p < 0.05)\) higher than in proliferating cells. The basal calcium concentrations were the same (data not shown).

Only in cells arrested by serum starvation in the G\(_0/G1\) phase was the AVP-induced increase in [Ca\(^{2+}\)], significantly reduced by pretreating the cells with PTX (see Fig. 3B). Therefore, the partial PTX sensitivity of the AVP-induced increase in [Ca\(^{2+}\)], can be attributed to cells grown arrested in the G\(_0/G1\) phase of the cell cycle and not to cells in the late G\(_1/S\) or G\(_2/M\) phase of the cell cycle.

To confirm the results of the PTX experiments with additional methods, we first studied whether the AVP-induced increase in [Ca\(^{2+}\)], in G\(_0/G1\) cells can be inhibited by microinjection of antibodies directed against the C-terminal domain of the G\(_{q}\), proteins, which prevent the interaction of the G protein with ligand-activated receptors. Cells were plated on coverslips and serum-starved for growth arrest in the G\(_0/G1\) phase of the cell cycle as described. Proliferating cells served as control. Cells were microinjected with an antibody binding to the C-terminal domain of all G\(_{q}\) subunits (AS 86) or antibodies binding to the C termini of G\(_{o}\), and G\(_{q}\), (AS 370). Control cells, on the same coverslip, were microinjected with an antibody against G\(_{o}\) (AS 404). The G\(_0/G1\) cells were compared with proliferating cells grown on separate coverslips. The AVP-induced increase in [Ca\(^{2+}\)], was determined in the antibodies injected cells by using a single cell calcium-imaging system (Fig. 4). As in the previous experiments, the AVP-induced increase in [Ca\(^{2+}\)], was significantly higher in G\(_0/G1\) cells than

![Image](120x579 to 226x729)
in proliferating cells. Microinjection of the $\alpha_{q11}$ antibody AS 370 resulted in a partial but significant inhibition of the AVP-induced increase in $[Ca^{2+}]_i$ in proliferating cells as well as in $G_i/G_o$ cells (see Fig. 4, dark bars). In contrast, microinjection of the $G_o$ antibody AS 86 resulted in a partial inhibition of AVP-induced increase in $[Ca^{2+}]_i$ only in $G_i/G_o$ cells, but not in proliferating cells (see Fig. 4, hatched bars). Microinjection of the $G_o$ antibody AS 404 had no effect compared with noninjected cells (see Fig. 4, open bars, and data not shown).

The expression of $G_{o12}$ and $G_{o3}$ had been shown in NIH-3T3 fibroblasts (32) and in membrane preparations derived from rat fetus (33), but in both cases no $G_{o1}$ expression could be detected. Since the antibodies used for immunoprecipitation or for microinjection cannot discriminate between the two members of the $G_o$ isoforms, $G_{o12}$ and $G_{o3}$, we assessed which of two $G_o$ isoforms are involved in the AVP-induced $Ca^{2+}$ release in Swiss 3T3 cells by microinjection of antisense oligonucleotides (for a review, see Ref. 34). To control whether the protein expression of $G_o$ and $G_{oq11}$ can be inhibited by microinjection of antisense oligonucleotides into Swiss 3T3 cells, antisense oligonucleotides directed against the mRNA of $G_{oq11}$ (anti-$G_{oq11}$) or all isoforms of $G_o$ (anti-$G_o$) were used. Forty-eight h after microinjection, the $G_{oq11}$ or $G_o$ proteins were determined semiquantitatively by indirect immunofluorescence (Fig. 5). A decrease in the expression of the respective $G_o$ protein subunits was detected in proliferating cells as well as in $G_i/G_o$ cells. The protein expression of the $\alpha_i$-subunits was reduced by about 35% in proliferating cells injected with anti-$\alpha_{i1}$ compared with noninjected control cells (see Fig. 5A, upper panel, 127 ± 9, n = 12, and 82 ± 5, n = 8, arbitrary units, respectively, $p < 0.01$), whereas in $G_i/G_o$ cells the $\alpha_i$-subunit protein expression was reduced by about 50% (see Fig. 5A, lower panel, 110 ± 12, n = 12, and 59 ± 3, n = 11, respectively, $p < 0.025$). Injection of anti-$\alpha_{q11.2}$ oligonucleotides into proliferating cells led to a reduction of the protein expression of the $\alpha_{q11}$-subunits by about 40% compared with noninjected control cells (see Fig. 5B, upper panel, 79 ± 5, n = 16, and 46 ± 9, n = 7, arbitrary units, respectively, $p < 0.005$), whereas in $G_i/G_o$ cells injected with anti-$\alpha_{q11.2}$ oligonucleotides the $\alpha_{q11}$-subunit expression was reduced by about 65% (see Fig. 5B, lower panel, 151 ± 12, n = 10, and 54 ± 5, n = 10, arbitrary units, respectively, $p < 0.001$).

