Receptor Species-dependent Desensitization Controls KCNQ1/KCNE1 K⁺ Channels as Downstream Effectors of Gq Protein-coupled Receptors*5

Marie-Cécile Kienitz1, Dilyana Vladimirova, Christian Müller, Lutz Pott, and Andreas Rinne
From the Institute of Physiology, Ruhr-University Bochum, Universitätstrasse 150, D-44780 Bochum, Germany

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Activation of Gq protein-coupled receptors (GqPCRs) might induce divergent cellular responses, related to receptor-specific activation of different branches of the Gq signaling pathway. Receptor-specific desensitization provides a mechanism of effector modulation by restricting the spatiotemporal activation of signaling components downstream of Gq. We quantified signaling events downstream of GqPCR activation with FRET-based biosensors in CHO and HEK 293 cells. KCNQ1/KCNE1 channels (Iks) were measured as a functional readout of receptor-specific activation. Activation of muscarinic M₁ receptors (M₁-Rs) caused robust and reversible inhibition of Iks. In contrast, activation of α₁B-adrnergic receptors (α₁B-ARs) induced transient inhibition of Iks which turned into delayed facilitation after agonist withdrawal. As a novel finding, we demonstrate that GqPCR-specific kinetics of Iks modulation are determined by receptor-specific desensitization, evident at the level of Gq activation, phosphatidylinositol 4,5-bisphosphate (PIP₂) depletion, and diacylglycerol production. Sustained Iks inhibition during M₁-R stimulation is attributed to robust membrane PIP₂ depletion, whereas the rapid desensitization of α₁B-AR delimits PIP₂ reduction and augments current activation by protein kinase C (PKC). Overexpression of Ca²⁺-independent PKCd did not affect the time course of α₁B-AR-induced diacylglycerol formation, excluding a contribution of PKCd to α₁B-AR desensitization. Pharmacological inhibition of Ca²⁺-dependent PKCd isoforms abolished fast α₁B receptor desensitization and augmented Iks reduction, but did not affect Iks facilitation. These data indicate a contribution of Ca²⁺-dependent PKCs to α₁B-AR desensitization, whereas Iks facilitation is induced by Ca²⁺-independent PKCd isoforms. In contrast, neither inhibition of Ca²⁺-dependent/Ca²⁺-independent isoforms nor overexpression of PKCd induced M₁ receptor desensitization, excluding a contribution of PKCd to M₁-R-induced Iks modulation.

The canonical signaling pathway of activated Goq subunits comprises stimulation of PLCβ2 isoforms, hydrolysis of PIP₂ to release inositol trisphosphate (IP₃) and diacylglycerol (DAG), and subsequent Ca²⁺ release from internal stores and DAG-mediated activation of protein kinase C (PKC) (1). More recent data (reviewed in Ref. 2) indicate that the linear pathway from GqPCR activation to Gq-induced PLC stimulation is inadequate to explain the broad range of (sometimes divergent) cellular responses. Functional diversity upon stimulation of Gq-coupled receptors might reflect differential coupling to Goq family members, different Gq efficacies for PLCβ activation, or activation of pathways independently of phosphoinositide hydrolysis. Furthermore, receptor-specific targeting of effector molecules has been shown to depend on the spatial proximity of GqPCR and effector protein (e.g. an ion channel) and on the mobility of signal molecules in the plasma membrane (3).

It is conceivable that GqPCRs can be distinguished by their efficiency to activate different branches of the Goq signaling pathway, either IP₃-Ca²⁺ or DAG-PKC, as recently shown for KCNQ2/3 channel modulation by P2Y₂-R and M₁-R (4, 5). Stimulation of distinct Gq-coupled receptors induces compartment-specific targeting of Goq effector proteins. As reviewed in Refs. 6–9, compartment-dependent translocation of PKC isoforms to the plasma membrane, the nuclear membrane, or the Golgi complex determines specific intracellular responses by placing PKC isoforms in proximity to their interaction partners. By using genetically encoded FRET-based sensors of organelle-specific PKC activity, recent studies provide evidence that location-specific DAG production enables recruitment of PKC isoforms to different intracellular membranes (10, 11). Spatially restricted activation of components downstream of Gq has been shown to modulate several types of ion channels in a receptor species-dependent fashion, e.g. N-type Ca²⁺ channels (12), G protein-activated inward-rectifying K⁺ (GIRK) channels (3), and KCNQ1/KCNE1 channels (13).

Apart from spatial organization of signaling components, temporal aspects of receptor activation or G protein-effector interactions determine receptor-specific kinetics of GqPCR signaling. Diversity of GPCR-induced cellular effects might result in different PKC isoforms. The different PKC isoforms respond to different activation events, and their spatiotemporal activation determines the downstream effector response.

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1 To whom correspondence should be addressed. Tel.: 49-234-3229200; Fax: 49-234-3214449; E-mail: cecile.kienitz@rub.de.
2 The abbreviations used are: PLC, phospholipase C; α₁₅-AR, α₁₅-adrenergic receptor; α₁₉-AR, α₁₉-adrenergic receptor; M₁-R, muscarinic M₁ receptor; cPKC, conventional protein kinase C; nPKC, novel protein kinase C; DAG, diacylglycerol; GPCR, G protein-coupled receptor; GqPCR, G protein-coupled receptor; PIP₂, phosphatidylinositol-4,5-bisphosphate; IP₃, inositol trisphosphate; GRK, G protein-coupled receptor kinase; DAGR, DAG reporter; aa, amino acid(s); ACh, acetylcholine; Phe, phenylephrine; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N′,N′,N′-tetraacetic acid; stauro, staurosporine; DN, dominant-negative.
from activation of multiple G proteins with varying efficacy and kinetics, inducing either fast cellular responses of limited extent through one type of G protein or a slow, but extended response through another (14). Moreover, as reviewed in Ref. 15, the affinity of activated G protein subunits to their specific effectors and their interaction kinetics determine the equilibrium of active and inactive trimeric G proteins and the dynamics of the G protein cycle.

