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Thyrotropin Receptor Stimulates Internalization-independent Persistent Phosphoinositide Signaling

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Abstract

The thyrotropin (TSH) receptor (TSHR) is known to acutely and persistently stimulate cAMP signaling and at higher TSH concentrations to acutely stimulate phosphoinositide signaling. We measured persistent signaling by stimulating TSHR-expressing HEK-EM293 cells with TSH and measuring cAMP or inositolmonophosphate (IP1) production, a measure of phosphoinositide signaling, 60 min or longer after TSH removal. In contrast to persistent cAMP production, persistent IP1 production increased progressively when TSH exposure was increased from 1 to 30 min whereas the rates of decay of persistent signaling were similar. Persistent IP1 and cAMP signaling were also caused by a small molecule agonist and a thyroid-stimulating antibody. TSH stimulated persistent IP1 production was inhibited by a small molecule inverse agonist and a neutral antagonist whereas the inverse agonist but not the neutral antagonist inhibited persistent cAMP production. As with persistent cAMP production, persistent IP1 production was not affected when TSHR internalization was inhibited or enhanced. Moreover, Alexa546-TSH-activated TSHR internalization was not accompanied by $G\alpha_q$ coupling protein internalization. Thus, transient exposure to high concentrations of TSH causes persistent phosphoinositide and cAMP signaling that is not dependent on internalization. To our knowledge, this is the first demonstration of persistent activation by any GPCR via the $G\alpha_q$ pathway and of two G protein-mediated pathways by any GPCR.

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Introduction

Although G protein-coupled receptors (GPCRs) are able to signal independently of G proteins, the major pathways for GPCR signaling involve coupling of the activated receptor to one or more G proteins (Woehler and Ponimaskin, 2009). The human thyrotropin (thyroid-stimulating hormone, TSH) receptor (TSHR) has been shown to couple to several G proteins (Laugwitz et al., 1996) including the stimulatory G protein (G_s), which activates adenylyl cyclase to produce cAMP (cAMP pathway), and $G_{q/11}$, which activates phospholipase C to produce inositol -1,4,5-trisphosphate (I-1,4,5-P_3) (phosphoinositide pathway). The G_s-mediated stimulation of cAMP formation has been regarded as the principal intracellular signaling mechanism mediating the action of TSH. However, recently Kero et al. (Kero et al., 2007) demonstrated that the G_q/G_{11}-mediated signaling pathway is required for TSH-induced thyroid hormone synthesis and release in the adult and that the lack of G_q/G_{11} leads to hypothyroidism. Additional support for an essential physiological role of Gq/11 proteins in mediating the regulation of thyroid function was demonstrated by a TSHR germline mutation that preferentially affected the phosphoinositide pathway (Grasberger et al., 2007).

Until recently, it was thought that GPCRs with dissociable agonists signal transiently and that the signaling pathway was rapidly desensitized by several mechanisms including receptor internalization (Hausdorff et al., 1990). However, over the last two years, it was shown that three GPCRs – TSHR (Calebiro et al., 2009; Neumann et al., 2010), the parathyroid hormone receptor (Ferrandon et al., 2009), which also couples to G_s, and the sphingosine-1-phosphate (S1P) receptor (S1P1) (Mullershausen et al., 2009), which couples to G_i to decrease cAMP production - exhibit persistent signaling even after the agonist has been removed. With these receptors, persistent signaling has been found to last more than several hours.

In this study, we sought to determine whether the TSHR signaled persistently via the phosphoinositide pathway. It was shown that higher concentrations of TSH are required to

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stimulate I-1,4,5-P_3 production than cAMP production (Van Sande et al., 1990). By using high concentrations of TSH, we were able to stimulate cAMP and phosphoinositide signaling simultaneously. We found that TSHR exhibits persistent activation of the phosphoinositide pathway as it does the cAMP pathway and that persistent phosphoinositide signaling occurs independently of internalization. We also show that persistent signaling can be caused by a small molecule agonist of TSHR and by a thyroid-stimulating antibody.

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Materials and Methods

Cell Culture and Transfection

The generation of a HEK-EM 293 cell lines stably expressing human TSHR (HEKTSHR cells) or human TRH receptor were described previously (Neumann et al., 2009; Engel et al., 2006). Cells were grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 10 μg/ml streptomycin (Life Technologies Corp., Carlsbad, CA, USA) and 250 μg/ml hygromycin B (Invitrogen, Carlsbad, CA, USA) at 37C in a humidified 5% CO_2 incubator.

Cells were transiently transfected with βArr2 or K44A (both kindly provided by Dr. Marc Caron, Duke University Medical Center, Durham, NC, USA), $G\alpha_q$-YFP chimera (Hughes et al., 2001) (kindly provided by Dr. Catherine Berlot, Weis Center for Research, Geisinger Clinic, Danville, PA, USA) in 24-well plates (7.5×10^4 cells per well) with 0.2 μg DNA/well or on poly-d-lysine coated coverglass culture dishes (MatTek Corp., Ashland, MA, USA) (10×10^3 cells/cm^2) with 0.3 μg DNA using FuGENE^{TM}6 reagent (Roche, Indianapolis, IN, USA). Experiments were performed 48 or 72 hr after transfection.

cAMP Production

Cells seeded into 24-well plates at a density of 2.2×10^5 cell/well were cultured for 24 h. Then they were washed three times with 0.5 ml of 37C HBSS and incubated in HBSS/10 mM HEPES, pH 7.4 for 30 min. Thereafter acute TSH stimulation was measured as cAMP production in cells incubated in a humidified incubator at 37C for 30 min in HBSS/HEPES containing increasing concentrations (0 to 300 mU/ml) of bovine TSH (bTSH, SIGMA, St. Louis, MO, USA) and 1 mM 3-isobutyl-1-methylxanthine (IBMX).

To determine the persistent effect of TSH, the small molecule ligand C2 and the thyroid-stimulating antibody M22 on cAMP production, cells were incubated with 100 mU/ml bTSH, 15

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or 30 μM C2 or 100 or 200 ng/ml M22 for 30 min ("pretreatment"). Thereafter the cells were washed three times with 0.5 ml ice cold HBSS followed by one wash at RT and incubated in 0.25 ml HBSS/HEPES at 37C. After 1 hr (standard "washout") or at the designated times (1, 2, 4, 6 h, and overnight) to determine the time course of loss of persistent signaling after the washes, the mediums were aspirated and the cells were incubated in 0.2 ml HBSS/HEPES containing 1 mM IBMX at 37C for 30-60 min ("production") (Fig. 2). Total cAMP production was measured by adding 0.2 ml lysis buffer (cAMP-Screen Direct^{TM} System, Applied Biosystems, Foster City, CA, USA) to the wells. cAMP content in the samples was determined according to the manufacturer's protocol. Chemiluminescence was measured in VICTOR3^{TM} V 1420 Multilabel Counter (Perkin Elmer, Waltham, MA, USA). To determine the time of acquisition of persistent signaling, the TSH "pretreatment" was performed for 0, 1, 2.5, 10 or 30 min.

IP1 Production

To determine the effects of acute and persistent TSH stimulation, total IP1 production was measured under the same conditions as described above for cAMP with the exception of using 50 mM LiCl instead of IBMX. The incubations were terminated by adding 0.05 ml lysis buffer (IP-One ELISA kit, CIS Bio International, France) to the wells. IP1 content in the samples was determined according to the manufacturer's protocol. Optical density was measured in SpectraMax Plus^{384} (Molecular Devices, Sunnyvale, CA, USA).

