Promiscuous Coupling of Receptors to Gq Class α Subunits and Effector Proteins in Pancreatic and Submandibular Gland Cells*

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Xin Xu‡, Jennifer T. Croy‡, Weizhang Zeng‡, Liping Zhao‡, Isabelle Davignon§, Serguei Popov§, Kan Yu§, Huiping Jiang¶, Stefan Offermanns¶, Shmuel Muallem‡**, and Thomas M. Wilkie‡‡‡

From the Department of Physiology and Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75335, ‡Astra Arcus USA, Inc., Rochester, New York 14602, and §Institute for Pharmacology, Klinikum Benjamin Franklin, Freie Universität Berlin 14195, Berlin, Germany

Mice with deficiencies in one or more Gq class α subunit genes were used to examine the role of the α subunit in regulating Ca2+ signaling in pancreatic and submandibular gland cells. Western blot analysis showed that these cells express three of the four Gq class subunits, Gq9, Gα11, and Gα14 but not Gα15. Surprisingly, all parameters of Ca2+ signaling were identical in cells from wild type and four lines of mutant mice: 1) Gα11−/−, 2) Gα11−/−/Gα14−/−, 3) Gα14−/−/Gα15−/−, and 4) Gq9−/−/Gα15−/−. These parameters included the K_app for several Gq class coupled receptors, induction of [Ca2+]i oscillations by weak stimulation, and a biphasic [Ca2+]i response by strong stimulation. Furthermore, Ca2+ release from internal stores and Ca2+ entry were not affected in cells from any of the mutant mice. We conclude that Gq9, Gα11, and Gα14 promiscuously couple several receptors (m3 muscarinic, bombesin, cholecystokinin, and α1 adrenergic) to effector proteins that activate both Ca2+ release from internal stores and Ca2+ entry.

Heterotrimeric guanine nucleotide-binding proteins (G proteins) couple seven transmembrane domain receptors and effector proteins to regulate the cytosolic concentration of second messengers such as cAMP, cGMP, and [Ca2+]i (1–3). Agonist-bound receptors catalyze the exchange of GTP for GDP on the Ga subunit to initiate intracellular responses to extracellular stimuli. The identity of the Gαβγ subunits regulates receptor coupling to effector proteins (1, 3). Most effector proteins are regulated by Gα, although Gαβγ also contribute to the selectivity of receptor-G protein coupling (4–6). Mammals express 20 distinct Gα proteins encoded by 16 genes that are grouped into four classes termed Gs, Gq, Gi, and G12 (7, 8) based on sequence comparisons and functional similarities of the α subunits within each class. For example, Gs class α subunits stimulate adenylyl cyclase to generate cAMP, whereas Gq class α subunits stimulate PLCβ1 to generate inositol 1,4,5-trisphosphate and mobilize Ca2+ from internal stores (1, 3, 4). The Gq class α subunits regulate a more diverse set of effector proteins (1, but signaling transduced by any of these proteins (with the exception of Goz) is inhibited by pertussis toxin-catalyzed ADP-ribosylation (1, 4). An effector protein of Go13 (in the G12 class) was recently identified as p115 RhoGEF (9). Thus, each class of Gα subunit has a unique role in regulating downstream effector proteins.

Signaling specificity among α subunits of the same class that have similar biochemical functions is not well understood in vivo. The Gq class includes four proteins expressed in mammals, termed Gq9, Gα11, Gα14, and Gα15/16 (8). Orthologs of Gq9, Gα11, and Gα14 expressed in human, mouse, cow, and dog are highly conserved (99, 97, and 96% identity, respectively), whereas paralogs of these Gq class genes (expressed in the same species) are less well conserved (10). This pattern of evolutionary conservation suggests that different Gq genes have distinct functions. However, reconstituted systems using recombinant proteins expressed in Sf9 cells showed that Goq, Gα11, and Gα14 stimulated PLCβ activity with similar potency (11). These Gq class proteins also had similar activities in transfected tissue culture cells (12). These apparent functional similarities are of interest because many cells express at least two and sometimes three members of the Gq class α subunits (3, 12, 14). Indeed, photolabeling with GTP azidoanilide showed that a single receptor type can simultaneously couple two different Gq class α subunits within the same cell (15–18).