Receptor-specific desensitization might provide a mechanism of effector modulation by restricting the spatiotemporal activation of downstream Gq signaling components. Receptor desensitization in the continuous presence of an agonist terminates the responsiveness of a cell by limiting second messenger formation. A recent study, investigating the role of PKC activation in regulating TRPC6 channel activity, provides evidence that desensitization of the histamine H1 receptor reduces TRPC6 channel activity by rapid termination of DAG production (16). However, whether receptor species-dependent differences in desensitization determine the time course of channel modulation during stimulation of distinct GqPCRs has not been elucidated. Rapid termination of second messenger production by receptor desensitization delimits activation of downstream effector proteins. Thus, it is conceivable that receptor desensitization reduces the efficiency to activate certain effector proteins but favors activation of other branches of Gq signaling pathways with either faster activation kinetics or higher second messenger affinity.

In the present study, we quantitatively analyzed signaling events downstream of distinct GqPCRs by means of FRET-based biosensors in a stable KCNQ1/KCNE1-transfected CHO cell line and in HEK 293 cells. I_{KS} amplitude during GqPCR stimulation was measured as a functional readout of receptor species-dependent activation of Gq effectors. Our study demonstrates for the first time significant receptor-dependent differences in the time course of Gq protein activation, DAG production (as a prerequisite of PKC activation), and PIP_{2} hydrolysis upon stimulation of either adrenergic α_{1B} or muscarinic M_{1} receptors. These fundamental differences are attributed to different time courses of receptor desensitization and, as a consequence, induce different time courses of I_{KS} modulation. Our data provide evidence that receptor-specific desensitization controls PIP_{2} reduction and recruitment of different PKC isoforms, thus resulting in different modes of fine-tuning of I_{KS} activity.

**Results**

**Receptor Species-dependent Desensitization at the Level of Gq Activation**—Activation of GqPCRs can be measured and quantified by using a FRET-based biosensor that monitors the Gq protein cycle (17). The expression of the Gq protein biosensor and either the α_{1B}-AR or M_{1}-R in HEK 293 cells allowed us to compare receptor species-dependent temporal properties of Gq protein activation.

As illustrated by the representative recordings in Fig. 1, A and D, increasing concentrations of phenylephrine (Phe) or acetylcholine (ACh) (ranging from 0.1 nM to 1 μM) result in an incremental increase of Gq activation as monitored by the stepwise decrease of the FRET ratio. The decrease in the FRET ratio (ΔF) was normalized to ΔF_{max} (the FRET signal obtained during a single application of 1 μM Phe or ACh), yielding concentration-response curves for phenylephrine (α_{1B}-AR)- and acetylcholine (M_{1}-R)-induced Gq activation (Fig. 1, B and E). Based on the concentration-response curves (revealing EC_{50} values of 37 nM (Phe, α_{1B}-AR) and 14 nM (ACh, M_{1}-R)), we applied 1 μM Phe as a saturating agonist concentration for α_{1B}-AR activation and 10 μM ACh for maximal M_{1}-R-induced Gq activation. During sustained α_{1B}-adrenergic receptor activation by phenylephrine, a gradual increase in the FRET ratio, i.e. a decline in Gq activation, was observed (Fig. 1C). The amount of desensitization was quantified by the ratio FRET_{30s after peak}/FRET_{peak}, indicating a reduction of the FRET ratio of 33 ± 4.5% (n = 8, Phe 1 μM) for the α_{1B}-AR (see also supplemental Fig. S1). In contrast, during stimulation of M_{1} receptors (n = 6, ACh 10 μM), the FRET ratio remained stable (Fig. 1F).

It is conceivable that the receptor-specific differences in Gq protein activation reflect either different rates of desensitization of α_{1B} and M_{1} receptors or less efficient coupling of α_{1B} receptors to Gqα subunits as compared with M_{1} receptors. We therefore investigated whether receptor species-dependent differences occurred downstream of Gq activation, using FRET sensors for DAG formation and PIP_{2} depletion.

**Analysis of Receptor Species-dependent Desensitization by Monitoring the Time Course of DAG Formation**—To analyze receptor species-dependent aspects of signaling downstream of the Gq protein, we analyzed the time course of DAG formation in HEK cells expressing either α_{1B}-AR or M_{1}-R and the fluorescent biosensor DAGR. DAGR reports formation of DAG by an increase in FRET ratio, and the time course of DAG formation can be used to analyze the rate of receptor desensitization during agonist exposure (18). The representative recordings in Fig. 2 show effects of GqPCR stimulation on DAG production during application of Phe (1 μM) and ACh (10 μM). Stimulation of both receptor species caused a comparable, rapid increase in FRET ratio, reflecting activation of PLC and formation of DAG at the plasma membrane. However, DAG production induced by α_{1B} receptors rapidly decayed during stimulation (Fig. 2A), an effect that was not observed for the M_{1}-R (Fig. 2B). The summarized data, expressed as the ratio FRET_{30s after peak}/FRET_{peak} in Fig. 2C, indicate a more than 50% reduction of the DAG signal during activation of α_{1B} receptors. In contrast, during stimulation of M_{1} receptors, we observed only a 10% reduction in DAG.