Acid Resistant Binding

To measure acid resistant binding, cells were incubated in binding buffer (HBSS/HEPES containing 2.5% milk powder and 0.2% BSA) with 60,000 cpm bovine ^{125}I-TSH (Brahms Aktiengesellschaft, Hennigsdorf, Germany) for 30 min at 37C and then washed three times with 0.5 ml ice cold HBSS or with ice cold acetic acid/Na acetate buffer, pH 2.8. Total binding was measured in the absence of unlabeled TSH and nonspecific binding in the presence of 100 mU/ml

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unlabeled bovine TSH. Percent acid resistant binding was calculated as 100 times specific binding (total minus nonspecific binding) after acid wash divided by specific binding after HBSS wash. Although it has traditionally been assumed that all acid resistant binding of ligands to receptors monitors internalized receptors, it has recently been shown that this is not the case because some acid resistant binding is present even when internalization is completely inhibited (Jones and Hinkle, 2008;Neumann et al., 2010).

Fluorescent Microscopy

Alexa Fluor 546-modified bovine TSH (Alexa-TSH) was synthesized as described previously (Neumann et al., 2010). Monolayers were washed twice with HEPES/HBSS and then blocked with HBSS containing 4% bovine serum albumin for 15 min at 37C. For binding, Alexa-TSH (10 µl) was diluted with 500 µl of blocking buffer, added to monolayer cultures and incubated for 30 min at 37C. Confocal (<0.5 µm) images were acquired on a ZEISS 510 NLO/Meta system, using a Plan-Apochromat 40X 1.3 objective and are representative of slices at midpoint cell thickness. Detector gains and microscope parameters remained unchanged throughout all experimental conditions. Quantitative colocalization analysis was performed using ZEISS Zen software. Each region of interest (ROI) within a confocal acquisition was assigned to include an entire cell. At least 5 unique images and 13 ROIs were analyzed for each experimental condition. Pearson's correlation coefficient (R_r) and Manders' overlap coefficient (R) were calculated (Zinchuk and Zinchuk, 2008).

Statistical analysis

The data were analyzed by Student's t-test or One-Way Anova; $P<0.05$ was considered significant.

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Results

We used IP1 production to quantify activation of the phosphoinositide pathway because I-1,4,5-P_3 is rapidly metabolized to IP1 and IP1 can be trapped in the presence of LiCl, which inhibits the IP1 monophosphatase. This is similar to quantifying the cAMP pathway by measuring cAMP in the presence of IBMX, which inhibits the cAMP phosphodiesterase. Figure 1 illustrates that TSHR activation acutely leads to stimulation of the production of cAMP and IP1 in HEKTSHR cells and confirms the previously reported observation that much lower concentrations of TSH stimulate cAMP production compared to those required for stimulation of IP1 production (Van Sande et al., 1990). TSH acutely stimulated cAMP production with an EC_{50} = 0.75 mU/ml and IP1 production with an estimated $EC_{50} \geq 71$ mU/ml. Indeed, the estimated EC_{50} for IP1 production is likely an underestimate as maximal stimulation may not have been achieved. We, therefore, needed to use high concentrations of TSH to stimulate both pathways simultaneously.

Figure 2 illustrates the experimental protocol we used to measure persistent TSH signaling. Cells were exposed to TSH ("pretreatment"), washed four times and incubated in buffer alone ("washout") and then incubated in buffer with IBMX or LiCl for measurement of cAMP or IP1 production, respectively. We had previously found that the level of activation of persistent cAMP signaling in HEKTSHR cells by 10 mU/ml TSH increased between 1 and 5 min and then remained constant for up to 30 min (Neumann et al., 2010). Figure 3 illustrates the time courses of acquisition of persistent signaling by the cAMP and IP1 pathways stimulated by 100 mU/ml TSH. This high concentration of TSH caused maximal persistent cAMP production after 1 min of stimulation. In contrast, the level of persistent IP1 production increased progressively with increasing time of exposure to 100 mU/ml TSH over the 30-min period. We showed previously that HEK293EM cells expressing human receptors for luteinizing hormone/chorionic gonadotropin or follicle-stimulating hormone do not exhibit persistent cAMP production

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(Neumann et al., 2010). We found that HEK293EM cells expressing human receptors for thyrotropin-releasing hormone do not exhibit persistent IP1 production (not shown). Thus, not all GPCRs exhibit persistent signaling in this cell model.

Figure 4 illustrates the time courses of loss of persistent cAMP and IP1 production stimulated by 100 mU/ml TSH. Both cAMP and IP1 production fell progressively at rates of approx. 3%/hr and had returned to pretreatment basal levels after 23 hr.

We determined whether other TSHR agonists – a small molecule agonist C2 (NCGC00161870) (Neumann et al., 2009) and a thyroid-stimulating antibody M22 (Sanders et al., 2004) – would cause persistent signaling. We used maximally effective doses of C2 and M22 as determined previously (Neumann et al., 2009; Sanders et al., 2004) and showed that they were maximally effective as the two doses gave similar levels of stimulation. We found qualitatively similar effects of C2 and M22 on persistent cAMP and IP1 signaling. Figure 5 illustrates that C2 and M22 are full agonists for cAMP production when added acutely and stimulated persistent cAMP signaling to similar levels as TSH. In contrast, C2 and M22 are partial agonists for acute IP1 production and stimulated persistent IP1 signaling to proportionately lower levels than TSH. Thus, C2 and M22 are functionally selective agonists (Strange, 2008) at TSHR and stimulate persistent signaling to levels concordant with their efficacies for acute stimulation.

We showed previously that a small molecule inverse agonist of TSHR NCGC00229600 (**1**) partially inhibited persistent cAMP signaling (Neumann et al., 2010). We have recently developed a small molecule neutral antagonist for TSHR NCGC00242595 (**2**). Supplementary Figure 1 illustrates the structure and synthetic scheme of **2** and Supplementary Figure 2 illustrates that **2** does not inhibit basal signaling by TSHR but inhibits TSH-stimulated signaling and is therefore a neutral antagonist. **2** inhibited cAMP production acutely stimulated by an EC_{50} dose of TSH by 55% with an EC_{50} of 2.7 μM. Figure 6 illustrates that **1** inhibited persistent cAMP production by 34% and persistent IP1 production by 89% whereas **2** had no effect on persistent

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cAMP production but inhibited persistent IP1 production by 84%. Thus, both **1** and **2** inhibited persistent IP1 production but only **1** inhibited persistent cAMP production.

As we had previously provided evidence that persistent cAMP production stimulated by TSH did not depend on TSHR internalization ((Neumann et al., 2010), but see Calebiro et al. (Calebiro et al., 2009), we determined whether persistent IP1 production was affected by TSHR internalization. We measured IP1 production in cells in which internalization was inhibited by expressing the dominant negative dynamin mutant protein (K44A) or was enhanced by expression of β-arrestin-2 (βArr2) in these cells (Neumann et al., 2010). Figure 7 illustrates that both expression of βArr2, which increased acid-resistant ^{125}I-bTSH binding from 12% (control) to 59%, and of K44A, which had no effect on acid-resistant ^{125}I-bTSH binding, had no effect on basal, on acute TSH stimulation of, on TSH-activated persistent stimulation of or on re-stimulation by TSH of IP1 production. (The lack of effect of K44A expression on acid resistant binding in these cells confirms our previous findings (Neumann et al., 2010) and the conclusion that not all acid resistant binding is caused by internalization (Jones and Hinkle, 2008)). Most importantly, inducing robust TSHR internalization with βArr2 (see below) did not affect TSH stimulation of the phosphoinositide pathway.