Next, we determined the AVP-induced increase in $[Ca^{2+}]_i$ in cells microinjected with anti-$G_{q11.1,2}$ and anti-$G_{o}$, which was sequence-specific. Microinjection of oligonucleotides (Fig. 6). Like microinjection of antibodies directed against $G_{q11}$, microinjection of anti-$G_{q11.2}$ into proliferating cells resulted in a weak inhibition of the AVP-induced increase in $[Ca^{2+}]_i$ (see Fig. 6A, left panel, open bars). In $G_i/G_o$ cells, the effect of the anti-$G_{q11.2}$ oligonucleotides was more pronounced (43% inhibition of the AVP-induced increase in $[Ca^{2+}]_i$) (Fig. 6A, right panel, open bars). Preincubation with PTX had no effect in proliferating cells (see Fig. 6A, left panel, filled bars). In $G_i/G_o$ cells, the AVP-induced increase in $[Ca^{2+}]_i$ was inhibited by about 50% upon pretreatment of the cells (see Fig. 6A, compare open to filled bars). Microinjection of anti-$G_{q11.2}$ in PTX-treated $G_i/G_o$ cells resulted in about 75% inhibition of the AVP-induced increase in $[Ca^{2+}]_i$, compared with noninjected and non-PTX-treated cells (see Fig. 6A, right panel, compare the filled hatched bar to the open bar). This indicates an additive effect of anti-$G_{q11}$ and $G_o$ in AVP-induced $Ca^{2+}$ signaling in $G_i/G_o$ Swiss 3T3 cells. Neither injection of anti-$G_o$ nor preincubation with PTX had an effect in proliferating cells (see Fig. 6B, left panel). In contrast, anti-$G_o$ injection as well as PTX treatment resulted in a similar inhibition of AVP-induced increase in $[Ca^{2+}]_i$ in $G_i/G_o$ cells (Fig. 6B, middle panel). Injection of a combination of anti-$G_{q11.2}$ and anti-$G_o$ caused an additive inhibition (about 80%) of the AVP-induced increase in $[Ca^{2+}]_i$, as demonstrated before with the combination of microinjection of anti-$G_{q11.2}$ and pretreatment of the cells with PTX (see Fig. 6A, right panel). The effect of the used antisense oligonucleotides was sequence-specific. Microinjection of oligonucleotides with sense and missense sequences corresponding to the anti-

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**Fig. 4.** AVP-induced increase in $[Ca^{2+}]_i$ in proliferating or $G_i/G_o$ Swiss 3T3 cells injected with polyclonal antibodies directed against the C termini of $G_{o12}, G_{o3}$, all $G_o$ subtypes, and $G_i$. Cells were plated on coverslips impregnated with squares for localization of injected cells. Proliferating and $G_i/G_o$ cells were cultured under the following culture conditions described under "Experimental Procedures." Cells were superfused with AVP (100 nM) 1 h after cytoplasmatic injection with the antibodies, and the AVP-induced increase in $[Ca^{2+}]_i$, was determined as described. Closed bars, cells injected with a $G_{oq11}$ antibody; hatched bars, cells injected with a $G_o$ antibody; open bars, cells injected with a $G_o$ antibody (control cells). Numbers in parentheses indicate the numbers of single cells measured. * values significantly different from those obtained in cells injected with the $G_o$ antibody ($p < 0.05$). For cell culture conditions, see "Experimental Procedures."