Receptor species-dependent differences in the time course of DAG reduction persisted across agonist concentrations. In the presence of nonsaturating agonist concentrations (e.g. 50 and 200 nM Phe or ACh), we observed a rapid decline of the DAG signal in α_{1B}-AR-, but not in M_{1}-R-expressing cells (see supplemental Fig. S2). The different time courses of DAG production in α_{1B}- and M_{1}-R-expressing HEK cells are likely to reflect receptor species-dependent differences in desensitization. Alternatively, differences in receptor expression levels may affect the time course of desensitization.

If this would be the case, any changes of GqPCR expression levels, controlled by adjusting the amount of transfected cDNA, might either increase or decrease the rate of desensitization of M_{1} or α_{1B} receptors. We therefore transfected HEK 293 cells...
with different amounts of receptor cDNA and subsequently monitored the time course of DAG formation during receptor stimulation. As shown in Fig. 2, neither increasing the expression level of M1 receptors nor decreasing the expression of \( \alpha_{1B} \) receptors significantly affected the receptor-specific desensitization properties.

Simultaneous Measurements of DAG Production and IKs Modulation in Stably Transfected KCNQ1/KCNE1 CHO Cells during Stimulation of \( \alpha_{1B}-\)AR and M1-R—Temporal aspects of GqPCR signaling were further analyzed in CHO cells stably transfected with KCNQ1/KCNE1 and transiently transfected with either \( \alpha_{1B} \) or M1 receptors and the DAG sensor DAGR. We simultaneously measured DAG production and modulation of IKs amplitude as a functional readout of GqPCR activation. Previous studies have reported conflicting results on IKs modulation by different Gq-coupled receptor species. Both inhibition and activation of KCNQ channels have been reported (13, 19, 20). Some of these receptor species-dependent differences in IKs modulation might be attributed to activation of different branches of downstream Gq signaling, e.g., enhanced PIP2 depletion or activation of different PKC isoforms.

The representative recordings in Fig. 3 show the relation between GqPCR desensitization (assessed with the DAGR FRET signal) and IKs current amplitudes in simultaneous recordings. Application of Phe (1 \( \mu \)M) in \( \alpha_{1B} \)-AR-expressing

\[ \text{FIGURE 1. Activation of } \alpha_{1B}\text{-AR caused acute desensitization of G protein signaling. A, C, D, and F, representative normalized FRET recordings from } \alpha_{1B}\text{-AR-} \]
\[ \text{(A and C) and M1-R-expressing (D and F) HEK 293 cells cotransfected with a FRET biosensor measuring Gq activation. Upon activation of Gq by } \alpha_{1B} \text{ or M1 receptor stimulation, a decrease in the FRET ratio reflects dissociation of Gq-YFP and Gq-Cerulean. B and E, concentration-response curves for } \alpha_{1B} \text{ (n = 6) and M1-R-induced Gq stimulation (n = 7). EC50 values of } \alpha_{1B} \text{- and M1-induced Gq activation were 37 and 14 nM. Upon application of saturating agonist concentrations (Phe, 1 \( \mu \)M; ACh, 10 \( \mu \)M), fast desensitization was prominent on the level of Gq activation in } \alpha_{1B}\text{-AR-} \]
\[ \text{(C) but not in M1-R-expressing cells (F). Periods of time plotted in blue correspond to times of exposure to agonists. Error bars indicate mean } \pm \text{ S.E. of n cells; } \text{P-values less than 0.05 were considered statistically significant.} \]
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FIGURE 2. Receptor-specific desensitization at the level of PLC activation and DAG formation is independent of the receptor expression level. A and B, representative FRET recordings from $\alpha_{1B}$-AR- or M$_1$-R-expressing HEK 293 cells cotransfected with the FRET biosensor DAGR to monitor changes in membrane DAG. Saturating concentrations of agonists were applied as indicated. Periods of time plotted in blue correspond to times of exposure to agonists. Note the rapid decay of the FRET ratio in the presence of agonist in $\alpha_{1B}$-AR- but not in M$_1$-R-expressing cells. C, summarized data of DAG reduction during agonist application (determined by the ratio $F_{\text{RET}}$ at 30 s after peak/$F_{\text{RET}}$ peak), indicating a more than 50% reduction of the DAG signal during activation of $\alpha_{1B}$ receptors ($n = 10$), but only a minor reduction (less than 10%) during stimulation of M$_1$ receptors ($n = 12$). Significant differences are indicated by asterisks. D and E, representative FRET recordings of HEK 293 cells transfected with 0.25 $\mu$g $\alpha_{1B}$-AR cDNA ($n = 7$) and DAGR during application of phenylephrine (1 $\mu$M). As shown by the summarized data in F comparing DAG reduction (measured 30 s after $F_{\text{RET}}$ peak), decreasing or increasing the expression level of $\alpha_{1B}$ receptors does not affect the rapid desensitization properties (0.1 $\mu$g ($n = 4$) or 1 $\mu$g of $\alpha_{1B}$-AR cDNA ($n = 7$)). G and H, representative FRET recordings of HEK 293 cells transfected with 0.5 $\mu$g (G, $n = 7$) or 1 $\mu$g of M$_1$-R cDNA (H, $n = 7$) and DAGR. Kinetics of DAG formation during application of acetylcholine (10 $\mu$M) were not affected by decreasing or increasing the expression level of M$_1$ receptors (see also summarized data in I, 0.1 $\mu$g, $n = 6$; 0.25 $\mu$g, $n = 5$). *, $p < 0.01$. n.s., not significant. Error bars indicate mean ± S.E. of $n$ cells.