To address questions of signaling specificity among Gq class α subunits, antisense oligonucleotides that targeted either Gα9 or Gα11 mRNA for degradation were injected into the nuclei of RBL-2H3 cells that overexpressed the m1 muscarinic receptor. These studies suggested that coupling of both Goq9 and Goq11 was required for stimulation of PLCβ and generation of a Ca2+ signal (6). The same approach was used in portal vein myocytes to conclude that α1 adrenergic receptor stimulation of Goq9 leads to Ca2+ release from internal stores, whereas stimulation of Goq11 was required for activation of store-operated Ca2+ entry (19). These results suggested that Goq9 and Goq11 coupled the same receptor type to stimulate distinct functions in smooth muscle cells.

The role of Goq has also been assessed in transgenic mice by expression of RNA antisense to Goq in liver and white adipose tissue (20). Goq deficiency induced in these tissues at birth increased body mass and hyperadiposity within 5 weeks that persisted throughout adulthood. We have altered expression of each of the four Gq class genes by another means, using gene

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** To whom correspondence should be addressed: UT Southwestern Medical Center, Dept. of Physiology, 5323 Harry Hines Blvd., Dallas, Texas 75395. Tel.: 214-648-2593; Fax: 214-648-8685; E-mail: smualle@mednet.swmed.edu.

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† The abbreviations used are: PLCβ, phospholipase C β; SMG, submandibular glands; WT, wild type; CCKS, cholecystokinin octa peptide.
targeting in embryonic stem cells to produce homozygous deficiencies in mice (21, 22). Each of the single knockout mice are viable and fertile. The most dramatic phenotypic defect was observed in Gq–/– mice, which are ataxic and have a bleeding disorder caused by a platelet aggregation defect (21, 23). Interestingly, the growth of Gq–/– mice is retarded relative to wild type litter mates, in contrast to increased body mass observed in transgenic mice expressing antisense Gq in specific tissues (20). Knockout mice were also used to begin an analysis of the shared and separate functions of Gq and Go11 during development. Fetuses with homozygous deficiencies of both Gq and Go11 (Gq+/–, Go11+/–) die during embryogenesis about 11 days post-coitum (22). Expression of a single wild type copy of either Gq or Go11 allows continued embryonic development, but neonatal pups die within an hour of birth (22). These results indicate that Gq and Go11 can each compensate for a deficiency in the other during development.

In the present work we used gene knockouts of Gq class α subunits in mice to re-evaluate the specificity of receptor coupling and their subsequent regulation of defined Ca2+ transport pathways in pancreatic and submandibular gland cells. These cells express three Gq class α subunits, Gq, Go11, and Go14. We found that all aspects of Ca2+ signaling were identical in cells from WT and all mutant mice, including Go11+/–/Go14+/– and Gq–/–/Go15–/– double knockouts. We conclude that the receptors examined in the present work (m3 muscarinic, bombesin, cholecystokinin, and α1 adrenergic) display promiscuity of coupling to Gq class α subunits and effector proteins that regulate all aspects of the Ca2+ signal in pancreatic and submandibular gland cells.

MATERIALS AND METHODS

Production of Knockout Mice—Mutant mice deficient in Gq, Go11, Go14, and Go15 were produced as detailed before (21, 22). Briefly, the murine genes were disrupted by homologous recombination in mouse embryonic stem cells. Targeting vectors were designed to replace specific exons with several translation termination codons present in the reverse orientation of the neomycin phosphotransferase gene. None of the targeted Gα proteins were detected in tissues from the corresponding homozygous null mutant mice (21, 22). The lines of knockout mice used in this study are viable and fertile. Go11+/–/Go14+/–, Go14+/–/Go15–/–, and Gq–/–/Go15–/– double homozygous null mice were obtained from intercrossing of the offspring of the single knockout mice. Two strains of double homozygous null mice were not available for this study. Gq–/–/Go11–/– mice could not be obtained as they die during embryogenesis (21), and Gq–/–/Go14–/– mice have not been obtained from intercrossing of the single knockouts because the genes are tandemly duplicated on mouse chromosome 19 (8) and are thus too close together to expect recombination to place both null mutations on the same chromosome. All WT and mutant mice were of 129/SvEv × C57BL/6 genetic background.