To further address the issue of whether TSHR internalization is necessary for persistent phosphoinositide pathway activation, we visualized TSH-bound TSHRs using Alexa Fluor 546-tagged TSH (Alexa-TSH) in HEKTSHR cells expressing $G\alpha_q$-YFP (Hughes et al., 2001) (Fig. 8). There was no apparent TSHR internalization in HEKTSHR cells nor in HEKTSHR cells expressing K44A (data not shown) but robust internalization in HEKTSHR cells expressing βArr2, as has been reported previously (Frenzel et al., 2006;Neumann et al., 2010). However, $G\alpha_q$-YFP remained at the surface of all cells including cells expressing βArr2 (Fig. 8). Quantitative analysis confirmed that there was a high level of colocalization of Alexa-TSH/TSHR and $G\alpha_q$-YFP on the surface of control cells not expressing βArr2 but no significant colocalization (Pearson's correlation coefficient) or a marked loss in colocalization (Manders's

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overlap coefficient) in cells expressing βArr2. Thus, HEKTSHR cells do not internalize TSH-bound TSHRs but can be made to internalize TSHRs after expression of βArr2 but $G\alpha_q$-YFP does not co-internalize with TSHRs. These findings, together with those in Figure 7, show that persistent IP1 production stimulated by TSHR does not require TSHR (or $G\alpha_q$) internalization.

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Discussion

We show herein that TSHR activation by TSH leads to persistent signaling by the phosphoinositide pathway as well as by the cAMP pathway, which was previously reported (Calebiro et al., 2009; Neumann et al., 2010). As was shown previously (Van Sande et al., 1990) and confirmed herein, the potency of TSH to activate the phosphoinositide pathway was approximately 100-fold lower than that needed to activate the cAMP pathway. Therefore, it was necessary to use high TSH concentrations to activate the cAMP and phosphoinositide pathways simultaneously. We measured persistent activation of the phosphoinositide pathway using a protocol similar to the one we used for measurement of persistent cAMP signaling (Neumann et al., 2010). For the cAMP pathway, we pretreated HEKTSHR cells with TSH, washed the cells to remove TSH and incubated the cells in medium without TSH for 1 hr. After incubation without TSH ("washout"), we measured cAMP production as the amount of cAMP accumulated during 30-60 min incubations in the presence of IBMX, which inhibits cAMP degradation (Figure 2). For the phosphoinositide pathway, we performed the same pretreatment, washes and washout incubation, and then added LiCl to inhibit IP1 degradation by the IP1 phosphatase because I-1,4,5-P_3 is rapidly metabolized to IP1 and there is no effective inhibitor of I-1,4,5-P_3 metabolism. We measured IP1 production as the amount of IP1 accumulated during a 30-60 min incubation in the presence of LiCl. We are, therefore, able to make direct quantitative comparisons between persistent signaling in these two pathways.

We showed that the time courses of acquisition of persistent signaling activated by TSH were different for the cAMP and phosphoinositide pathways. Acquisition of persistent cAMP production was very rapid whereas persistent IP1 production was progressive over 30 min exposure to TSH. We think these different findings were most likely caused by the need to occupy a smaller fraction of TSHRs to generate maximal cAMP production and a higher fraction of TSHRs to generate maximal IP1 production. Indeed, our finding that persistent cAMP

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production was not increased (Neumann et al., 2010) but IP1 production was increased further upon re-exposure to TSH (Fig. 7) may have been because not all TSHR binding sites were occupied during the pretreatment exposure to TSH. By contrast, decay of persistent cAMP and IP1 production were similar (3%/hr) and may reflect an intrinsic mechanism of inactivation of TSH/TSHR complexes.

In the previous reports of persistent cAMP signaling by activated TSHRs the only agonist used was TSH (Calebiro et al., 2009; Neumann et al., 2010). In this study, we activated TSHR with TSH, a small molecule agonist C2 (Neumann et al., 2009) and a thyroid-stimulating antibody M22 (Sanders et al., 2004). We found qualitatively similar effects of C2 and M22 on persistent cAMP and IP1 signaling. Both C2 and M22 are full agonists for cAMP production when added acutely and stimulated persistent cAMP signaling to similar levels as TSH whereas C2 and M22 are partial agonists for acute IP1 production and stimulated persistent IP1 signaling to proportionately lower levels than TSH. Thus, both C2 and M22 exhibit functionally selective acute signaling (Strange, 2008). It had been shown previously that some thyroid-stimulating antibodies exhibit functionally selective signaling at TSHR (Morshed et al., 2010; Morshed et al., 2009). Of note, there was a clear parallelism between the efficacies of these agonists to acutely and persistently stimulate cAMP and IP1 production. These findings are consistent with the idea that the activatory conformation achieved after binding a given agonist to stimulate second messenger formation acutely is retained during persistent signaling. One hypothesis that is compatible with this idea is that the persistently signaling complex is comprised of agonist/activated TSHR/Gα subunit. Another possibility is that the persistently signaling complex is activated TSHR/Gα subunit but does not contain agonist.

To attempt to distinguish between these possibilities we took advantage of our recent discovery of a small molecule neutral antagonist of TSHR **2** (Supplementary Figures 1 and 2). In our previous report (Neumann et al., 2010), we used another structurally related small molecule inverse agonist (NCGC 00161856) and showed that it inhibited persistent cAMP production.

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These previous data allowed us to conclude that activated TSHR was necessary for persistent cAMP production but could not allow the determination of whether TSH was still present in the complex because an inverse agonist may inhibit signaling by competition of agonist binding to receptors and by unoccupied receptors. Herein we showed that both **1** and **2** markedly inhibited persistent IP1 production. Therefore, if **2** is a neutral antagonist at TSHR for IP1 production as it is for cAMP production, which is difficult to determine because basal IP1 production stimulated by TSHR is very low, then we may conclude that the complex that persistently signals includes the agonist-occupied, activated TSHR. We confirmed that **1** inhibits persistent cAMP production but found that **2** did not. The explanation for the finding that 2 inhibits persistent IP1 signaling but not persistent cAMP signaling may be because a lower fraction of TSHRs need be bound by TSH to cause persistent cAMP signaling and a higher fraction for IP1 signaling. Another possibility is that there may be "spare receptors" for cAMP but not for IP1 signaling. We are currently performing experiments that may distinguish between these possibilities.