cells resulted in $I_{Ks}$ inhibition caused by depletion of membrane PIP$_2$ (13), followed by a sustained increase in current amplitude after agonist withdrawal (Fig. 3, A and C). This delayed increase in current was lacking after M$_1$-R stimulation. On the other hand, the initial inhibition of $I_{Ks}$ during agonist application appeared to be more pronounced upon stimulation of M$_1$ receptors as compared with $\alpha_{1B}$-adrenergic receptors (Fig. 3, A and C). The corresponding DAG dynamics in $\alpha_{1B}$-AR-expressing cells showed a rapid decline during agonist application and had almost returned to basal levels when the $\alpha_{1B}$-AR-induced $I_{Ks}$ increase occurred (Fig. 3, B and D). In contrast, stimulation of M$_1$ receptors induced a more pronounced $I_{Ks}$ inhibition (about 30% of the initial current amplitude in the absence of agonist) as compared with stimulation of $\alpha_{1B}$ receptors (about 15%, Fig. 3C). DAG production persisted in the presence of acetylcholine (Fig. 3, B and D), but failed to stimulate $I_{Ks}$ after
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agonist withdrawal. (Fig. 3, A and C). These data suggest that receptor species-dependent differences in $G_q$ effector signaling account for divergent $I_{Ks}$ modulation, supporting the idea that desensitization of different receptor species determines temporal aspects of downstream $G_q$ signaling pathways.

Qualitatively, the temporal aspects of DAG formation during $G_q$PCR activation are identical in CHO and HEK cells (compare representative traces in Figs. 2 and 3B; see also supplemental Fig. S3), indicating that different time courses of DAG production are receptor species-dependent, but independent

FIGURE 3. Receptor-specific desensitization determines the time course of $I_{Ks}$ modulation. A and B, simultaneous recordings of $I_{Ks}$ (A) and DAG production (indicated by an increase in the FRET ratio (B)) in stable KCNQ1/KCNE1 CHO cells expressing either $\alpha_{1B}$-AR or M1-R and the FRET biosensor DAGR. $I_{Ks}$ was evoked by depolarizing pulses from $-80$ mV to $+60$ mV (duration 5 s, 0.05 s$^{-1}$). An individual $I_{Ks}$ recording (marked by an asterisk in A) is shown as an inset. Application of Phe (1 mM) in $\alpha_{1B}$-AR-expressing cells resulted in $I_{Ks}$ inhibition and a subsequent increase after agonist withdrawal. Stimulation of M1 receptors with acetylcholine (10 mM) induced a more pronounced $I_{Ks}$ inhibition, but failed to increase $I_{Ks}$ amplitude. C, time course of normalized $I_{Ks}$ ($I/I_0$) during agonist exposure ($n = 9$ for $\alpha_{1B}$-AR- and $n = 8$ or M1-R-expressing cells). The last $I_{Ks}$ current before agonist application was set as $I_0$. Dotted lines indicate the extent of current inhibition. D, summarized data of DAG production (normalized FRET ratio), measured simultaneously to $I_{Ks}$. Error bars indicate mean ± S.E. of $n$ cells.
of the cellular background. Moreover, if the receptor-specific time course of the DAG signal reflects intrinsic receptor properties, receptor species-dependent desensitization should be evident at other branches of the G protein signaling pathway, e.g. the level of PLC activation and depletion of membrane PIP2.

**Stimulation of α1b Receptors in CHO and HEK Cells Induced a Membrane PIP2 Depletion That Rapidly Recovered in the Presence of Phenylnephrine**—We investigated the depletion of membrane PIP2 following α1b or M1 receptor stimulation in CHO and HEK cells by utilizing a biosensor that directly reports the depletion of membrane PIP2 during GqPCR/PLC activation with a decrease in FRET ratio (21, 22). As illustrated in a representative FRET recording in Fig. 4A, stimulation of α1b receptors in CHO cells caused a rapid decrease in FRET ratio, reflecting membrane PIP2 depletion. This α1b-AR-mediated depletion showed a biphasic time course: during application of phenylephrine (1 μM), FRET slowly decreased for about 20 s, followed by a rapid decay. Remarkably, the FRET ratio rapidly returned to baseline levels, indicating PIP2 replenishment during sustained receptor stimulation. In contrast, exposure of M1-R-transfected CHO cells to acetylcholine (10 μM) resulted in a fully reversible decrease in the FRET ratio (Fig. 4B) with a rapid onset of PIP2 depletion that was persistent in the presence of the agonist. Comparing membrane PIP2 depletion in HEK 293 cells during α1b and M1 receptor stimulation yielded analogous results, i.e. transient reduction and replenishment of PIP2 in the presence of phenylephrine and persistent PIP2 depletion during application of acetylcholine (Fig. 4, D and E). The summarized FRET30 s after peak/FRET peak ratios in Fig. 4, C and F, indicate a reduction of 56% of the PIP2 signal in CHO cells and a reduction of 32% in HEK 293 cells during activation of α1b receptors and a minor reduction (less than 5%) during stimulation of M1 receptors in both cell types.