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**Fig. 5.** Inhibition of $G_{q11}$ and $G_o$ protein expression in Swiss 3T3 cells injected with anti-$G_{q11.2}$ and anti-$G_{o}$, oligonucleotides. A, shown are proliferating (upper panel) and $G_i/G_o$ (lower panel) Swiss 3T3 cells not injected or injected with anti-$G_{q11.2}$ antibody AS 266, and data not shown). B, shown are proliferating (upper panel) and $G_i/G_o$ (lower panel) Swiss 3T3 cells not injected or injected with anti-$G_{q11.2}$. All cells on a coverslip were stained 48 h after injection with rabbit anti-$G_o$ antiseraum AS266 (1:100) specific for $G_o$, or AS 370 (1:100) specific for $G_{q11}$ and visualized by staining with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:1000).
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Fig. 6. AVP-induced increase in $[Ca^{2+}]$, in Swiss 3T3 cells cultured under various conditions or injected with antisense oligonucleotides directed against the mRNAs of G protein $\alpha$-subunits. Swiss 3T3 cells were plated on coverslips imprinted with squares for localization of injected cells. Cells were superfused with AVP (100 nM) 48 h after intranuclear injection with the antisense oligonucleotides, and the AVP-induced increase in $[Ca^{2+}]$, was determined as described. Antisense oligonucleotide-injected and -noninjected cells were grown on the same coverslips. Where indicated, cells were incubated for 24 h prior to calcium measurement with 100 ng/ml pertussis toxin. Numbers in parentheses indicate the numbers of single cells measured. * values significantly different from those obtained in noninjected cells (p < 0.05). For cell culture conditions, see “Experimental Procedures.” A, AVP-induced increase in proliferating and G0/G1, Swiss 3T3 cells injected with $\alpha_{1a12}$ antisense oligonucleotides or treated with PTX. Bars show mean values ± S.E. of AVP-induced increase in $[Ca^{2+}]$, of anti-$\alpha_{1a12}$-injected cells (shaded open or filled bars), noninjected cells (open bars), or PTX-treated cells (filled bars). B, AVP-induced increase in $[Ca^{2+}]$, in proliferating and G0/G1, Swiss 3T3 cells injected with anti-$\alpha_{1a12}$, or a mixture of anti-$\alpha_{1a12}$ and anti-$\alpha_{1a11}$ (hatched bars), treated with PTX (filled bars) or noninjected control (open bars).

Fig. 7. AVP-induced increase in $[Ca^{2+}]$, in Swiss 3T3 cells synchronized in the G0/G1 phase of the cell cycle and injected with specific antisense oligonucleotides directed against sequences of the mRNAs of the three G0 subtypes. Swiss 3T3 cells were plated on coverslips imprinted with squares for localization of injected cells. Cells were superfused with AVP (100 nM) 48 h after intranuclear injection with the antisense oligonucleotides, and the AVP-induced increase in $[Ca^{2+}]$, was determined as described. Bars show mean values ± S.E. of AVP-induced increase in $[Ca^{2+}]$, of antisense oligonucleotide-injected cells (filled bars) and noninjected cells (open bars), which were grown in the same coverslips outside of the square containing the corresponding injected cells. Numbers in parentheses indicate the numbers of single cells measured. * values significantly different from those obtained in noninjected cells (p < 0.05). For cell culture conditions, see “Experimental Procedures.”

