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Alternate Coupling of Receptors to G_s and G_i in Pancreatic and Submandibular Gland Cells*

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Many G_s-coupled receptors can activate both cAMP and Ca^{2+} signaling pathways. Three mechanisms for dual activation have been proposed. One is receptor coupling to both G_s and G_{15} (a G_q class heterotrimeric G protein) to initiate independent signaling cascades that elevate intracellular levels of cAMP and Ca^{+2}, respectively. The other two mechanisms involve cAMP-dependent protein kinase-mediated activation of phospholipase Cβ either directly or by switching receptor coupling from G_s to G_i. These mechanisms were primarily inferred from studies with transfected cell lines. In native cells we found that two G_s-coupled receptors (the vasoactive intestinal peptide and β-adrenergic receptors) in pancreatic acinar and submandibular gland duct cells, respectively, evoke a Ca^{2+} signal by a mechanism involving both G_s and G_i. This inference was based on the inhibitory action of antibodies specific for $G\alpha_s$, $G\alpha_i$, and phosphatidylinositol 4,5-bisphosphate, pertussis toxin, RGS4, a fragment of β-adrenergic receptor kinase and inhibitors of cAMP-dependent protein kinase. By contrast, Ca^{2+} signaling evoked by G_s-coupled receptor agonists was not blocked by G_q class-specific antibodies and was unaffected in $G\alpha_{15}$ −/− knockout mice. We conclude that sequential activation of G_s and G_i, mediated by cAMP-dependent protein kinase, may represent a general mechanism in native cells for dual stimulation of signaling pathways by G_s-coupled receptors.

A family of heterotrimeric guanine nucleotide-binding proteins (G proteins) transduces a variety of signals across the plasma membrane by sequential interactions with receptor and effector proteins (*e.g.* second messenger-generating enzymes and ion channels). These interactions result from guanine nucleotide-driven conformational changes in G protein α subunits (1). Agonist-bound receptors catalyze the exchange of GDP for GTP on the α subunits of their cognate G proteins to promote dissociation of α from a high affinity complex of $\beta\gamma$ subunits. Dissociated subunits are competent to modulate the activity of effectors. GTP hydrolysis ultimately returns Gα to the GDP-bound state, thus allowing reformation of inactive heterotrimer. Sixteen distinct genes encode G protein α subunits in mammals. The family is commonly divided into four classes based on amino acid sequence identity and function: G_s, G_i, G_q, and G_{12}. Members of a newly identified family of regulators of G protein signaling (RGS proteins)[1] have been shown to stimulate the GTPase activity of G_i and G_q class α subunits, thus attenuating signaling (2).

One of the more thoroughly characterized examples of G protein-mediated signal transduction is carried out by the hormone-sensitive adenylyl cyclase system. Relevant receptors communicate with homologous G proteins, one of which (G_s) activates adenylyl cyclase while others (G_i) inhibit the enzyme (1). The second messenger (cAMP) mediates diverse cellular responses, primarily by activating cAMP-dependent protein kinase (PKA). In the case of Ca^{2+}-mobilizing agonists, G protein activation is followed by stimulation of phospholipase Cβ (PLCβ) to generate IP_3 in the cytosol, which initiates the $[Ca^{2+}]_i$ signal by release of Ca^{2+} from internal stores (1, 3). PLCβ can be activated by each of the four G_q class α subunits or by G$\beta\gamma$ subunits released from G_i class proteins (4). Only G_i-mediated PLCβ activation is inhibited by pertussis toxin (4). In this study we sought to learn the mechanism by which G_s-coupled receptors evoke Ca^{2+} signaling.

Several G_s-coupled receptors can activate dual signaling cascades. For example, increases in both cAMP and $[Ca^{2+}]_i$ have been observed by histamine acting on H_2 receptors in parietal cells (5), parathyroid hormone acting on osteoblasts (6), and isoprenaline acting on cardiac myocytes (7) or salivary gland cells (8, 9). In contrast to the simple paradigm that each receptor molecule can activate a single class of G protein (10), activation of more than one signaling cascade could be due to coupling of one receptor type to two classes of G proteins. This model is supported by experiments in heterologous expression systems. Overexpression of histaminergic H_2 (11), parathyroid hormone (12), luteinizing hormone (13), P2Y11 (14), vasopressin V2, dopamine D1A, and adenosine A2A (15) receptors resulted in stimulation of adenylyl cyclase and PLCβ. The β-adrenergic receptor (which is considered to be a classical G_s-coupled receptor) and the vasopressin V2, dopamine D1A, and adenosine A2A can functionally interact with the G_q family member, G_{15}, when both proteins are overexpressed in COS cells (15, 16).

An alternate mechanism for stimulation of Ca^{2+} signaling by

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[1] The abbreviations used are: RGS, regulator of G protein signaling; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol trisphosphate; PLCβ, phospholipase Cβ; PKA, cAMP-dependent protein kinase; VIP, vasoactive intestinal peptide; Iso, isoprenaline; βARK1, β-adrenergic receptor kinase 1; PTX, pertussis toxin; SMG, submandibular gland; SLO, streptolysin O toxin; R_s, Gs-coupled receptor; R_q, G_q-coupled receptor; Rp-8-CPT-cAMP-S, 8-(4-chlorophenylthio)adenosine cyclic 3′,5′-phosphorothioate; $[Ca^{2+}]_i$, intracellular Ca^{2+}.