To address whether internalization of the α1b receptor causes its fast desensitization, we analyzed the time courses of arrestin binding as well as of subsequent receptor internalization. Binding of arrestins represents the initial step to prime a GPCR for endocytosis. Of note, the recruitment of arrestins to the receptor prevents G protein binding (23). Thus, recruitment of arrestins to the receptor prevents G protein binding (23). Thus, recruitment of arrestins could contribute to desensitization of Gq signaling. To estimate the kinetics of arrestin binding for stimulated α1b-ARs and M1-ARs, we used a FRET assay that monitors the recruitment of Turquoise-labeled β-arrestin to receptors that were labeled with YFP at their C terminus (Fig. 5A) (24). The biosensor revealed that recruitment of arrestin occurred fast and with similar kinetics for both receptor subtypes (Fig. 5, B and C). Therefore, arrestin binding cannot explain the differences in desensitization kinetics of both receptors. Furthermore, the internalization of α1b-AR during long-term exposure to Phe was even slower; in the absence of agonist, we observed a proper membrane localization of α1b-AR-YFP-IL3 (Fig. 5D, panel a). During exposure to 10 μM Phe, internalization of the receptor was evident by a reduction in plasma membrane fluorescence and formation of intracellular punctae, starting between 15 and 30 min of incubation time (Fig. 5D, panels d–g).
This is in line with the time course for internalization of this receptor in a previous study (25) and is significantly slower (up to 15 min) than the time course of acute desensitization observed in our experiments (e.g. see Fig. 2 with complete desensitization of \( \alpha_{1B} \)-AR within 60 s of agonist application). These results confirm that neither recruitment of arrestins nor an internalization of the \( \alpha_{1B} \)-AR caused its acute desensitization.

We further investigated recovery from \( \alpha_{1B} \)-AR desensitization by consecutive applications of phenylephrine (1 \( \mu \)M) with variable time intervals. The second Phe-induced DAG signal was normalized to the first DAG signal and plotted against recovery time (defined as the time interval between termination of the first DAG signal and increase of the second signal). Data were fitted with a monoexponential function.
zation and recovery from desensitization are most likely related to direct modifications of the receptor protein, such as phosphorylation/dephosphorylation.

**Contribution of PKC Isoforms to Acute α_{1B} Receptor Desensitization**—Receptor phosphorylation has been shown to be the earliest biochemical event in homologous and heterologous α_{1B} receptor desensitization either by G protein-coupled receptor kinases (GRK) or by PKC (26). Both conventional PKC isoforms (cPKCs, Ca^{2+}- and DAG-dependent) and novel PKC isoforms (nPKCs, DAG-dependent, but Ca^{2+}-independent) are downstream effectors of Gq-coupled receptor signaling pathways (27), but their contribution to homologous desensitization of α_{1B}-R is still undefined. We aimed to investigate the contribution of different PKC isoforms to homologous GqPCR desensitization either by isoform-specific pharmacological PKC inhibition or by manipulating PKC expression levels.

In a first series of experiments, the contribution of PKC to homologous α_{1B} receptor desensitization was analyzed in HEK cells cotransfected with α_{1B}-AR and DAGR by application of the protein kinase inhibitor staurosporine. In the continuous presence of staurosporine (100 nM, 2-h incubation time, with staurosporine in the recording solution), the acute phase of α_{1B} receptor desensitization was completely abrogated (see the representative FRET recordings in Fig. 6, A and B). As indicated by the summarized data in Fig. 6G, comparing the ratio FRET_{30 s after peak}/FRET_{peak} in the presence or absence of staurosporine, reduction of the DAG signal during agonist application was markedly diminished by staurosporine.

Staurosporine is a nonspecific protein kinase inhibitor that does not discriminate between PKC isoforms. To evaluate the contribution of different PKC isoforms to homologous desensitization of α_{1B} receptors, we either inhibited cPKCs (Fig. 6, E and F) or coexpressed the nPKC isoform PKCδ as WT PKCδ (Fig. 6C) or as the inactive mutant PKCδ DN (Fig. 6D). Activation of α_{1B} receptors in the presence of either WT PKCδ or PKCδ DN resulted in DAG formation that rapidly declined to a similar rate as compared with control conditions (see also Fig. 6G), suggesting that PKCδ does not contribute to the acute phase of α_{1B} Receptor desensitization.

To reduce activation of cPKCs, we dialyzed α_{1B}-AR/DAGR-cotransfected HEK cells with the Ca^{2+} chelator BAPTA (5 mM) via the patch pipette and measured the time course of DAG decay in the presence of 1 μM Phe. As depicted in Fig. 6, E and G, buffering the increase in [Ca^{2+}], by BAPTA abolished the acute phase of α_{1B} receptor desensitization. The PKC inhibitor Gö6976 inhibits the cPKC isoforms PKCα and PKCβII with an IC_{50} of 2.3 and 6.2 nM without affecting the activity of PKCδ even at high concentrations in the micromolar range (28), thus providing a reliable pharmacological tool to disrupt the activity of cPKCs. In the presence of 10 nM Gö6976 (2-h incubation plus Gö6976 in the recording solution), application of Phe induced DAG formation that did not decline in the presence of agonist, indicating that inhibition of cPKCs eliminated acute desensitization (see also Fig. 6G).