In the initial reports (Calebiro et al., 2009; Ferrandon et al., 2009; Mullershausen et al., 2009), persistent signaling by GPCRs with dissociable agonists was shown to correlate with their internalization. Indeed, for the parathyroid hormone receptor (Ferrandon et al., 2009), parathyroid hormone, which stimulated internalization, caused persistent cAMP signaling whereas parathyroid hormone-related peptide, which did not cause internalization, did not. Evidence was also presented that the GPCRs co-localized with G_s and adenylyl cyclase intracellularly within an early endosomal compartment. These authors, therefore, hypothesized that persistent cAMP signaling was mediated by activated receptors within intracellular compartments. This mechanism was likened to the well established findings that some tyrosine kinase receptors signal intracellularly (Barbieri et al., 2004) and that some GPCRs signal via interactions with arrestin proteins that may be in intracellular vesicles (Hanyaloglu and Von Zastrow, 2008). However, no direct evidence showing that persistent GPCR signaling was dependent on internalization was presented. We recently reported that the TSHR could cause persistent cAMP signaling in

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HEKTSHR cells in which internalization did not occur or was inhibited, and that persistent cAMP signaling was not increased in HEKTSHR cells in which internalization was induced by exogenously expressing βArr2 (Neumann et al., 2010). We concluded, therefore, that persistent cAMP signaling was not dependent on internalization. Indeed, we showed that a small molecule antagonist of TSHR (Neumann et al., 2010) could inhibit persistent signaling and allow re-added TSH to activate the receptor. We, therefore, hypothesized that persistently signaling TSHRs were not in endosomal vesicles but were in a compartment that was accessible to the TSHR antagonist and TSH. Herein we present evidence that persistent signaling via the phosphoinositide pathway is independent of internalization also. We show that inhibiting or increasing internalization does not affect persistent IP1 production stimulated by TSH and that in cells in which TSHR internalization is increased there is no detectable internalization of $G\alpha_q$-YFP.

Even though the concentrations of TSH needed to stimulate the phosphoinositide pathway are higher than the levels found in the blood of normal subjects these levels may be found in tissues in which TSH-producing cells and TSHR-expressing cells are found together, such as the pituitary gland (Prummel et al., 2000) and epidermis (Cianfarani et al., 2010;Bodo et al., 2010). Moreover, thyrostimulin, another heterodimeric glycoprotein activator of TSHR that is thought to act in a paracrine fashion (Nakabayashi et al., 2002; Sun et al., 2010), may achieve concentrations sufficient to stimulate TSHR-mediated phosphoinositide signaling locally. Thyrostimulin has been shown to activate cAMP and calcium signaling, which is most likely initiated by coupling of TSHR to G_q (Nagasaki et al., 2006). Strong support for the idea that TSHR signaling via the phosphoinositide pathway is physiologically relevant was provided by the recent demonstrations that the G_q/G_{11}-mediated signaling pathway is required for TSH-induced thyroid hormone synthesis and release in the adult and that the lack of G_q/G_{11} leads to hypothyroidism (Kero et al., 2007) and the finding that a TSHR germline mutation that preferentially affected the phosphoinositide pathway affected thyroid gland function (Grasberger

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et al., 2007). Thus, we think that phosphoinositide signaling mediated by activation of TSHR is physiologically relevant.

In conclusion, we showed that TSHR activation by TSH leads to persistent signaling by both the cAMP (Calebiro et al., 2009; Neumann et al., 2010) and phosphoinositide pathways. To our knowledge, this is the first demonstration of persistent activation by any GPCR via the G_q pathway and of persistent signaling of two G protein-mediated pathways by any GPCR.

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Author Contributions

Participated in research design: Boutin, Allen, Geras-Raaka, Neumann, Gershengorn

Conducted experiments: Boutin, Geras-Raaka, Gershengorn

Contributed new reagents or analytic tools: Allen, Geras-Raaka, Huang, Gershengorn

Performed data analysis: Boutin, Geras-Raaka, Gershengorn

Wrote or contributed to the writing: Boutin, Geras-Raaka, Neumann, Gershengorn

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Footnotes

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Legends for figures

Figure 1. Acute stimulation of cAMP and IP1 production by TSH.

HEKTSHR cells were acutely exposed to the noted concentrations of TSH in HBSS/HEPES with 1 mM IBMX (cAMP) or 50 mM LiCl (IP1) at 37C. After 60 min, the incubation was stopped and total cAMP or IP1 levels were measured by ELISA as described in Materials and Methods. The apparent EC_{50}s for cAMP and IP1 production were 0.75 and >71 mU/ml, respectively (p<0.001). The data are from one of three experiments with duplicate samples and are presented as mean±range.

Figure 2. Schematic of the protocol to measure persistent TSH signaling.

Cells, which were seeded and cultured for 24 h, were incubated with 100 mU/ml bTSH for 1-30 min ("pretreatment"). Thereafter the cells were washed and incubated in HBSS/HEPES at 37C for 60-1320 min ("washout"). Thereafter the mediums were aspirated and the cells were incubated in HBSS/HEPES containing IBMX (cAMP) or LiCl (IP1) for 30-60 min and total cAMP or IP1levels were measured ("production").

Figure 3. Time course of acquisition of persistent cAMP and IP1 production caused by TSH.

HEKTSHR cells were incubated with 100 mU/ml TSH for the pretreatment times indicated at 37C. After 0 (control), 1, 2.5, 5, 10 or 30 min of exposure to TSH ("pretreatment"), the cells were washed and then incubated in HBSS/HEPES for 60 min ("washout"). Thereafter, the cells were incubated in HBSS/HEPES with IBMX (cAMP) or LiCl (IP1). After an additional 30 min ("production"), the incubations were stopped and total cAMP and IP1 levels were measured. cAMP and IP1 production increased persistently after 1 min of exposure (p<0.01) and the levels of IP1 production increased progressively up to 10 min of exposure (p<0.01). The bars are the mean±range of duplicate measurements in one of three experiments.

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Figure 4. Time course of decay of persistent cAMP and IP1 production caused by TSH.

HEKTSHR cells were incubated without or with 100 mU/ml TSH at 37C. After 30 min ("pretreatment"), the cells were washed and then incubated in HBSS/HEPES for 60 min ("washout"). At the times indicated after washout, the buffers were aspirated and the cells were incubated in HBSS/HEPES with IBMX (cAMP) or LiCl (IP1) for 30 min ("production"). The incubations were stopped and total cAMP or IP1 levels were measured. The individual levels of production in the three experiments were: for cAMP 130, 105, and 10 pmol/well and for IP1 were 460, 102 and 345 nmol/well. The decreases in IP1 production were progressive beginning after 120 min ($p<0.01$). The data are from three experiments with duplicate or triplicate samples and are presented as mean±SE.

Figure 5. Comparison of persistent signaling caused by TSH, C2 and M22.

HEKTSHR cells were incubated without or with 100 mU/ml TSH, 15 or 30 μM C2 or 100 or 200 ng/ml M22 at 37C. After 30 min ("pretreatment"), the cells were washed and then incubated in HBSS/HEPES for 60 min ("washout"). At the times indicated after washout, the buffers were aspirated and the cells were incubated in HBSS/HEPES with IBMX (cAMP) or LiCl (IP1) for 60 min ("production"). The incubations were stopped and total cAMP or IP1 levels were measured. TSH, C2 and M22 caused persistent cAMP and IP1 production ($p<0.001$). The data are from one of three experiments with duplicate samples and are presented as mean±SD.

Figure 6. Inhibition of persistent signaling by **1** and **2**.