The activity of G proteins is not only determined by the rate of receptor-initiated GTP exchange but also by the rate of GTP hydrolysis. Therefore, we additionally determined the expression of members of a recently discovered family of GTPase-activating proteins termed RGS. We did not detect RGS1, -2, -4, and -7 in Swiss 3T3 cell membranes (data not shown), but expression of RGS3, -5, and -16 was verified (Fig. 8B). The expression of RGS3 was similar in G0/G1, and proliferating cells. In contrast, the expression of RGS3 and RGS16 was increased in G0/G1 cells compared with proliferating cells, paralleling the increased expression of G0/1 (see Fig. 8B).

**DISCUSSION**

Although the ability of G proteins to stimulate cell proliferation is well known, little work has been done on GPCR function during different phases of the cell cycle. A cell cycle-dependent switch of the receptor-G protein coupling from $G_s$ to $G_i$ sense or missense sequences, which showed no effects (data not shown).

Finally, we addressed the question of which mechanism may be responsible for this additional coupling of the $V_{1a}$ receptor to another G protein besides $G_{q/11}$. One mechanism reported to facilitate the dual coupling of a GPCR to G proteins is the altered balance in the expression of receptors or G proteins. For this reason, we studied the $V_{1a}$ receptor density and the protein expression of $G_{q/11}$ or $G_{q/3}$ in proliferating and G0/G1, Swiss 3T3 cells. Cell membranes were prepared from both cell type and used either for determination of radioligand binding of an iodinated AVP antagonist or for immunoblotting of $G_{q/11}$ or $G_{q/3}$ protein. Using the linear $V_{1a}$ receptor antagonist, $[125]$-phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH₂, maximal binding and affinity were determined in both membrane preparations. The $K_D$ values obtained in membranes of G0/G1 cells and proliferating cells were identical. The number of binding sites, however, was reduced by 30% in membranes from G0/G1 cells compared with proliferating cells (Table I). $G_{q/3}$ protein expression was similar in proliferating or G0/G1 cells. In contrast, the expression of $G_{q/11}$ protein was about 2-fold higher in G0/G1 cells compared with proliferating cells (Fig. 8A).

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**TABLE I**

Binding affinities and binding sites of a linear vasopressin antagonist for membranes derived from proliferating and Gₛ/G₁ Swiss 3T3 cells

Specific binding was measured in membranes prepared from proliferating Swiss 3T3 cells and Swiss 3T3 cells arrested in the Gₛ/G₁ phase of the cell cycle using 1-300 pmol of the linear vasopressin antagonist 125I-phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH₂. The values indicate the mean ± S.D. of five independent experiments performed with four different membrane preparations. For binding and cell culture conditions, see “Experimental Procedures.”

|          | Bₘₐₓ ± S.D. | Kᵦ ± S.D. |
|----------|-------------|-----------|
| Proliferating cells | 398 ± 101 | 34.2 ± 9.3 |
| Gₛ/G₁ cells | 281 ± 90* | 34.2 ± 11.2 |

* Value significantly different (p ≤ 0.1) from value obtained in membranes of proliferating cells.

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**FIG. 8. Expression pattern of G protein α-subunits and RGS proteins in Gₛ/G₁ and proliferating Swiss 3T3 cells.** Membrane proteins of the proliferating Swiss 3T3 cells, cells synchronized in the Gₛ/G₁ phase of the cell cycle, or recombinant RGS5 and RGS16 were separated by SDS-PAGE and blotted to nitrocellulose membranes. For detection of the G protein α-subunits, 50 μg of membrane protein were loaded per lane, and the proteins were separated in a 12% SDS gel. For detection of the RGS proteins, 100 μg of membrane protein or 100 ng of recombinant protein were loaded per lane and the proteins separated in a 15% SDS gel. The bound specific antibody were detected with a second antibody coupled to horseradish peroxidase and by using a chemiluminescence detection system. A, nitrocellulose membranes were incubated with antibodies specific for Ga₁₁ or for Ga₅ subunits as indicated at the right. B, the nitrocellulose membranes were incubated with antibodies specific for RGS3, 5, and 16 as indicated at the right. Numbers on the left indicate molecular masses of marker proteins.