**Overexpression of WT PKCδ Induced Rapid Desensitization of α_{1A}-AR-induced DAG Formation**—The consistent kinetics of α_{1B}-AR-induced DAG signaling upon overexpression of WT PKCδ might exclude a contribution of PKCδ to acute α_{1B} receptor desensitization. To exclude inadequate PKCδ overexpression above the endogenous protein levels, we tested the impact of PKCδ overexpression on the function of the closely related α_{1A}-AR. We investigated DAG kinetics in α_{1A}-AR-expressing HEK cells either under control conditions (Fig. 7A) or during coexpression of the WT PKCδ (Fig. 7B) or during expression of the catalytically inactive mutant PKCδ DN (Fig. 7C). The representative FRET recording in Fig. 7A shows a slow decline of the DAG signal during stimulation of α_{1A}-AR with Phe (1 μM). The summarized data in Fig. 7F, expressed as the ratio FRET_{30 s after peak}/FRET_{peak}, indicate a minor reduction of about 12% during activation of α_{1A} receptors under control conditions, but a rapid decline of DAG production upon overexpression of WT PKCδ (Fig. 7B, see also Fig. 7F). The pronounced reduction of DAG signaling (more than 40%) reflects PKCδ-induced α_{1A}-AR desensitization, which was only observed in the presence of coexpressed PKCδ. To exclude an artifact of protein overexpression, we cotransfected α_{1A}-AR-expressing cells with the PKCδ DN and monitored DAG production. In the presence of the mutant PKCδ DN, DAG kinetics resemble those observed under control conditions (Fig. 7, C and F), supporting the previous finding that the inactive kinase PKCδ DN specifically inhibits PKCδ-induced signaling pathways (29).

To investigate the contribution of endogenous PKC isoforms to α_{1A}-AR desensitization, we monitored the time course of the α_{1A}-AR-induced DAG signal in the continuous presence of staurosporine (Fig. 7D) or Gö6976 (Fig. 7E). As shown by the summarized data in Fig. 7F, comparing the ratio FRET_{30 s after peak}/FRET_{peak}, reduction of the DAG signal during agonist application was significantly diminished by staurosporine, but not by Gö6976.

These data indicate a receptor species-dependent and PKC isoform-specific regulation. Inhibition of cPKCs with Gö6976 abrogates desensitization of α_{1B}-ARs, but not of α_{1A}-ARs. Furthermore, although α_{1A}-AR signaling was regulated by PKCδ, there was no direct evidence for a regulation of α_{1B}-AR by PKCδ.

**Signaling of M_{1}-R Was Not Affected by Intracellular Kinases**—Agonist-induced desensitization of muscarinic M₁ receptor requires GRK-dependent receptor phosphorylation and recruitment of β-arrestins, resulting in clathrin-dependent receptor internalization (30) (for review, see Ref. 31). Stimulation of PKC has been shown to exert opposing effects on M₁ receptor activity, e.g. enhanced acute receptor desensitization (32), a loss of cell surface M₁ receptors, or even no effect on M₁ receptor desensitization or internalization (31). Analogous to the experiments on α_{1B}-R/DAGR-cotransfected cells (Fig. 6), we investigated the contribution of different PKC isoforms to M₁-R signaling by pharmacological inhibition of PKC or coexpression of the wild-type PKCδ. As depicted in Fig. 8, the time course of DAG formation during ACh exposure (10 μM) in the presence or absence of the PKC inhibitors staurosporine and Gö6976 and upon overexpression of PKCδ was indistinguishable, indicating that M₁-R signaling at the level of PLC activation is not modulated by PKC. We did not investigate whether increasing the PKCδ expression level had long-term effects on the number of M₁ receptors at the cell surface. However, during
FIGURE 6. Rapid desensitization of $\alpha_{1b}$-AR is abolished by inhibition of PKC. A and B, FRET recordings from $\alpha_{1b}$-AR-expressing HEK 293 cells cotransfected with DAGR in the presence (B) or absence (A) of staurosporine (100 nM, incubation time 2 h, stauro-containing bath solution was used throughout the experiment). C–F, rapid desensitization of $\alpha_{1b}$-AR was abolished by staurosporine, BAPTA (E), and Gö6976 (10 nM, incubation time 2 h and throughout the experiment (F)), but not by cotransfection with wild-type PKCδ (C) or the inactive mutant PKCδ-DN (D). G, summarized data comparing DAG reduction (ratio FRET$_{30\text{ s after peak}}$/FRET$_{\text{peak}}$) ($n = 12$ for $\alpha_{1b}$-AR − stauro, $n = 7$ for $\alpha_{1b}$-AR + stauro, $n = 10$ for $\alpha_{1b}$-AR + WT PKCδ, $n = 16$ for PKCδ-DN, $n = 7$ for BAPTA, and $n = 9$ for $\alpha_{1b}$-AR + Gö6976). n.s. = not significant. *, $p < 0.01$. Error bars indicate mean ± S.E. of $n$ cells.
and their contribution to IKs modulation are mainly elucidated (20), but to date, temporal aspects of cPKC and nPKC activation and a PKC-induced feedback modulation of receptor activity. Apart from modulating homologous receptor desensitization, and summarized data in (9B) ratio in Fig. 9 (showing the time courses of Dak signals on an expanded time scale), the attenuation of α1b-AR desensitization became evident during sustained agonist application. For example, after 40 s of agonist

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our experiments (with a duration of 300 – 600 s), receptor internalization is unlikely to occur because we did not observe a PKC-induced reduction in M1 receptor responsiveness. Pharmacological PKC Inhibition Reduced the Acute Desensitization of α1b-AR and Modulated IKs Facilitation—Recent studies on GqPCR-induced modulation of IKs suggest fundamental differences in the biophysical channel modulation by Ca2+-dependent and Ca2+-independent PKC isoforms (13, 20), but to date, temporal aspects of cPKC and nPKC activation and their contribution to IKs modulation are mainly elucidated from kinetic models (13).

Our data suggest that receptor species-dependent differences in desensitization account for a receptor-specific recruitment of different PKC isoforms downstream of PIP2 depletion and a PKC-induced feedback modulation of receptor activity. Apart from modulating homologous receptor desensitization, the recruitment of different PKC isoforms during GqPCR activation might shape the temporal signaling properties of downstream Gq signaling effectors.