HEKTSHR cells were incubated without or with 100 mU/ml TSH at 37C. After 30 min ("pretreatment"), the cells were washed and then incubated in HBSS/HEPES for 45 min (first "washout" period). After 45 min, the cells were incubated in HBSS/HEPES with no addition or with 30 μM **1** or 30 μM **2** for 15 min (second "washout" phase). After both washout phases, the

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buffers were aspirated and the cells were incubated in HBSS/HEPES with IBMX (cAMP) or LiCl (IP1) without (No addition) or with **1** or **2** for 60 min ("production" phase). The incubations were stopped and total cAMP or IP1 levels were measured. **1** but not **2** inhibited persistent cAMP production ($p<0.001$). **1** and **2** inhibited persistent IP1 production ($p<0.001$). The data are from one of three experiments with duplicate or triplicate samples and are presented as mean±SD.

Figure 7. Dominant negative dynamin mutant protein (K44A) or βArrestin2 (βArr2) had no effect on persistent IP1 production.

HEKTSHR cells were transfected with empty plasmid (Mock), plasmid to express βArr2 or plasmid to express K44A.

A. Acid resistant ^{125}I-TSH binding. After 48 h, specific ^{125}I-TSH binding (HBSS wash) and ^{125}I-TSH binding after acid wash were measured and acid resistant binding calculated as described in Materials and Methods. βArr2 increased acid resistant binding ($p<0.001$) whereas K44A had no effect ($p>0.1$). The bars are the mean±SE of duplicate measurements in three experiments.

B. IP1 production. After 48 h, the cells were incubated without (Control) or with 100 mU/ml TSH at 37C. After 30 min ("pretreatment"), the cells were washed and incubated in HBSS/HEPES at 37C ("washout"). At 60 min after the washes, the cells were incubated in HBSS/HEPES with LiCl alone or LiCl and 100 mU/ml TSH at 37C for 30 min ("production"). The cells were lysed and IP1 content was measured in the cell lysates. Control-Control: no pretreatment-no treatment during production phase; Control- TSH; no pretreatment-TSH added during production phase; TSH-Control: TSH pretreatment-no treatment during production phase (persistent signaling); TSH-TSH: TSH pretreatment-TSH treatment during production phase. There was no increase in IP1 production in cells expressing βArr2 ($p>0.1$) nor a decrease in IP1

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production in cells expressing K44A (p>0.1). The bars are the mean±SE of duplicate measurements in three experiments.

Figure 8. $G\alpha_q$-YFP does not internalize with TSHRs. HEKTSHR cells were transfected to transiently express βArr2 and $G\alpha_q$-YFP. After 48 h, the cells were incubated with Alexa-TSH for 30 min at 37C. Images were acquired as described in Materials and Methods. The micrographs shown are representative of slices at the midpoint of the cell thickness. Left: Control. Right: βArr2. Alexa-TSH: red. $G\alpha_q$-YFP: green. Scale bar – 10 μm.

Below are the values from the quantitative colocalization analysis. Both coefficients were different in cells expressing βArr2 (p<0.001). n = number of cells.