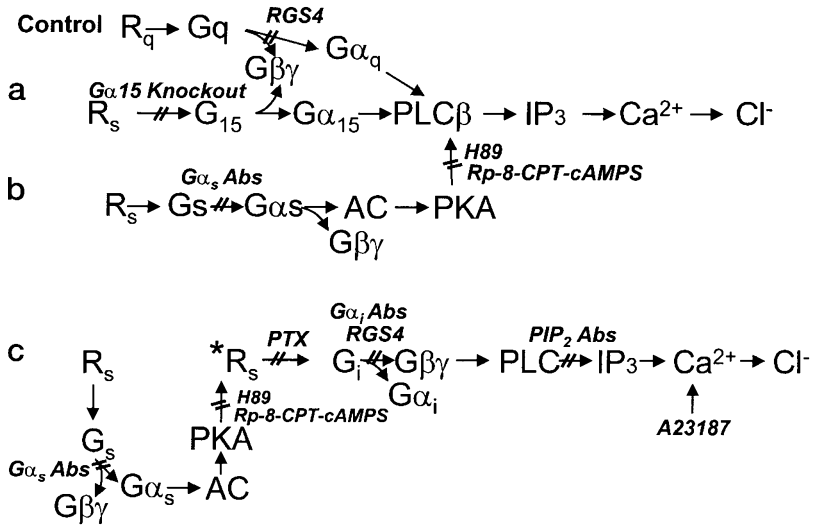

FIG. 1. **Signaling pathways tested in this study.** *Double slashes* through *arrows* in the pathway signify inhibition by the indicated agents. A positive control for Ca^{2+} release was tested by carbachol stimulation of G_q-coupled muscarinic m3 receptors (R_q), which stimulate PLCβ via G_q class α subunits. Ca^{2+} signaling in this work is followed by measuring the activity of Ca^{2+}-activated Cl^- current. *a,* G_s-coupled receptors (R_s) such as the VIP or β-adrenergic receptor might activate the G_q class heterotrimeric G protein, $G\alpha_{15}$. A wide variety of G_s-coupled receptors can couple to G_{15}, activate PLCβ to produce IP_3, and release Ca^{2+} from intracellular stores (15, 16). This potential pathway would be absent from $G\alpha_{15}$ knockout mice. *b,* R_s activation of G_s and stimulation of adenylyl cyclase (*AC*) to increase production of cAMP, activates PKA which could activate PLCβ. *c,* agonist stimulation of R_s typically activates G_s and stimulates AC to increase production of cAMP (inhibited by carboxyl-terminal $G\alpha_s$ antibodies). PKA activity is required (directly or indirectly) for generation of $*R_s$, thereby eliciting a switch (17) or an augmentation to account for activation of G_i by G_s-coupled receptor agonists. G$\beta\gamma$ released by activation of G_i could stimulate PLCβ activity, produce IP_3, and release Ca^{2+} from intracellular stores. PTX and antibodies (*Abs*) to the carboxyl terminus of $G\alpha_i$ inhibit receptor activation of G_i. PIP_2 antibodies prevent PLCβ hydrolysis of PIP_2 and production of IP_3. The Ca^{2+} ionophore, A23187, bypasses the need for IP_3 production needed for Ca^{2+} release from intracellular stores.

G_s-coupled receptors is activation of PLCβ by PKA. In several cell types, increasing cellular cAMP with forskolin (5, 8, 9) or membrane permeable cAMP analogues (5) increased $[Ca^{2+}]_i$ similar to stimulation of G_s-coupled receptors. In a recent study we showed that stimulation of submandibular gland (SMG) duct cells with forskolin results in PLCβ-mediated and IP_3-dependent Ca^{2+} release from internal stores (9). These findings suggest that, at least in some cell types, stimulation of PKA can activate PLCβ to generate a Ca^{2+} signal.

Phosphorylation-dependent switching of receptor specificity for G proteins is another mechanism by which a single receptor could activate more than one G protein (17). As outlined recently by Lefkowitz (18), receptor-dependent activation of G_s stimulates adenylyl cyclase, generates cAMP, and activates PKA. Phosphorylation of the receptor by PKA is proposed to switch its coupling specificity from G_s to G_i. Receptor-dependent activation of G_i could thus release sufficient G$\beta\gamma$ to activate PLCβ. Activation of PLCβ generates IP_3 (which releases Ca^{2+} from internal stores) and diacylglycerol to activate protein kinase C. Hence, PKA-dependent switching of receptor coupling to different classes of G proteins (the G_s/G_i switching model) is a potential mechanism for activation of multiple signal transduction cascades by the same receptor.

In the work presented here we sought to determine if any of the above models applied to classical G_s-coupled receptors that evoke Ca^{2+} signals in cells freshly isolated from native tissues. We used vasoactive intestinal peptide (VIP) stimulation of pancreatic acinar cells and isoprenaline (Iso) stimulation of SMG duct cells to show that switching or augmentation of receptor coupling to G_i could account for activation of cAMP and Ca^{2+} signaling systems *in vivo*.