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protein, and a subsequent switch from the adenyl cyclase to the Ca²⁺/protein kinase C signal pathway was reported for the calcitonin receptor in a pig kidney cell line (35). For the parathyroid hormone receptor, an additional coupling to Gᵢ proteins and a subsequent increase in Ca²⁺ influx depending on cell cycle was shown in osteogenic sarcoma cells (36). Recently, a switch of the β₂-adrenoreceptor in coupling from Gₛ to G₁ was shown to be related to phosphorylation of the receptor by protein kinase A (37).

Here we describe an enhanced AVP-induced Ca²⁺ response in quiescent Swiss 3T3 cells. A similar effect was observed by others who found in quiescent Swiss 3T3 cells an increased Ca²⁺ mobilization and phosphorylation of proteins induced by bradykinin (38). Part of the enhanced AVP response was blocked by preincubation of the cells with PTX, suggesting an additional recruitment of a Gᵢ protein by AVP receptors (see Fig. 2). The additional coupling of vasopressin receptors to a Gᵢ protein was confirmed by microinjection of an anti-α₁c, anti-serum and anti-α₁c, antisense oligonucleotides (see Figs. 4 and 6). In addition, AVP-induced activation of G₁ proteins could be detected by the photoaffinity labeling technique in cell membranes (see Fig. 1). By microinjection of subtype-specific antisense oligonucleotides against Ga₁₂, Ga₁₅, and Ga₁₆, we identified the G protein α-subunit involved in the PTX-sensitive Ca²⁺ response as Ga₁₆ (see Fig. 7).

The coupling of vasopressin receptors to the Ga₁₆ was absent when the quiescent cells were stimulated for proliferation (see Figs. 1 and 2). In order to attribute the coupling of the AVP receptors to Ga₁₆ to a definite cell cycle state, we synchronized the cell proliferation in different phases of the cell cycle (see Fig. 4A). Although 62% of the proliferating cells and 75% of the quiescent cells were found to be in the Gₛ/G₁ phase, only in growth-arrested cells (92% in the Gₛ/G₁ phase), an increased, partially PTX-sensitive calcium response induced by AVP was detected (see Fig. 4B). By the methodology used, we could not, however, distinguish what percentage of the cells were in the Gₛ or the G₁ phase of the cell cycle. Thus, a feasible explanation for this discrepancy might be that in the growth-arrested cells, most of these cells were in the Gₛ phase. In contrast, the percentage of Gₛ/G₁ cells in proliferating and quiescent-treated cells might represent mainly cells in the G₁ phase. Therefore, the PTX-sensitive AVP response might only occur in the Gₛ phase.

Having established the additional coupling of AVP receptors to Ga₁₆ in quiescent Swiss 3T3 fibroblasts, we also addressed the question how this coupling might be achieved on the molecular level. The most simple explanation would be the presence of two different receptor subtypes in the quiescent Swiss 3T3 cells. Indeed, the mRNA for the V₁₆ receptor was found in some nonneuronal tissues (39), but binding studies, using peptide and nonpeptide ligands specific for the rat and the human V₁₆ receptor, demonstrated only one binding site specific for V₁₆ receptor ligands in Swiss 3T3 cells (21). Nevertheless, we had to exclude the possibility that in our Swiss 3T3 cells the partial PTX sensitivity of the AVP response was based on expression of a vasopressin receptor subtype other than the V₁₆ subtype. Our results obtained with the V₁₆ receptor-specific linear peptide receptor antagonist [1-β-mercaptopropyl-β-cyclopentamethylene proipionic acid]-O-Me-Tyr²,Arg₈vasopressin show that only the V₁₆ receptor subtype is involved in the functional coupling of the AVP receptor to the Ca²⁺ response in these cells in all phases of the cell cycle.