Figure 1

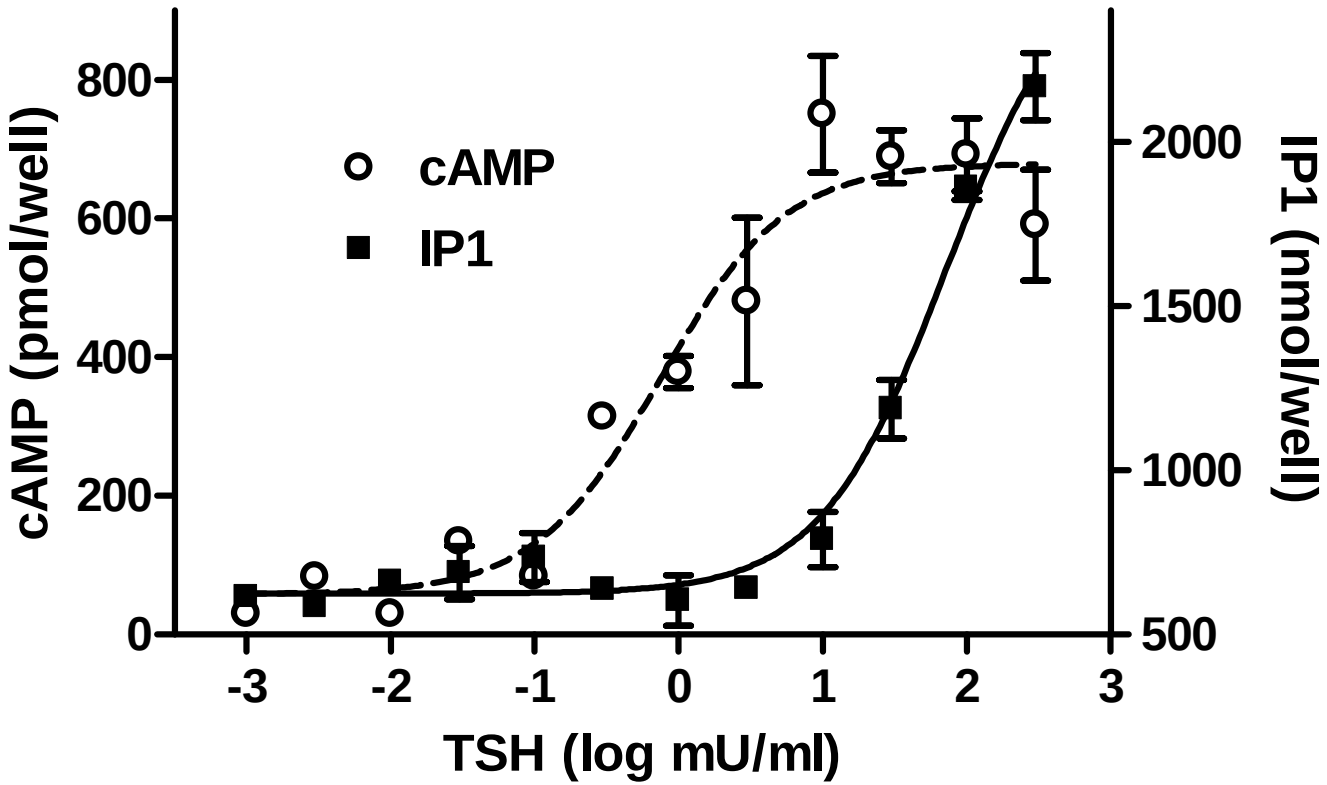


Figure 2

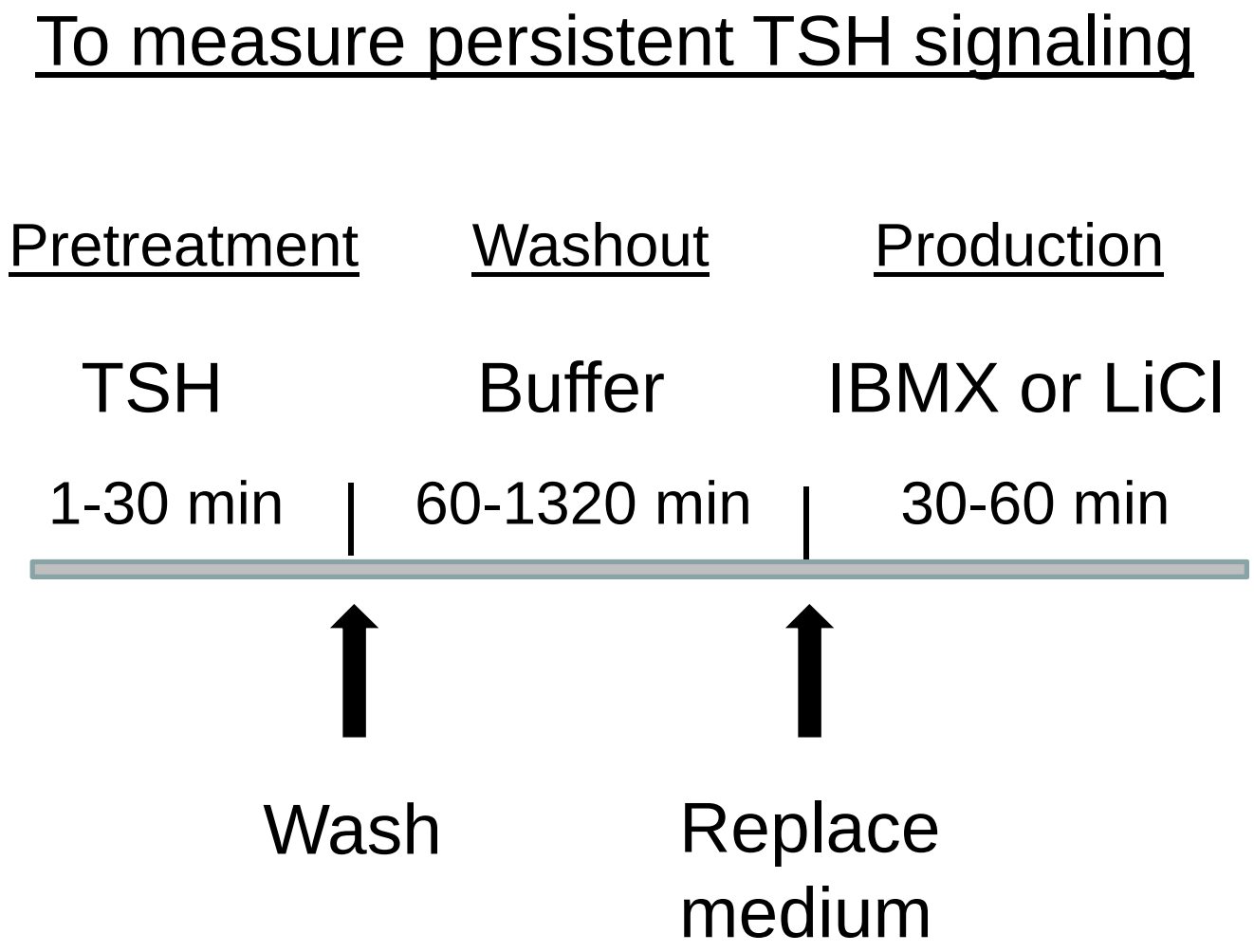


Figure 3

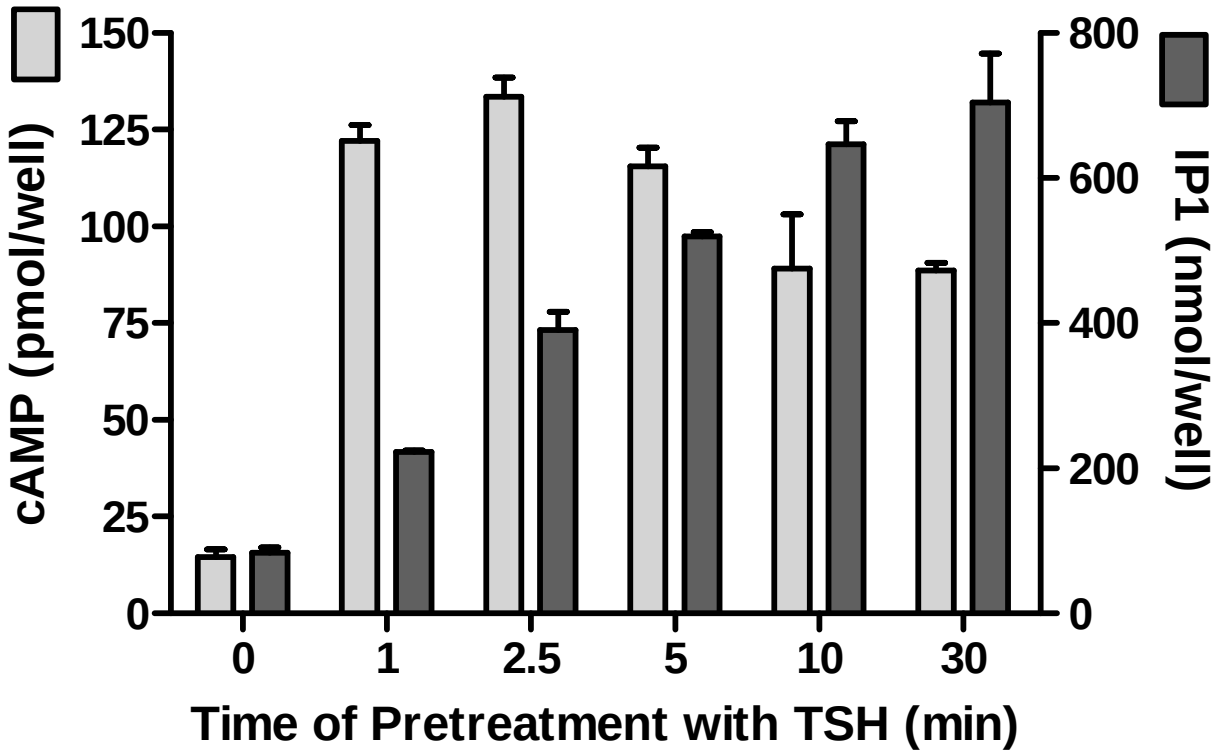