To address this, we analyzed the modulation of IKs amplitude in KCNQ1/KCNE1 CHO cells and monitored α1b-AR function simultaneously (DAGR-FRET biosensor) in the presence of PKC inhibitors. As shown previously in Fig. 3A, the α1b-AR-induced inhibition of IKs in the absence of PKC inhibitors was transient and turned into delayed facilitation after agonist withdrawal. Nonspecific inhibition of PKC isoforms with staurosporine reduced the rapid decay of DAG formation in the presence of phenylephrine (Fig. 9, B and D). Furthermore, PKC inhibition with staurosporine increased α1b-AR-induced IKs reduction and abolished IKs facilitation (Fig. 9, A and C).

In the presence of Gö6976, the DAG signal during stimulation of α1b-AR receptors was significantly prolonged (see FRET ratio in Fig. 9B and summarized data in 9D), indicating a reduction of homologous α1b-AR desensitization. As depicted in Fig. 9E (showing the time courses of α1b-AR-induced DAG signals on an expanded time scale), the attenuation of α1b-AR desensitization became evident during sustained agonist application. For example, after 40 s of agonist

**FIGURE 7.** DAG formation in α1a-AR-expressing HEK cells cotransfected with WT PKCδ or the inactive mutant PKCδ DN. A–E, FRET recordings from α1a-AR/DAGR-expressing HEK 293 cells under control conditions (A), cotransfected with WT PKCδ (B) or the inactive mutant PKCδ DN (C), or in the presence of staurosporine (100 nM, D), or Gö6976 (10 nM, E). Rapid desensitization of α1a-AR was induced by overexpression of WT PKCδ, but not by the inactive mutant PKCδ DN. Incubation of α1a-AR/DAGR-expressing cells with staurosporine (D), but not with Gö6976, significantly reduced α1a-AR desensitization. F, summarized data comparing DAG reduction (ratio FRET30 s after peak/FRETpeak) for α1a-AR (n = 17), α1a-AR + WT PKCδ (n = 7), α1a-AR + PKCδ DN (n = 8), α1a-AR + stauro (n = 7), and α1a-AR + Gö6976 (n = 10). n.s. = not significant. *, p < 0.01. Error bars indicate mean ± S.E. of n cells.
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**FIGURE 8. Signaling of $M_1$-R is not affected by intracellular kinases.** A–D, FRET recordings from $M_1$-R-expressing HEK 293 cells cotransfected with DAGR in the presence (B) or absence (A) of staurosporine. Neither incubation with staurosporine nor Gö6976 (D) nor cotransfection with wild-type PKCδ (C) affected the time course of DAG production. E, summarized data comparing DAG reduction (measured 30 s after FRET$_{peak}$) ($n = 10$ for $M_1$-R + stauro, $n = 6$ for $M_1$-R + stauro, $n = 14$ for $M_1$-R + WT PKCδ, and $n = 5$ for $M_1$-R + Gö6976). n.s. = not significant. Error bars indicate mean ± S.E. of n cells.

application (Fig. 9E at $t = 145$ s), the Phe-induced DAG signal was decreased to 38% in the absence, but only to 65% in the presence of Gö6976. Moreover, pharmacological inhibition of Ca$^{2+}$-dependent PKC isoforms increased the $\alpha_{1B}$-AR-induced $I_{Ks}$ reduction (Fig. 9C), but did not abolish $I_{Ks}$ facilitation after agonist withdrawal. These data indicate a contribution of different PKC isoforms to the time course of $\alpha_{1B}$-AR-induced $I_{Ks}$ modulation. Activation of Ca$^{2+}$-dependent PKC isoforms induces rapid $\alpha_{1B}$-AR desensitization, which delimits PIP$_2$ depletion, resulting in moderate $I_{Ks}$ inhibition. Disruption of cPKC activation prolongs $\alpha_{1B}$-AR-induced PIP$_2$ depletion, thus augmenting $I_{Ks}$ inhibition (compare the extent of current inhibition indicated by dotted lines in Figs. 3C, left panel, and 9C). However, a contribution of cPKCs to the current increase after agonist withdrawal can be excluded, because $I_{Ks}$ facilitation was still observed during pharmacological inhibition of cPKCs. In contrast, inhibition of cPKCs and nPKCs by staurosporine abolished $I_{Ks}$ facilitation, suggesting that activation of Ca$^{2+}$-independent PKC isoforms mediates $I_{Ks}$ increase.