EXPERIMENTAL PROCEDURES

Reagents—Affinity purified B087, C260, and C267 polyclonal antibodies specific for $G\alpha_{i1}$ and $G\alpha_{i2}$ ($G\alpha_{i1,2}$), $G\alpha_{i3}$, and $G\alpha_o$ ($G\alpha_{i3,0}$) and $G\alpha_s$, respectively (19), and anti-$G\alpha_q$ IgG (20, 21) were prepared as described. Monoclonal antibody against PIP_2 was purchased from Preseptive Diagnostics. Pertussis toxin (PTX) (from List Biological Laboratories) was reconstituted into distilled H_2O and diluted into a pipette solution containing 0.5 mM dithiothreitol. A glutathione-tagged fragment of β-adrenergic receptor kinase (βARK1) was kindly provided by Dr. Robert Lefkowitz (Duke University, Durham, NC). His-tagged RGS4 was expressed in *Escherichia coli* and purified as described (22). Stock solutions of all antibodies, the βARK1 fragment, and RGS4 were dialyzed against a solution containing 100 mM KCl and 10 mM HEPES (pH 7.2 with NaOH) and stored at −20 °C until dilution into the pipette solution. H89 and Rp-8-CPT-cAMP-S were obtained from Biomole and BioLog, respectively. The pipette solution contained (in mM): 150 KCl, 10 HEPES (pH 7.2 with NaOH), 2 $MgCl_2$, 1 ATP, and 0.1 EGTA. The standard bath solution A contained (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES (pH 7.2 with NaOH), and 10 glucose. When this solution was supplemented with 10 mM pyruvate, 1 mg/ml bovine serum albumin, and 0.02% soybean trypsin inhibitor, it was abbreviated PSA.

Cell Preparation—Production of $G\alpha_{15}$ (−/−)-mutant mice was described (22, 23). Single pancreatic acinar and submandibular gland (SMG) duct cells from wild type (WT) and $G\alpha_{15}$ (−/−)-mice were prepared by standard collagenase and trypsin digestion procedures (24, 25). In brief, mice were sacrificed by exposure to a methoxyflurane-saturated atmosphere. The pancreas and SMG were removed and cleaned by injection of PSA. Minced tissues were incubated in a PSA solution containing 0.1 mg/ml collagenase (type CLSP, Worthington) before a short treatment with a trypsin/EDTA solution to release single cells. The cells were washed with PSA and kept on ice until use.

Current Recording—The Ca^{2+}-activated Cl^- current of pancreatic acinar and SMG duct cells was recorded as detailed (21), using the whole cell configuration of the patch clamp technique (26). The cells were dialyzed with the pipette solution for 8–10 min before the first stimulation to allow equilibration of proteins and antibodies when included in the pipette solution. Membrane potential was held at −40 mV to record the inward current. The output signal recorded with a pClamp 6 and DigiData 1200 interface was filtered at 20 Hz. Due to significant variations in the current magnitude between preparations, results are given primarily as the number of responding cells. For each protocol similar results were obtained with cells from at least three mice.

RESULTS AND DISCUSSION

Fig. 1 summarizes the signaling pathways by which G_s-coupled receptors (R_s) may trigger a Ca^{2+} signal. Stimulation of a G_q-coupled cholinergic receptor with carbachol (R_q) was

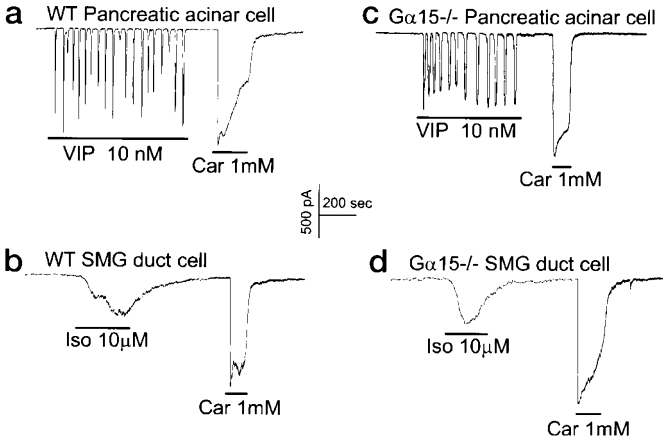

FIG. 2. **Activation of Ca^{2+}-dependent Cl^- current by VIP and Iso in cells from wild type and $G\alpha_{15}$ (−/−)-mice.** Pancreatic acinar (*a* and *c*) or SMG duct cells (*b* and *d*) from wild type (*WT*, *a* and *b*) and $G\alpha_{15}$ (−/−) (*c* and *d*) mice were dialyzed with the standard pipette solution for at least 10 min before cell stimulation. As indicated by the *bars*, pancreatic acinar cells were stimulated by 10 nM VIP, which induced $[Ca^{2+}]_i$ oscillations, and then with 1 mM carbachol, which induced a biphasic response. SMG duct cells were stimulated with 10 μM Iso and then 1 mM carbachol (*Car*). Both agonists induced a biphasic response. The number of observations under each condition is given in the text.

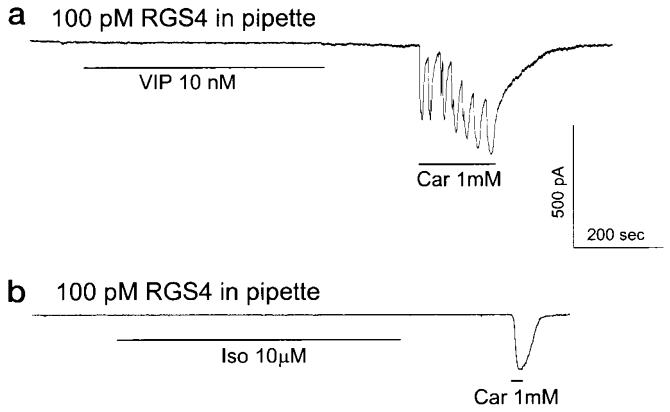

FIG. 3. **Effect of RGS4 on Ca^{2+} signaling.** Pancreatic acinar (*a*) and SMG duct cells (*b*) were dialyzed with a pipette solution containing 100 pM recombinant RGS4. Cells were stimulated with VIP, Iso, or carbachol (*Car*), as indicated.

used as a positive control. Three mechanisms were tested: (*a*) activation of $G\alpha_{15}$ by R_s, (*b*) direct activation of PLCβ by PKA, and (*c*) switching or augmentation of coupling specificity of R_s from G_s to G_i. We tested these mechanisms using two G_s-coupled receptors which evoke different types of Ca^{2+} signals: pancreatic acinar cells stimulated with VIP and SMG duct cells stimulated with Iso. Ca^{2+} signaling was followed by measuring the activity of the Ca^{2+}-activated Cl^- current in each cell type. Previous work showed that pancreatic acinar and SMG cells express the Ca^{2+}-activated Cl^- channel (21, 25, 27) and this current faithfully reflects changes in $[Ca^{2+}]_i$ (21, 27).