Figure 4

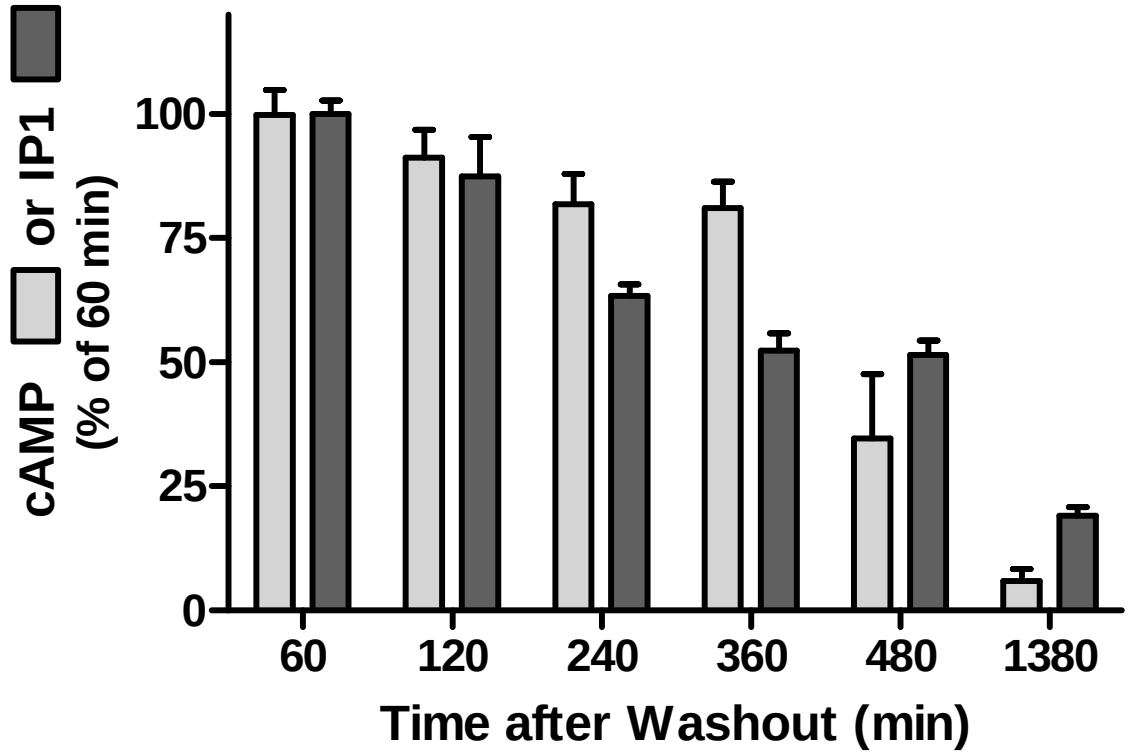


Figure 5

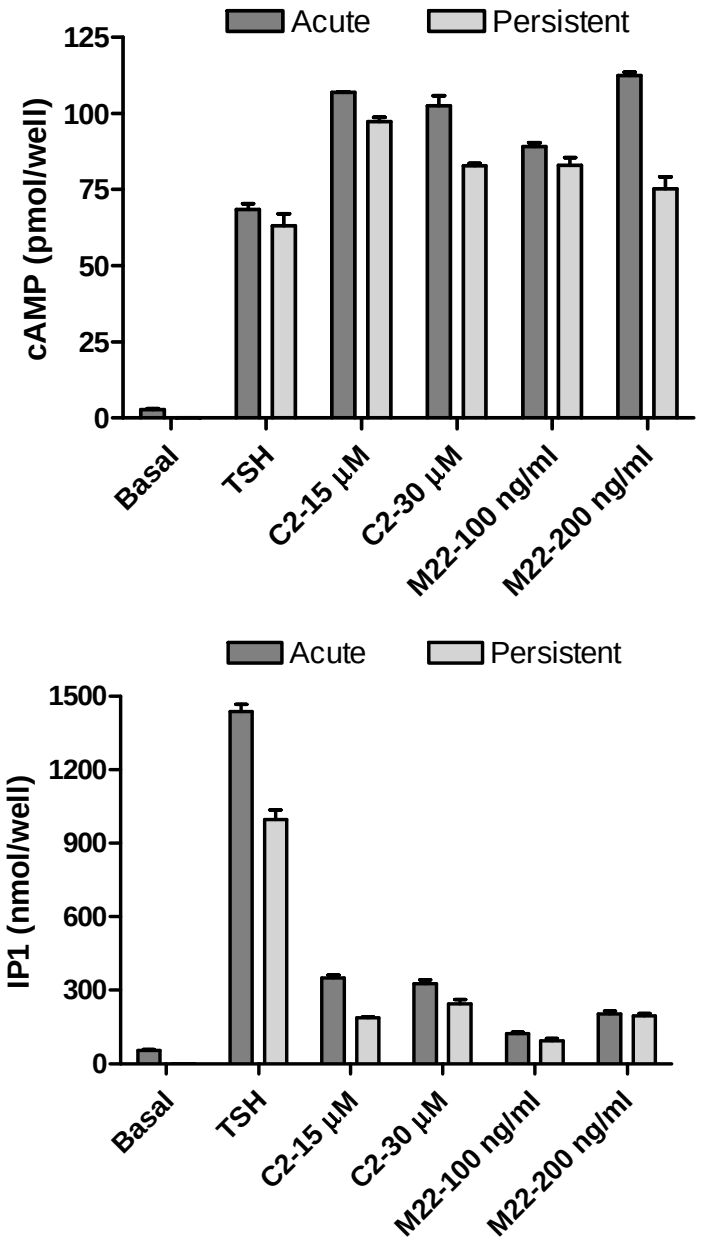
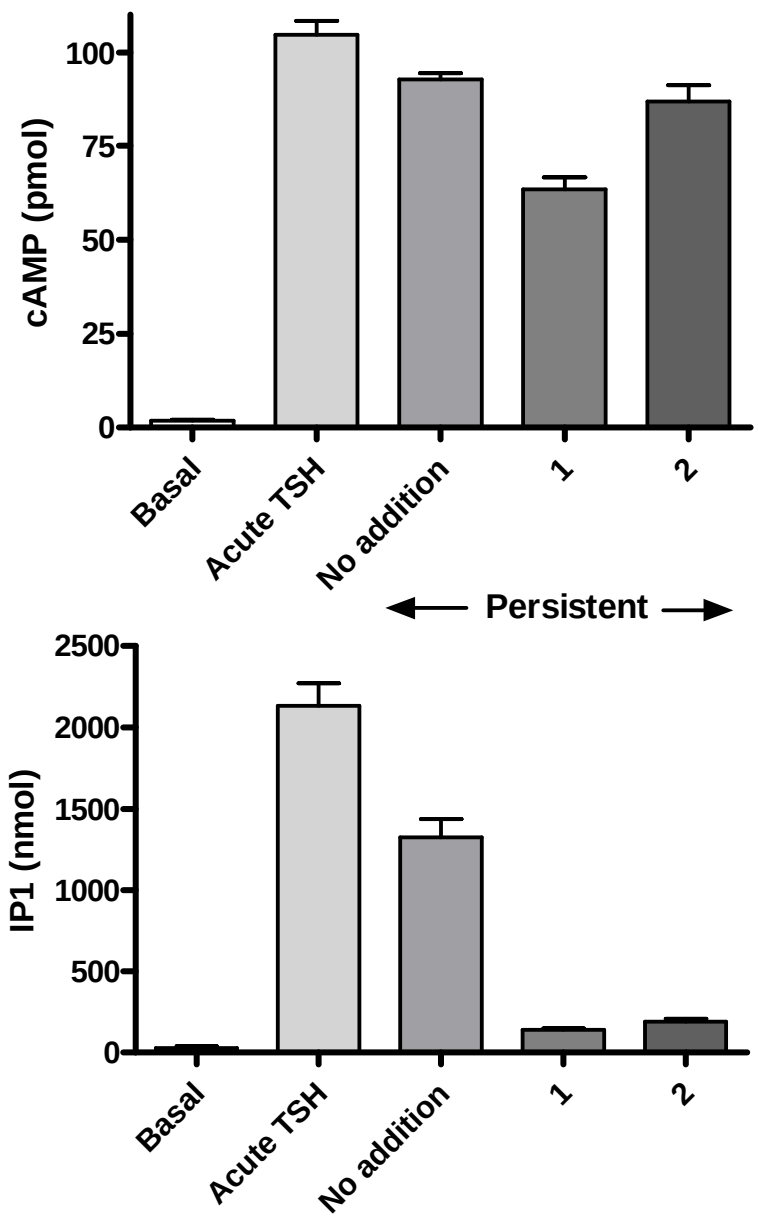