A second possibility would be an alteration in the receptor/G protein stochiometry. Recombinantly expressed V₁₆ receptor has been reported to couple solely to members of the G₅ family in Chinese hamster ovary cells when expressed at low levels. At higher expression levels, Gₛ and G₅ were additionally recruited by V₁₆ receptors (40). A similar effect has been shown for some primarily Gₛ coupled receptors (e.g. LH, V₂₅, and β-adrenergic receptors). Dual coupling of these receptors to adenyl cyclase and PLC-β also occurs with increasing numbers of expressed receptors (41). Note here that in these studies the numbers of receptors varied by a factor of 5 or more. In our hands, the number of the AVP-binding sites in Gₛ/G₁ cells was reduced to
70% compared with the binding sites in proliferating cells (see Table 1). Although we cannot exclude this possibility, it is rather unlikely that this small decrease is responsible for the additional coupling of the V1a receptors.

The balance of the receptor and the adjacent signal transduction molecules is also determined by the expression levels of the involved G proteins. Thus, an increased expression of Gbg protein would be another possible scenario. We could not detect a change in protein expression of Gbg. On the contrary, we found a 2-fold increased expression of Gbg protein in Gbg/Gbg cells (see Fig. 5A). This increase was accompanied by a higher binding of the nonhydrolyzable GTP analog aGTP in the absence and presence of AVP (see Fig. 1). Nevertheless, the higher expression and activation of Gbg did not result in an enhanced PTX-insensitive Ca^2+ response. The PTX-insensitive Δ[Ca^2+]i was even reduced by about 35% in quiescent cells compared with the proliferating controls (see Figs. 2 and 3). This discrepancy might be explained by the higher expression of RGS5 and RGSS16 found in the quiescent cells. Both RGS proteins catalytically enhance the GTPase activity of Gbg as well as Gbg proteins and, thus, negatively regulate signals mediated by these G proteins (for reviews, see Refs. 42–45). In addition, RGSS16 has been shown to regulate Gi3-mediated signals in a receptor-selective manner (46, 47). Therefore, it is feasible that the up-regulation of RGS5 and -16 is responsible for the diminished Gbg-mediated Ca^2+ responses, although the expression and activation of Gbg is enhanced in quiescent cells.

Interestingly, the tumor suppressor p53, a critical regulator of growth arrest, induces the expression of RGSS, including (48). Therefore, the question arises whether the up-regulation of the RGS proteins is involved in the additional coupling of the V1a receptor to Gbg in the quiescent cells. As already mentioned above and shown for RGSS6 in the study of Buckbinder et al. (48), these RGS proteins negatively regulate Gi-mediated pathways. We cannot estimate the impact of the up-regulation of RGSS5 and -16 on Gbg-mediated signaling, since we have no PTX-sensitive signal in the proliferating cells. Nevertheless, it is difficult to imagine how up-regulation of per se negative regulators of Gi-mediated signals can cause an additional coupling of the V1a receptor to Gbg.

One model of receptor/G protein interaction is based on preformed complexes of receptors, G proteins, and effectors preformed in microdomains at the plasma membrane as a possible mechanism of coupling specificity (49). A co-localization of the V1a receptor with Gbg and Gbg was described in hepatocytes (50). The AVP-induced DNA synthesis in Swiss 3T3 cells was reported to be partially sensitive to PTX (24), suggesting that in these cells the V1a receptor couples to Gi proteins of the Gbg and the Gi family. Eventually, the here described additional recruitment of Gbg by the V1a receptor might represent an activation of such a preformed signal transduction complex in Swiss 3T3 cells. The changes in receptor numbers, which we found in Gi/Gbg cells compared with proliferating cells, are very small, but the amount of expressed membrane proteins does not necessarily reflect the availability of these proteins in microdomains in the membranes in which the signal transduction complexes assemble. Therefore, it is possible that even small changes in the expression of endogenous effectors might have functional consequences.