Western Analysis—Membrane proteins collected from the indicated tissues were assayed for expression of Goq, Go11, Go14, and Go15 in WT and knockout mice by Western blot as described (24). In all experiments membrane proteins isolated from tissues that express abundant amounts of a given Gα subunit were included as positive controls; Goq and Go11 (brain), Go14 (testis), and Go15 (spleen; results not shown, but see Refs. 13, 21, and 22).

Preparation of Cells—Pancreatic acini and submandibular gland (SMG) duct and acini were prepared by standard collagenase digestion procedures as described (25, 26). The cells were suspended in solution A containing 140 mm NaCl, 5 mm KCl, 10 mm Hepes (pH 7.4), 1 mm MgCl2, 1 mm CaCl2, and 10 mm glucose. The cells were kept on ice until use.

Measurement of [Ca2+]i—Pancreatic and SMG cells from WT and knockout mice were incubated in solution A containing 5 mm Fura 2/AM for 20–30 min at 37 °C, washed twice with solution A, and kept on ice until used. Cells were plated on glass coverslips that formed the bottom of a perfusion chamber and were allowed to attach during a 2-min incubation at room temperature before the start of perfusion. The cells were continuously perfused during fluorescence recording with solution A or, as indicated, CaCl2-deficient solution A that contained 0.1 mm EGTA (Ca2+-free). Fluorescence was recorded with an image acquisition system from Photon Technology International. The image ratios, recorded at excitation wavelengths of 355 and 380 nm, were calibrated and analyzed exactly as described (25, 26).

Agonist Potency in WT and Mutant Mice—Potency of agonist stimulation (Kapp) in pancreatic acini from WT and mutant mice was evaluated from the effect on [Ca2+]i, of five agonist concentrations expected to be below, at, and above the EC50. Separate preparations of pancreatic acini from WT and each of the mutant mice (see Table I) were used in each experiment to obtain between 2 and 6 estimates of Kapp for each of the agonists used. At each agonist concentration [Ca2+]i, was analyzed from at least 5 acinar clusters composed of 8–15 cells and 3–5 individual cells that were present in the same recording field. Initially [Ca2+]i, was analyzed in individual cells and individual clusters to examine cell to cell and cluster to cluster variations. No striking differences were noted between the responses of individual cells or acini from the different mice. Therefore the results from all cells and clusters of common genotype in each experiment were combined at each agonist concentration. These combined results were used to determine the average increase in [Ca2+]i, caused by any agonist concentration. Because low concentrations of agonists usually induce [Ca2+]i, oscillations, whose frequency and sometimes amplitude depends on agonist concentration (2), independent verification of Kapp was obtained by measuring the lag time to the first noted increase in [Ca2+]i. Previous studies showed that the lag time to the first response depends on agonist concentration (27). The combined responses to all concentrations of a given agonist were displayed on an expanded time scale, and the lag time was taken as the time between the agonist entering the perfusion chamber and the time [Ca2+]i, started to increase. The time of agonist entry into the chamber was obtained by subtracting the dead volume of the perfusion line from the time of solution changes. Peak [Ca2+]i, or decreasing lag time were plotted as a function of agonist concentration, and Kapp values were determined by fitting the results to a Hill equation of the form

\[
y = Y_{\text{max}}(x/K_{\text{app}} + x)^n/n\]

where \(Y_{\text{max}}\) is the extrapolated maximal [Ca2+]i, increase or minimal lag time, \(x\) is the agonist concentration, and \(n\) is the Hill coefficient. Kapp determined from the [Ca2+]i, increase and lag time agreed very well (within 10% for a given experiment) and were used to obtain an average value for each experiment. The values obtained from all experiments using separate acinar preparations were used to calculate the mean +/− S.E. Statistical significance was evaluated by analysis of variance.