Figure 6



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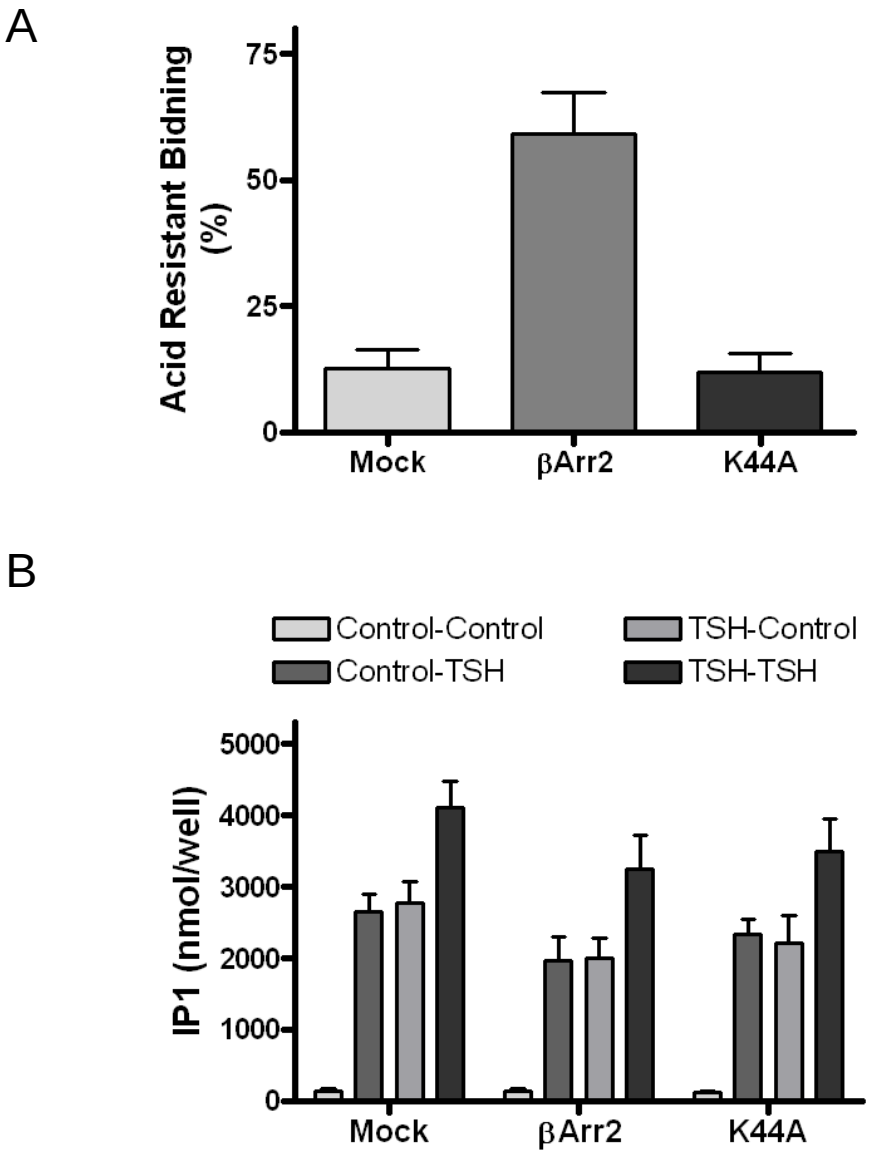
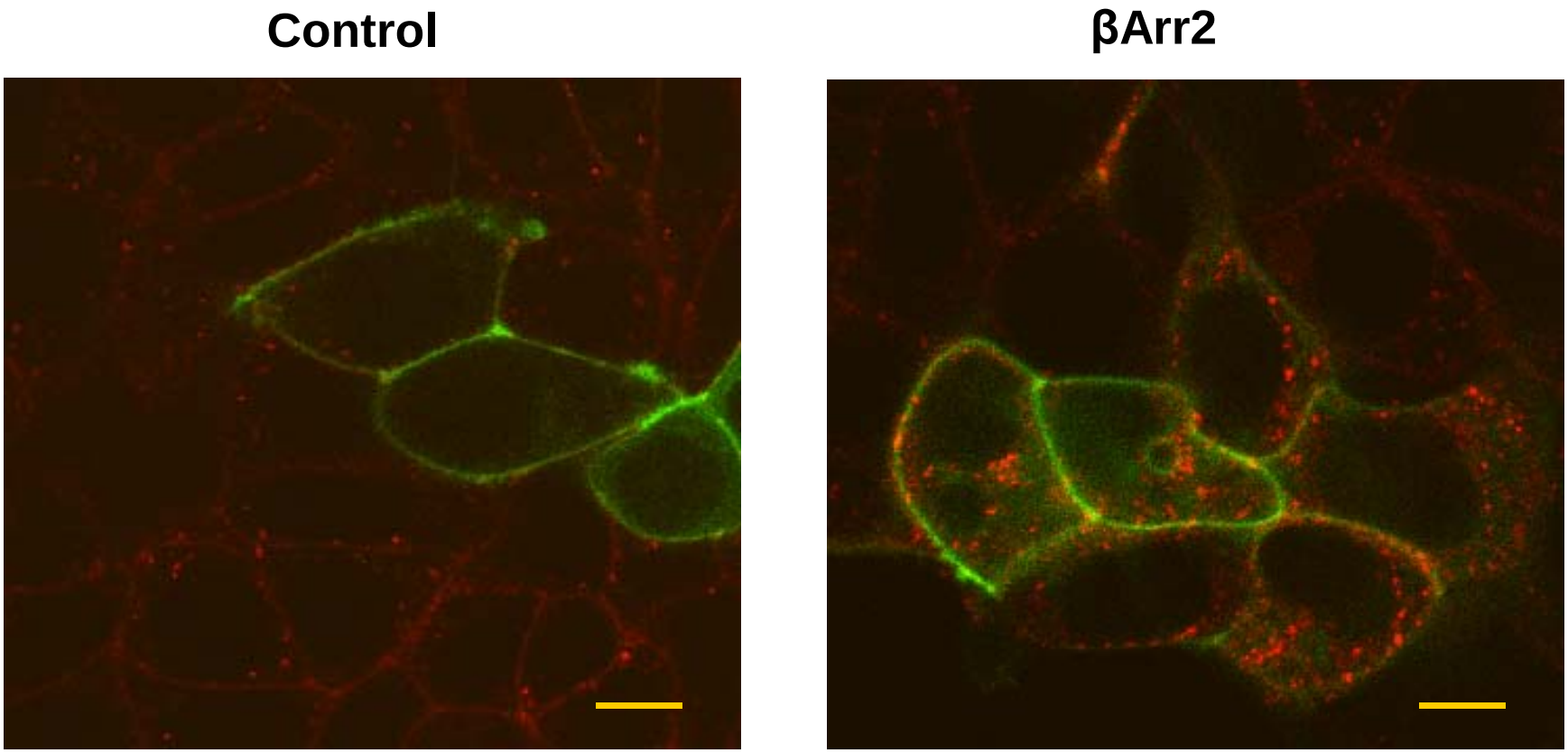


Figure 8



	Pearson's correlation coefficient	Manders' overlap coefficient
Control (n=13)	0.42±0.16	0.74±0.05
βArr2 (n=33)	0.00±0.06	0.48±0.07