Therefore, one can anticipate a model that includes two functional classes of vasopressin V1a receptors in Swiss 3T3 cells. The first is precoupled with Gbg proteins and responsible for the AVP response in proliferating cells and partially in quiescent cells. The second class precoupled with Gbg3 proteins can only be recruited for the AVP response in quiescent Swiss 3T3 cells. We and others (22) were not able to detect any activation of Gi proteins in proliferating cells by the sensitive method of photoaffinity labeling with aGTP (see Fig. 1). Thus, when the Swiss 3T3 cells are stimulated to proliferate, this subpopulation of V1a receptors is obviously desensitized on the level of receptor-G protein interaction or even removed from the cell surface. Desensitization of the V1a receptor is accompanied by phosphorylation of the receptor molecule (51), but the functional consequence of this event remains to be elucidated. We did not study the phosphorylation state of the V1a receptor in Swiss 3T3 cells in the various phases of the cell cycle. Therefore, we can only speculate on such a mechanism.

Taken together, our data provide evidence for a dual coupling of the V1a receptor to PLC-β isoforms dependent on the proliferation state of the cells. The main axis of PLC-β activation is mediated by Gbg. In the Gi/Gbg phase of the cell cycle, which is physiologically the resting state of differentiated tissue, there is an additional coupling of the V1a receptor to Gbg, leading to activation of PLC-β isoforms by βγ subunits released upon activation. While AVP acts as mitogenic signal for Swiss 3T3 cells (52), activation of PLC-β is not sufficient for stimulation of DNA synthesis (53), and AVP-induced DNA synthesis was reported to be partially sensitive to preincubation of cells with PTX (24). Considering that many mitogenic signals were found to be dependent on PTX-sensitive G proteins, our results may indicate additional mechanisms, such as enhanced calcium release, βγ (derived from Gbg)-mediated activation of MAP kinases, or phosphoinositide-3-kinase, to be involved in mitogenic signaling of the V1a receptor.