RESULTS AND DISCUSSION

Analysis of Gq Class α Subunit Expression—Expression of Gq class α subunits in the mouse pancreas and SMG was determined by Western blot analysis using antisera raised against peptide sequences unique to each of the four proteins (24). Fig. 1 shows that Goq, Go11, and Go14 were the predominant Gq class α subunits expressed in the pancreas and SMG of WT mice. Pancreatic acini expressed higher levels of Go14 than SMG cells, whereas SMG cells expressed higher levels of Goq and Go11. Go15 was either too low to be detected or was not expressed in either cell type. Furthermore, Western analysis showed that Goq was equally abundant in WT, Go11+/–, Go14+/–, and Go15–/– pancreas and SMG cells and, as expected, was absent from Goq–/– cells. Similarly, expression of other Gq class α subunits was absent in the corresponding knockout mouse. Single knockout mice were bred to generate the needed knockout lines that could be obtained, Go11+/–/Go14+/–, Go14+/–/Go15–/–, and Goq–/–/Go15–/–. It is important to note in the single and double knockout mice that expression of the normal Gq class genes was monitored relative to WT mice (21, 22). For example, in pancreatic acini and SMG cells of the Go11+/–/Go14+/– mice, Goq was expressed at normal levels and was the only Gq class α subunit available to couple receptors activated by the various agonists that were used. These mice, together with the Goq–/–/Go15–/– and the single knockout mice, allow clear analysis of specialized functions of Goq, Go11, and Go14 in Ca2+ signaling.

Ca2+ Signaling Is Identical in WT and Mutant Mice—Previous reports using an antisense oligonucleotide approach (6, 19) would predict that deletion of either Goq or Go11 would be sufficient to reduce or eliminate Ca2+ signaling by receptors coupled to Gq class proteins. That this was not likely to be the
case was evident from the mild phenotype of each single knock-put line (21, 22). Direct evidence for the promiscuous coupling of receptors to different Gq class α subunits was obtained by measuring the Ca^{2+} response of pancreatic acini to stimulation of three distinct receptors.

Fig. 2 shows representative examples of cell responses to weak and intense agonist stimulation. Weak stimulation with low agonist concentration commonly evokes [Ca^{2+}]_{i} oscillations in virtually all cell types (2), including pancreatic acini (28, 29). Figs. 2, a–d, shows that stimulation with 0.25 nM bombesin induced similar [Ca^{2+}]_{i} oscillations in acini from WT and all mutant mice. [Ca^{2+}]_{i} oscillations were also induced by low concentrations of carbachol and CCK8 (not shown). A typical biphasic response to intense stimulation with 10 nM CCK8 is shown in Figs. 2, e–h. Again, the response to CCK8 was similar in acini from all mice (Fig. 2), and similar responses were recorded in acini from all mice stimulated with 1 mM carbachol or 0.1 μM bombesin (not shown). Hence, the pattern of the [Ca^{2+}]_{i} responses at low and high agonist concentrations were not altered by deletion of any of the indicated single or pairs of Gq class α subunits. This indicates that (α) all three receptors can productively couple to Goq, Ga11, and/or Ga14 and (β) receptor-dependent activation of each α subunit equally activated all components of the Ca^{2+} signal downstream of the α subunits.

A rigorous test of the specificity and strength of coupling between receptors and α subunits can be obtained by measuring the apparent affinity (K_{app}) for a given agonist. Therefore, pancreatic acini from WT and the mutant mice were used to estimate K_{app} for each of the agonists. K_{app} was determined by measuring the effect of at least five concentrations of carbachol between 1 and 100 μM, bombesin between 0.1 and 300 nM, and CCK8 between 0.01 and 10 nM, which covered the entire range of effective concentrations for each agonist. At least five acinar clusters were used to estimate the averaged [Ca^{2+}]_{i} increase at each agonist concentration. As can be seen from the results in Table I, the K_{app} for all agonists remained the same in all mutant mice. In all cases the absolute increase in [Ca^{2+}]_{i} evoked by each agonist was the same as that measured in acini from WT mice (see also Fig. 3).