Fig. 2*a* shows that stimulation of pancreatic acinar cells with a saturating concentration of VIP-induced $[Ca^{2+}]_i$ oscillations which lasted for the duration of cell stimulation, as previously reported (28). Maximal stimulation of the G_q-coupled muscarinic m3 receptor with 1 mM carbachol in the same cells resulted in a typical biphasic response of a spike and a plateau. This response was highly reproducible in mouse pancreatic acinar cells; similar responses were observed in 15/15 cells from 13 mice. Fig. 2*b* shows that stimulation of SMG duct cells with the β-adrenergic agonist Iso caused a sustained increase in the Ca^{2+}-activated Cl^- current with no apparent oscillations. Following removal of Iso, stimulation with carbachol caused a large biphasic response. The Cl^- current responses are similar in shape and time course to the previously reported changes in $[Ca^{2+}]_i$ caused by these agonists in SMG cells (8, 9). Among cells which responded to carbachol, prior stimulation with Iso elicited a response similar to that in Fig. 2*b* in 19/25 SMG duct cells from 17 mice.

β-Adrenergic, vasopressin V2, dopamine D1A, and adenosine A2A receptors overexpressed in COS cells can couple to $G\alpha_{15}$, but not other members of the G_q class, and stimulate PLCβ activity (15, 16). This would suggest that $G\alpha_{15}$ has the unique ability to couple to receptors which are usually coupled to G_s. Currently, there are no good biochemical tools to specifically evaluate $G\alpha_{15}$ function in native cells. Genetics provide an alternative approach. We measured the effect of VIP and Iso on Ca^{2+} signaling in cells prepared from mutant $G\alpha_{15}$ (−/−)-mice to rule out the possibility that $G\alpha_{15}$ contributes to Ca^{2+} signaling by G_s-coupled receptors in SMG and pancreatic acinar cells. Fig. 2*c* shows that VIP- and carbachol-induced Ca^{2+} signaling was completely normal in pancreatic acini for $G\alpha_{15}$ (−/−)-mice. The same results were obtained in six out of six experiments with acini from six mice. Fig. 2*d* shows that Iso- and carbachol-induced Ca^{2+} signaling was normal in SMG duct cells from $G\alpha_{15}$ (−/−)-mice. Similar results were obtained in four out of six experiments with SMG ducts prepared from the six mice that were used to study the response of pancreatic acinar cells. These findings exclude coupling to $G\alpha_{15}$ as obligatory for activation of Ca^{2+} signaling by the G_s-coupled receptors. Coupling of R_s to other members of the G_q class is also excluded by experiments with antibodies described below.

Experiments with RGS4 supplied our first evidence that activation of Ca^{2+} signaling by VIP and Iso involves more than activation of G_s. RGS4 accelerates GTP hydrolysis by G_q and G_i class α subunits but not $G\alpha_s$ (29, 30). In Fig. 3, infusion of 100 pM RGS4 through a patch pipette into pancreatic acinar (Fig. 3*a*) or SMG duct (Fig. 3*b*) cells completely inhibited the Ca^{2+} response to VIP and Iso, respectively. The control shows that the response to subsequent stimulation with carbachol was markedly reduced, as we reported recently (23). Measurement of cAMP production in streptolysin *O*-permeabilized cells showed that inhibition of Ca^{2+} signaling by RGS4 was not due to inhibition of cAMP production by the G_s-coupled receptors (not shown). The results with RGS4 exclude model b of Fig. 1 as the mechanism by which R_s evokes a Ca^{2+} signal.

In the next set of experiments we systematically tested the model for PKA-dependent G_s/G_i switching (or augmentation) of receptor specificity shown in Fig. 1*c* (17, 18). We first tested if stimulation of G_s is obligatory for launching a Ca^{2+} signal by the VIP and Iso receptors. This was achieved by introducing antibodies specific for $G\alpha_s$ into the cells through a patch pipette. Antibodies to the carboxyl terminus of $G\alpha_s$ were used because they have been reported to block receptor-mediated activation of adenylyl cyclase (31). Fig. 4 shows that the antibodies specific for $G\alpha_s$ inhibited Ca^{2+} oscillations induced by VIP stimulation of pancreatic acinar cells and the Ca^{2+} signal stimulated by Iso acting on SMG duct cells without affecting the oscillations or the biphasic response evoked by stimulation of the G_q-coupled m3 receptor with carbachol. Similar findings were observed in 4 additional acinar and 3 additional duct cells. As discussed below, infusion of $G\alpha_q$ specific antibodies did not effect VIP- or Iso-evoked Ca^{2+} signaling. Therefore, G_s stimulation was essential for launching a Ca^{2+} signal by the two classical G_s-coupled receptors.