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REFERENCES

1. Birnbaumer, L. (1992) Cell 71, 1069–1072
2. Lolait, S. J., O’Carroll, A. M., McBride, O. W., König, M., Morel, A., and Brownstein, M. J. (1992) Nature 357, 336–339
3. Thibonnier, M., Auzan, C., Madhun, Z., Wilkins, P., Berri-Mattaira, L., and Clauser, E. (1994) J. Biol. Chem. 269, 3304–3310
4. Baertschi, A. J., and Friedli, M. (1985) Endocrinology 116, 499–502
5. Jourd, S., Guillard, S. V., Guillon, G., Marie, J., Schoeneng, P., Muller, A. F., Manning, M., and Sawyer, W. H. (1986) Mol. Pharmacol. 30, 171–177
6. Sugimoto, T., Saito, M., Mochizuki, S., Watanabe, Y., Hashimoto, S., and Kawashima, H. (1994) J. Biol. Chem. 269, 27088–27092
7. Howard, J., and Wheatley, M. (1995) Gen. Pharmacol. 26, 1143–1152
8. Barberis, C., Morin, D., Durrous, T., Mouillar, B., Guillot, G., Meyer, S., Mibert, M., Tribollet, E., and Manning, M. (1999) Drug News Perspect. 12, 279–292
9. Exton, J. H. (1997) Eur. J. Biochem. 243, 10–20
10. Cockcroft, S., and Thomas, G. M. (1992) Biochem. J. 288, 1–14
11. Camps, M., Carozzi, A., Sabnab, S., Scheer, A., Parker, P. J., and Gierschik, P. (1992) Nature 360, 684–686
12. Katz, A., Wu, D., and Simon, M. I. (1992) Nature 360, 686–689
13. Park, D., Jhon, D.-J., Lee, C.-L., Lee, K.-H., and Rhee, S. G. (1993) J. Biol. Chem. 268, 4573–4576
14. Jhon, D.-Y., Lee, H.-H., Park, D., Lee, C.-W., Lee, K.-H., Yoo, O. J., and Rhee, S. G. (1993) J. Biol. Chem. 268, 6654–6661
15. Kozasa, T., Hepler, J. R., Sternweis, P. C., and Gilman, A. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9176–9180
16. Jiang, H., Wu, D., and Simon, M. I. (1994) J. Biol. Chem. 269, 7593–7596
17. Levy, A., Lightman, S. L., Hoyland, J., and Mason, W. T. (1990) Clin. Endocrinol. 33, 73–79
18. Thibonnier, M. (1992) Regul. Pept. 38, 1–11
19. Wang, R. L., Smrcka, A. V., Sternweis, P. C., and Exton, J. H. (1991) J. Biol. Chem. 266, 11409–11412
20. Thibonnier, M., Boyer, A. L., and Leng, Z. (1993) Regul. Pept. 45, 79–84
21. Serredel-Le Gal, C., Bourrie, B., Raufaste, D., Carayon, P., Garcia, C., Maffrand, J. P., Le Fur, G., and Casellas, P. (1994) Biochem. Pharmacol. 47, 633–641
22. Offermanns, S., Heier, E., Spicher, K., and Schultz, G. (1994) FEBS Lett. 349, 201–204
23. Thibonnier, M., Berri-Mattaira, L. N., Dulin, N., Conarty, D. M., and Mattera, R. (1998) Prog. Brain Res. 119, 147–161
24. Taylor, C. W., Blakely, D. M., Cortez, A. N., Berridge, M. J., and Brown, K. D. (1988) Biochem. J. 249, 917–920
25. Spicher, K., Kalkbrenner, F., Zobel, A., Harhammer, R., Nurnberg, B., Stein, A., and Schultz, G. (1994) Biochem. Biophys. Res. Commun. 198, 906–914
26. Offermanns, S., Schultz, G., and Rosenthal, W. (1991) Methods Enzymol. 195, 286–301
27. Dippel, E., Kalkbrenner, F., Wittig, B., and Schultz, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1391–1396
28. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
29. Neill, J. D., Duck, L. W., Sellers, J. C., Musgrove, L. C., Scheschonka, A., Druey, K. M., and Kehrl, J. H. (1997) Endocrinology 138, 843–846
30. Dulin, N. O., Sorokin, A., Reed, E., Elliott, S., Kehrl, J. H., and Dunn, M. J. (1999) Mol. Cell. Biol. 19, 714–723
31. Zhu, X., Gilbert, S., Birnbaumer, M., and Birnbaumer, L. (1997) Endocrinology 138, 4109–4122
32. Duzic, E., Coupry, I., Downing, S., and Lanier, S. M. (1991) J. Biol. Chem. 267, 9844–9851
33. Zhang, J., and Pratt, R. E. (1996) J. Biol. Chem. 271, 15026–15033
34. Bizzarri, C., and Civitelli, R. (1994) Endocrinology 134, 133–140
35. Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1997) Nature 390, 88–91
36. Buckbinder, L., Velasco-Miguel, S., Chen, Y., Xu, N., Talbott, R., Gelbert, L., Gao, J., Seizinger, B. R., Gutkind, J. S., and Kley, N. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7868–7872
37. Leib科学家, and Rozengurt, E. (1998) J. Biol. Chem. 273, 7155–7161
38. Millar, J. B., and Rozengurt, E. (1999) J. Biol. Chem. 274, 1284–1287
39. Hill, J. C., Birnbaumer, M., and Birnbaumer, L. (1997) Mol. Pharmacol. 51, 217–224
40. Neubig, R. R. (1994) FASEB J. 8, 939–946
41. Trakova, Z., Kumar, A., Watson, A. J., and Soloff, M. S. (1997) Mol. Pharmacol. 51, 217–224
42. Inamorati, G., Sadeghi, H., and Birnbaumer, M. (1998) J. Biol. Chem. 273, 7155–7161
43. Millar, J. B., and Rozengurt, E. (1999) J. Biol. Chem. 275, 19973–19979