Many tissues and cell types express both Goq and Ga11. Expression of Ga14 and Ga15 is more restricted (13). The cells used in the present studies express Goq, Ga11, and Ga14 at high levels. However, deletion of Ga14 alone or in combination with other Gq class α subunits did not produce dramatic phe-
Promiscuity of Gq Class α Subunits

Table I

$K_{app}$ for carbachol, bombesin, and CCK to induce [Ca$^{2+}$]i, increase in pancreatic acini of WT and mutant mice

| Agonist          | WT               | Ga11−/−          | Ga11−/−/Ga14−/− | Goq−/−/Go15−/− |
|------------------|------------------|------------------|-----------------|---------------|
| Carbachol (μM)   | 5.73 ± 0.28 (4)  | 6.03 ± 0.31 (3)  | 5.5±6.7 (2)     | 5.44 ± 0.46 (6) |
| Bombesin (nM)    | 1.24 ± 0.07 (4)  | 1.11 ± 0.10 (3)  | 1.05/13 (2)     | 1.42 ± 0.13 (3) |
| CCK (nM)         | 0.27 ± 0.01 (4)  | 0.25 ± 0.01 (3)  | 0.22/0.24 (2)   | 0.29 ± 0.01 (6) |

**FIG. 3.** Measurement of Ca$^{2+}$ release and influx in submandibular gland cells from WT and mutant mice. Submandibular gland cells from WT (a and e) and mutant mice (b–d, f–h) were stimulated with 10 μM epinephrine (Epi, a–d) or 1 mM carbachol (Car, e–h). As indicated, the cells were perfused with a solution containing 1 mM CaCl$_2$, which was then replaced with a Ca$^{2+}$-free solution containing 0.1 mM EGTA during the time indicated by the bars. The peak and plateau of [Ca$^{2+}$], measured after epinephrine or carbachol stimulation of SMG cells are summarized in Table II.

**Studies**, suppression of Goq or Ga11 alone was as efficient as suppression of both subunits in inhibiting Ca$^{2+}$ signaling. A possible explanation for the different effects obtained by the two approaches may be the extent to which active Gq class α subunits are removed by the two techniques. Preliminary results with embryonic cells from mutant mice that did not express Goq and Ga11 expressed only one copy of either gene showed that Ca$^{2+}$ signaling in response to angiotensin II was absent in embryonic cardiomyocytes from double knockout mice and significantly reduced (but not absent) in cells containing only one active allele of either gene (22). Signaling in cells with two or more active copies of Goq and/or Ga11 was apparently normal. This may suggest that cells express a 2–3-fold excess Gq class α subunits capable of promiscuous coupling to receptors that activate PLC$\beta$, but only part of this Gq class α subunit pool is active at any time. Application of antisense oligonucleotides for two days may have depleted the targeted pool but not allowed sufficient time to activate the pool of α subunits held in reserve. In the mutant mice we have analyzed, all active receptors may be coupled by the remaining Gq class α subunits, but the inactive pool of Goq may be diminished. Alternatively, signaling specificity may be different in smooth muscle and secretory gland cells. However, we did not observe defects in smooth muscle function in vivo in Gq class knockout mice. For example, blood pressure in Ga11−/− mice is normal. Independent of the reason for discrepancies between knockout and antisense approaches, our results strongly suggest that Goq, Goa11, and Goa14 promiscuously couple several receptors to the effector and regulatory proteins that mediate Ca$^{2+}$ signaling.