If PKA-dependent phosphorylation were involved, then inhibition of PKA activity should block G_s- but not G_q-dependent signaling (Fig. 1*c*). The R_s in both cell types met this criterion

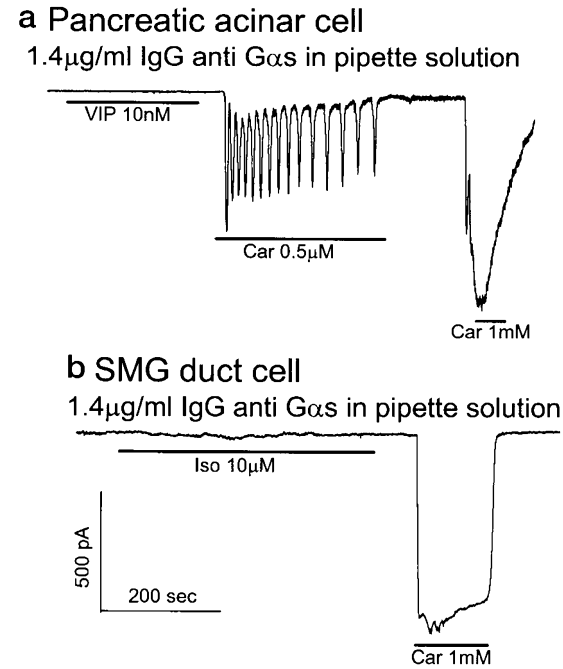

FIG. 4. **$G\alpha_s$ antibodies inhibit effect of VIP and Iso on $[Ca^{2+}]_i$.** Pancreatic acinar (*a*) or SMG duct (*b*) cells were dialyzed with a pipette solution containing antibodies against $G\alpha_s$ for at least 10 min before stimulation with the respective agonist as indicated by the *bars*. Controls for these experiments are shown in Figs. 6, 8, and 9. The number of similar observations are indicated in the text.

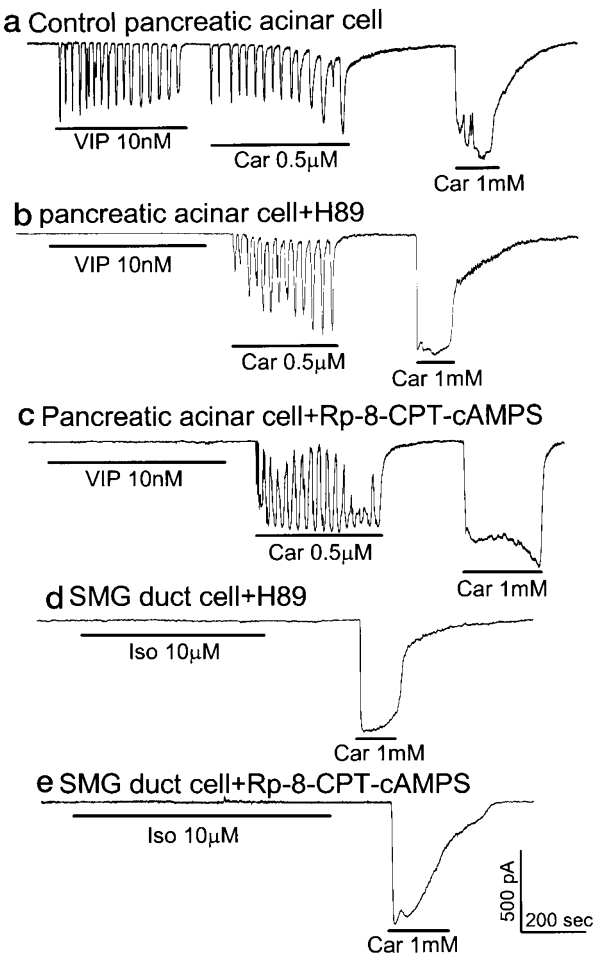

FIG. 5. **Effect of PKA inhibitors on Ca^{2+} signaling.** Pancreatic acinar (*a-c*) or SMG duct cells (*d* and *e*) were dialyzed with the standard pipette solution. In *c* and *e* the pipette solution contained 10 μM Rp-8-CPT-cAMP-S. The bath was perfused with solution A (*a*) that also contained 10 μM H89 (*b* and *d*). After about 10 min incubation with H89, pancreatic acinar cells were stimulated with 10 nM VIP, then with the submaximal concentration of 0.5 μM carbachol (*Car*) to induce G_q-dependent oscillations, and finally with the supermaximal concentration of 1 mM carbachol to induce a biphasic response. SMG duct cells were stimulated with 10 μM Iso and then 1 mM carbachol. The number of observations under each condition is given in the text.

as shown in Fig. 5. In control experiments, Ca^{2+} oscillations were initiated by stimulation of pancreatic acinar cells with VIP. After termination of VIP stimulation by removing the agonist, very similar oscillations were initiated by stimulating the same cells with low concentrations of carbachol, which acts through the G_q-coupled muscarinic receptor. Finally, the cell was stimulated with a supermaximal concentration of carbachol (Fig. 5). Similar results were obtained in 14 cells. In four separate experiments, the VIP response was completely abolished when pancreatic acinar cells were treated with 10 μM H89, a selective and potent inhibitor of PKA (32), whereas the ability of a low concentration of carbachol to induce oscillations or of a supermaximal concentration to induce a biphasic response was unaltered (Fig. 5*b*). Similarly, treatment of SMG duct cells with 10 μM H89 abolished Iso-dependent $[Ca^{2+}]_i$ increase, without affecting the carbachol-dependent response (Fig. 5*d*). Inhibition of the response to Iso was observed in all 6 SMG duct cells treated with H89. The requirement for PKA stimulation was further verified by testing the effect of the potent and selective inhibitor of PKA, Rp-8-CPT-cAMP-S. Infusing the cells with 10 μM Rp-8-CPT-cAMP-S through the pipette abolished the response to VIP ($n = 7$) and Iso ($n = 5$) in all cells tested (Fig. 5, *c* and *e*). Again, control experiments in the same cells showed that all forms of G_q-dependent responses were unaffected by inhibition of PKA with Rp-8-CPT-cAMP-S. These inhibitory effects of the two PKA inhibitors argue against the possibilities that unregulated VIP or β-adrenergic receptors are coupled directly to G_i (33) or that $G\alpha_s$ directly modulates Ca^{2+} channels (34) in these systems.

To directly address a role for G_i in Ca^{2+} signaling by VIP and Iso we measured the effect of infusing the cells with PTX or antibodies specific for certain members of the G_i subclass of α subunits. Preliminary studies showed that concentrations of PTX below 20 ng/ml in the pipette solution did not consistently inhibit VIP-induced signaling. At concentrations above 50 ng/ml, PTX rapidly caused a large, time-dependent, nonselective increase in membrane conductance, as if PTX caused cell permeabilization. We therefore limited our testing to the effect of 20 ng/ml PTX on Ca^{2+} signaling in pancreatic acinar cells. Fig. 6 shows that treatment with PTX inhibited VIP but not carbachol-dependent Ca^{2+} signaling. Similar results were obtained in four experiments. In 13 additional experiments, PTX-treated acinar cells lysed before the experimental protocol could be completed. We were unable to find a concentration of PTX that inhibited the Iso response in SMG duct cell without causing cell lysis.

Antibodies generated against peptides representing the carboxyl termini of $G\alpha_i$ and $G\alpha_q$ subunits inhibit receptor-initiated activation of these G proteins (20, 21, 35). The results obtained by infusing antibodies into pancreatic acinar cells are illustrated in Fig. 7. Two types of polyclonal antibodies against G_i were used, one recognizing $G\alpha_{i3}$ and $G\alpha_o$ or one specific for $G\alpha_{i1}$ and $G\alpha_{i2}$ (19). Fig. 7*a* shows that infusing 17.5 μg/ml antibodies specific for $G\alpha_{i3}$ and $G\alpha_o$ had no effect on Ca^{2+} signaling induced by G_s- or G_q-coupled receptors. Similar results were obtained in four cells. However, these antibodies were not without effect, as seen for SMG cells (described below). Fig. 7*b* shows that infusing pancreatic acinar cells with 9 μg/ml $G\alpha_{i1,i2}$-

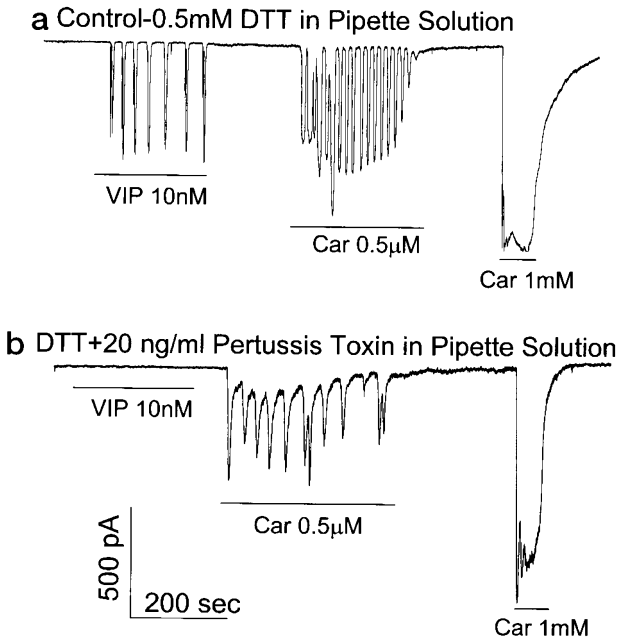

FIG. 6. **PTX inhibits VIP-mediated Ca^{2+} signaling without affecting the response to carbachol.** Pancreatic acinar cells were dialyzed for 7 min with pipette solutions containing 0.5 mM dithiothreitol (*DTT*) (*a* and *b*) and 20 ng/ml PTX (*b*) before stimulation with 10 nM VIP, 0.5 μM carbachol (*Car*), or 1 mM carbachol as indicated by the *bars*. The number of similar observations is given in the text.

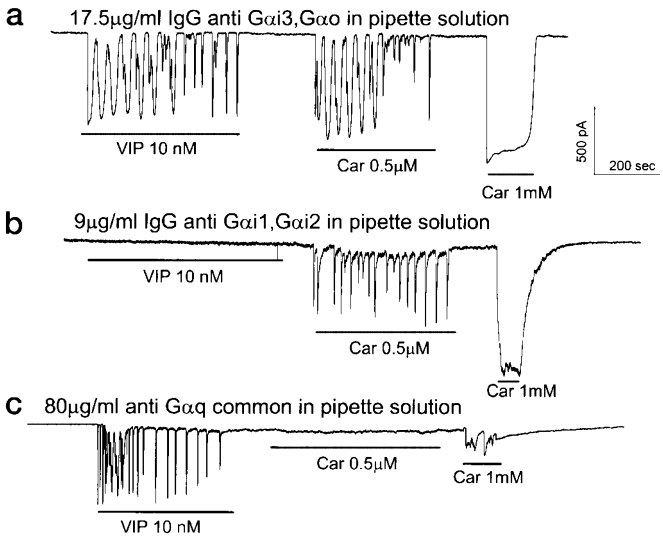

FIG. 7. **G_i-dependent and G_q-independent effect of VIP on $[Ca^{2+}]_i$.** Pancreatic acinar cells were dialyzed for at least 10 min with pipette solutions containing 17.5 μg/ml $G\alpha_{i3,o}$ antibodies (*a*), 9 μg/ml $G\alpha_{i1,i2}$ antibodies, or 80 μg/ml IgG anti-$G\alpha_{q,11}$ common antibodies before stimulation with 10 nM VIP, 0.5 μM carbachol (*Car*), or 1 mM carbachol. The number of similar observations is given in the text.