Gq Class α Subunits and Ca$^{2+}$ Influx—Another aspect of specificity in Gq class-mediated signaling that we examined was the activation of specific Ca$^{2+}$ transport pathways by certain Gq class α subunits. Previous studies (19) used cells expressing α1 adrenergic receptors to examine the role of the Gq class α subunits in Ca$^{2+}$ signaling. Therefore, our initial experiments to analyze Ca$^{2+}$ release and Ca$^{2+}$ entry were performed with SMG duct and acinar cells because they also express the α1 adrenergic receptor. Furthermore, Ca$^{2+}$-mobilizing agonists cause a marked activation of Ca$^{2+}$ influx in SMG cells (26, 30, 31). Fig. 3 shows the biphasic response of SMG duct and acinar cells from WT and mutant mice to maximal stimulation with epinephrine or carbachol, respectively. Table II summarizes the effect of these agonists on the peak and plateau increases in [Ca$^{2+}$]. Epinephrine stimulation of duct cells and carbachol stimulation of acinar cells are the most potent Ca$^{2+}$-mobilizing agonists acting on these cells (26, 30, 31). It is clear that Ca$^{2+}$ release from internal stores (initial peak) and Ca$^{2+}$ influx (plateau) were the same in cells from WT and all mutant mice. Removal and re-addition of Ca$^{2+}$ to the incubation medium further emphasized the similar extent of Ca$^{2+}$ entry and the similarity of all other aspects of Ca$^{2+}$ signaling in WT and mutant mice. Acinar cells from these preparations that were present in the same recording field as duct cells also responded to α1 adrenergic stimulation with partial depletion of Ca$^{2+}$ stores and showed identical profiles of Ca$^{2+}$ release and Ca$^{2+}$ entry between cells from WT and mutant mice (not shown). Ca$^{2+}$ release and Ca$^{2+}$ entry were also examined in pancreatic acinar cells using the protocol described above. We could not detect any differences in the kinetics or extent of Ca$^{2+}$ release or Ca$^{2+}$ entry between pancreatic acini from WT or mutant mice (see legend to Table I).

The results presented in Fig. 3 and Table II reveal another discrepancy between depleting Gq class α subunits by injecting antisense oligonucleotides (19) and gene knockouts. Suppression of Ga11 with antisense oligonucleotides had no effect on Ca$^{2+}$ release from internal stores but completely eliminated Ca$^{2+}$ entry stimulated by α1 adrenergic receptors (19). By contrast, depletion of Goq completely inhibited Ca$^{2+}$ release from internal stores, whereas epinephrine-stimulated Ca$^{2+}$ entry remained intact (19). This would suggest that Goq regulates Ca$^{2+}$ release, and Goa11 directly activates the Ca$^{2+}$ influx pathway. However, our results and previous studies using different approaches (11, 12) demonstrate that Goa11 activates Ca$^{2+}$ entry.
PLCβ. Furthermore, all parameters of Ca\(^{2+}\) signaling were identical in WT, Goq\(^{-/-}\), and Goa11/−/−/Goa14/−/− mice, thus excluding a novel function for Goa11 in signal transduction evoked by the agonists that were used. Hence, our findings indicate that receptor-dependent activation of Goa or Goa11 similarly activates PLCβ to generate inositol 1,4,5-trisphosphate, release Ca\(^{2+}\) from internal stores, and activate Ca\(^{2+}\) entry. This does not exclude the possibility that the Ca\(^{2+}\) influx pathway is also directly or indirectly regulated by G protein α subunits. Our previous work showed that constitutively active Goa-regulated Ca\(^{2+}\) influx is downstream of Ca\(^{2+}\) release (32). Furthermore, mammalian channel related to Drosophila trp appears to be directly activated by Goa11 (33). Thus, if Goq class α subunits regulate Ca\(^{2+}\) influx, our results clearly show that all members of the class can equally regulate the pathway.

In summary, we used gene knockouts in mice to show that Gq class α subunits promiscuously couple several different receptors in three cell types. Pancreatic acini (25) and SMG cells (34) respond to the agonists used in the present studies by generating agonist-specific Ca\(^{2+}\) waves. It is clear from the present work that the identity of the Gq class α subunit does not determine the receptor-specific pattern of Ca\(^{2+}\) waves. This highlights the importance of our recent observation that RGS4 inhibits Ca\(^{2+}\) signaling in a receptor-specific manner (35), thus contributing to agonist specificity in Ca\(^{2+}\) signaling.

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