specific antibodies completely inhibited the response to VIP without affecting the response to carbachol. Similar results were observed in six cells. An important control is shown in Fig. 7*c*. In contrast to the effect of G_i-specific antibodies, infusing the cells with $G\alpha_{q,11}$ antibodies (at sufficient concentration to abolish the oscillation and largely inhibit the sustained response to carbachol) had no effect on the ability of VIP to induce oscillations. In seven experiments with cells infused with 80 μg/ml anti-$G\alpha_q$ IgG the response to VIP remained normal, while the response to the low concentration of carbachol was abolished and the response to supermaximal concentration of carbachol was inhibited by 83 ± 7%.

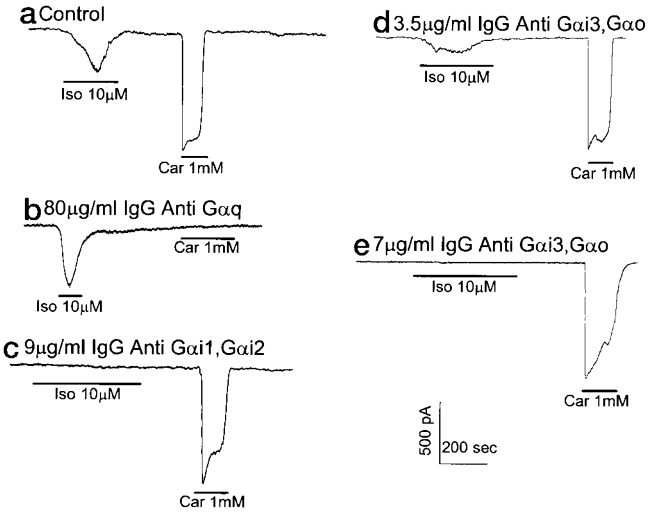

FIG. 8. **G_i-dependent and G_q-independent effect of Iso on $[Ca^{2+}]_i$.** SMG duct cells were dialyzed for at least 10 min with standard pipette solution containing 173 μg/ml of the IgG fraction from preimmune serum (*a*, control), 80 μg/ml IgG anti-$G\alpha_q$ common (*b*), 9 μg/ml $G\alpha_{i1,i2}$ antibodies (*c*), and 3.5 μg/ml (*d*), or 7 μg/ml (*e*) $G\alpha_{i3,o}$ antibodies prior to stimulation with 10 μM Iso or 1 mM carbachol (*Car*). The number of similar observations is given in the text.

Activation of G_i by Iso is further suggested by the results for SMG duct cells shown in Fig. 8. In six cells infused with $G\alpha_{q,11}$ reactive IgG, the response to supermaximal concentrations of carbachol was reduced by 91 ± 6% while the response to Iso was not affected (Fig. 8*b*). Unlike the findings in pancreatic acinar cells stimulated with VIP, both G_i antibody preparations effectively inhibited the response to Iso in SMG duct cells. $G\alpha_{i1,i2}$-specific antibodies, at a concentration of 9 μg/ml, completely inhibited the Ca^{2+} response to Iso (Fig. 8*c*). Infusion of only 3.5 μg/ml $G\alpha_{i3,o}$ antibodies completely inhibited the response to Iso in two cells and partially (63 ± 14%) in three cells (Fig. 8*d*). At a concentration of 7 μg/ml the anti-$G\alpha_{i3,o}$ completely inhibited the response to Iso in five cells (Fig. 8*e*).

The findings in Figs. 7 and 8 provide strong evidence that activation of Ca^{2+} signaling by G_s-coupled receptors is independent of members of the G_q class. The inhibitory G_q antibodies used in the present work recognizes the predominant G_q class α subunits expressed in these cells, $G\alpha_q$, $G\alpha_{11}$, and $G\alpha_{14}$ (22). Furthermore, these antibodies were shown to inhibit Ca^{2+} signaling evoked by several G_q-coupled receptors in pancreatic (21) and other cell types (36, 37). At a concentration inhibiting the oscillatory and the biphasic response to cholinergic stimulation, the antibodies had no apparent effect on the response to either VIP or Iso. This data supports the conclusion that inhibition of VIP- and Iso-induced Ca^{2+} signaling by RGS4 was due to acceleration of GTPase activity of a G_i class α subunit(s).

The use of PTX and $G\alpha_i$ antibodies indicates that receptor-mediated activation of G_i was required for activation of Ca^{2+} signaling by VIP or Iso. It is notable that both G_i antibody preparations inhibited Iso-stimulated Ca^{2+} signaling in SMG duct cells whereas only the $G\alpha_{i1,i2}$-specific preparation was effective for inhibiting VIP-stimulated signaling in the pancreatic acinar cells. This minor difference between the two systems may be attributed to cell type-specific expression patterns of $G\alpha_i$ isoforms or the degree of $G\alpha_i$ selectivity exhibited by putative PKA-phosphorylated VIP and β-adrenergic receptors. It is puzzling that the β-adrenergic Ca^{2+} response is inhibited completely by either G_i antibody preparation. If the β-adrenergic receptor couples to all members of the G_i class, then each antibody preparation would be expected to only partially inhibit and a mixture of the antibodies to completely inhibit

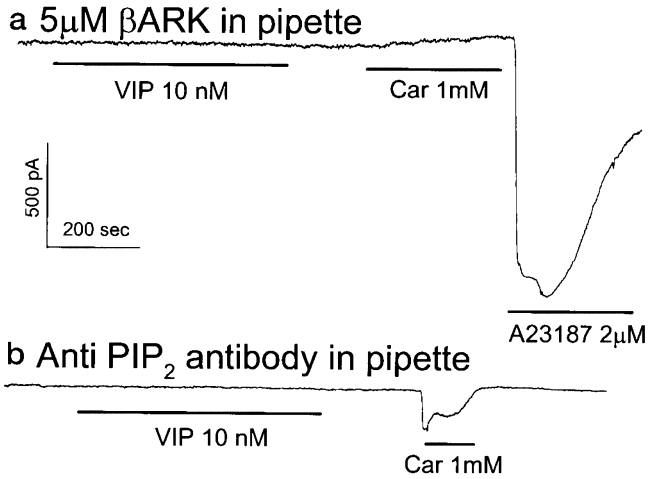

FIG. 9. **Inhibition of VIP-induced Ca^{2+} signaling by βARK1 and PIP_2 antibodies.** Pancreatic acinar cells were dialyzed with pipette solutions containing 5 μM recombinant βARK1 fragment (*a*) or PIP_2 antibodies (*b*) prior to stimulation with 10 nM VIP and 1 mM carbachol (*Car*). As indicated, $[Ca^{2+}]_i$ was increased by the addition of A23187 to the cell in *a*. The number of similar observations is given in the text.

signaling by these receptors. The complete inhibition of signaling by either antibody preparation suggests that partial inhibition of IP_3 production by stimulation of the β-adrenergic receptor had reduced IP_3 below a threshold level needed to trigger Ca^{2+} release. This interpretation is supported by previous work showing that Iso released Ca^{2+} from the IP_3 mobilizable Ca^{2+} pool (9) without causing a detectable increase in global IP_3 concentration (8).

In the G_s/G_i switching model, activated receptor, phosphorylated by PKA, couples to G_i (18). This predicts that Gβγ released from G_i could activate PLCβ. Thus, inhibition of Gβγ or PLCβ activity is expected to inhibit the effect of the G_s-coupled receptors on $[Ca^{2+}]_i$. To test these predictions, we measured the effect of the Gβγ scavenging protein βARK1 (21, 38) and of the inhibitory PIP_2 antibody (39, 40) on VIP-dependent Ca^{2+} signaling. Fig. 9*a* shows that infusing 5 μM βARK1 into pancreatic acinar cells completely inhibited the response to VIP. As we (21) and others (37) reported earlier, βARK1 also inhibited the response to stimulation of the G_q-coupled muscarinic receptor. Inhibition by βARK1 was upstream of the Ca^{2+} increase because elevation of $[Ca^{2+}]_i$ with A23187 strongly activated the Cl^- current. Results similar to those in Fig. 9*a*, including the positive control with A23187, were obtained in five experiments. Fig. 9*b* shows that cytoplasmic PIP_2 antibodies completely inhibited the response to VIP and reduced the response to carbachol by 88 ± 11% (n = 7). These experiments indicate that both VIP and carbachol stimulate PLCβ to cause the hydrolysis of PIP_2.

In summary, our examination of the $[Ca^{2+}]_i$ increase triggered by G_s-coupled receptors supports a model for switching or augmentation of receptor coupling to extend to G_i in native cells freshly isolated from tissue. We conclude that the pathway involves activation of G_s and PKA, receptor stimulation of G_i, and activation of PLCβ by Gβγ (derived from G_i). We acknowledge that the PKA substrate(s) responsible for activation of G_i are not known but, as suggested by the switching model (17, 18), they could be the same receptors that were initially coupled only to G_s. We use caution, however, in referring to the Ca^{2+} pathway (Fig. 1*c*), as a receptor switching model. PKA-dependent phosphorylation of the VIP or β-adrenergic receptors could allow G_i to replace G_s but the data are also consistent with broadening of receptor coupling to G_s plus G_i. One mode for augmentation of receptor coupling can be envisioned if it is assumed that most β-adrenergic or VIP receptors are productively coupled to G_s but a smaller subpopulation are poised to couple to G_i. Effective G_i coupling would occur only when the receptors are phosphorylated by PKA. Because expression of a mutant (phosphorylation negative) β-adrenergic receptor prevented PKA-dependent activation of G_i in HEK 293 cells (17), it is unlikely that phosphorylation of proteins downstream of the VIP or β-adrenergic receptors are responsible for activation of G_i in pancreatic acinar or submandibular gland cells. An alternative to the assumption that mutant receptor is unable to couple to G_i (17) is that the mutant cannot regulate its interaction with an RGS protein that may ordinarily suppress G_i activation stimulated by the β-adrenergic receptor. A role for regulation of RGS protein function by receptor phosphorylation is attractive, not only because RGS proteins exhibit selectivity among receptor signaling complexes (23, 41, 42), but also because phosphorylation is not necessary for purified β-adrenergic receptors to activate G_i *in vitro* (33). Additional experimental tools are needed to distinguish between these and other potential mechanisms. Independent of the mode of coupling it is clear that in pancreatic acinar and submandibular cells G_s-coupled receptors activate Ca^{2+} signaling by coupling to G_i and this coupling requires activation of G_s.

An equally important conclusion is that VIP and β-adrenergic receptor regulation of Ca^{2+} release is completely independent of G_q class proteins. The observation that PKA-dependent switching/augmentation in receptor/G protein coupling occurs in two different native cell types via two different receptors (that generate different types of Ca^{2+} signals) suggests a generalization of the mechanism by which G_s-coupled receptors generate a second signal to activate a distinct signaling cascade.

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