*The Rapid Decline of DAG Formation Is Reduced in HEK Cells Expressing the $\alpha_{1B}/\alpha_{1A}$-CT chimera as Compared with $\alpha_{1B}$-AR-induced DAG Signals—Phosphorylation sites at the C terminus of $\alpha_{1B}$-AR have been shown to be critically involved in receptor desensitization (33–35) and receptor endocytosis (36). These phosphorylation sites are not conserved among the other $\alpha$-adrenergic receptor subtypes. As shown previously, $\alpha_{1A}$-ARs are less sensitive to agonist-induced desensitization and are phosphorylated to a lesser extent as compared with $\alpha_{1B}$-AR (34). Because former studies were not designed to analyze dynamic temporal aspects of desensitization, we coexpressed chimeric $\alpha_{1B}$-AR carrying the C terminus of $\alpha_{1A}$-AR ($\alpha_{1B}/\alpha_{1A}$-CT chimera) together with DAGR in HEK 293 cells and compared the agonist-induced DAG dynamics among wild-type $\alpha_{1B}$-AR, $\alpha_{1A}$-AR, and the $\alpha_{1B}/\alpha_{1A}$-CT chimera (Fig. 10). The representative FRET recordings in Fig. 10 show the effects of $G_q$PCR stimulation on DAG dynamics during application of Phe (1 $\mu$M). DAG production in HEK 293 cells rapidly decayed during stimulation of $\alpha_{1B}$ receptors (Fig. 10A), but was significantly prolonged in $\alpha_{1A}$-AR- and $\alpha_{1B}/\alpha_{1A}$-CT chimera-expressing cells (Fig. 10, B and C). The summarized data, expressed as the ratio FRET$_{30}$s after peak/FRET$_{peak}$ in Fig. 10E, indicate a more than 50% reduction of the DAG signal during activation of $\alpha_{1B}$ receptors in line with the data shown in Fig. 2, and a significantly smaller reduction during stimulation of $\alpha_{1A}$ receptors (about 15%, the same data as depicted in Fig. 7D) and $\alpha_{1B}/\alpha_{1A}$-CT chimera receptors (about 27%).
During sustained agonist application (>60 s), the DAG signal declined almost to baseline in α₁B-AR-expressing cells, but decayed with a slower time course during stimulation of α₁A-AR- and α₁B/α₁A-CT chimera-AR. As indicated by the summarized data in Fig. 10F, comparing the ratio $F_{\text{RET,60s after peak}}/F_{\text{RET,peak}}$, the desensitization characteristics of α₁B/α₁A-CT chimera-expressing cells resemble those observed in wild-type α₁A-AR-expressing cells, although desensitization of α₁B/
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![Graphs showing desensitization controls](image)

**FIGURE 10.** Rapid decline of DAG formation is reduced in HEK cells expressing the $\alpha_{1B}/\alpha_{1A}$-CT chimera as compared with $\alpha_{1B}$-AR-induced DAG signals. A–D, FRET recordings of DAG production in $\alpha_{1B}$-AR-expressing HEK 293 cells (A) cotransfected with DAGR or $\alpha_{1A}$-AR/DAGR (B) or $\alpha_{1B}/\alpha_{1A}$-CT chimera/DAGR (C) or $\alpha_{1B}/\alpha_{1A}$-CT chimera/DAGR/WT PKCδ-expressing cells (D). Phenylephrine (1 µM) was applied as indicated. Periods of time plotted in blue correspond to times of exposure to agonist. Note the rapid decay of the FRET ratio in the presence of agonist in $\alpha_{1B}$-AR but not in $\alpha_{1A}$-AR or $\alpha_{1B}/\alpha_{1A}$-CT chimera-expressing cells. E and F, summarized data of DAG reduction during agonist application (determined by the ratio FRET30 s after peak/FRETpeak (E) or FRET60 s after peak/FRETpeak (F)), indicating a more than 50% reduction of the DAG signal (within 30 s) during activation of $\alpha_{1A}$ receptors (n = 7), a reduction of about 27% during stimulation of the $\alpha_{1B}/\alpha_{1A}$-CT chimera (n = 11), and a reduction of 58% in $\alpha_{1B}/\alpha_{1A}$-CT chimera/DAGR/WT PKCδ-expressing cells (n = 6). After 60 s of agonist application, the DAG signal decreased by about 90% in $\alpha_{1B}$-AR-expressing HEK 293 cells, by 34% in $\alpha_{1A}$-AR-expressing cells, by 50% in $\alpha_{1B}/\alpha_{1A}$-CT chimera/DAGR-expressing cells, and by 89% in $\alpha_{1B}/\alpha_{1A}$-CT chimera/DAGR/WT PKCδ-expressing cells. n.s. = not significant. *p < 0.01. Error bars indicate mean ± S.E. of n cells.

$\alpha_{1A}$-CT chimera-AR occurred slightly faster than those of wild-type $\alpha_{1A}$-AR (Fig. 10F). Furthermore, as for the wild-type $\alpha_{1A}$-AR, overexpression of WT PKCδ induced pronounced desensitization of $\alpha_{1B}/\alpha_{1A}$-CT chimera receptors as indicated by the rapid decline of the DAG signal (Fig. 10D).

Because the time course of desensitization induced by the chimera is an intermediate between the $\alpha_{1B}$-AR and $\alpha_{1A}$-AR desensitization phenotypes, it is proposed that additional regions, apart from the C terminus, contribute to $\alpha_{1B}$ receptor desensitization. Although the identification of additional mechanisms of $\alpha_{1B}$-AR desensitization is beyond the scope of the present study, the significant reduction of desensitization in chimeric $\alpha_{1B}$ receptors carrying the $\alpha_{1A}$-AR C terminus emphasizes that the number of phosphorylation sites (i.e., the degree of receptor phosphorylation) is directly related to the extent of acute desensitization of the receptors.

**Discussion**

Spatial and temporal organization of signaling components downstream of $G_q$ activation is related to distinct cellular responses upon activation of different $G_q$PCRs (2). Receptor-specific cellular events might be induced by promiscuous coupling of GPCRs to $G_q$ family members, interaction of $G_q$ with GRK2 or regulators of G protein Signaling (RGS) proteins, or different affinities of $G_q$ effector activation (2). Furthermore, functional diversity upon stimulation of $G_q$PCRs can be determined by spatial proximity of $G_q$PCRs and signal molecules (3),
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Mutational studies on hamster $\alpha_{1B}$-AR indicate that PKC activation and phosphorylation of residues within the C-tail promote rapid receptor internalization (43). More recent studies propose that interactions of $\alpha_{1B}$-AR with $\beta$-arrestin and the clathrin adaptor complex AP2 (36) or with Rab4 and Rab5 proteins that are associated with early endosomes (25) regulate receptor internalization. All receptor modifications stated above may result in termination of receptor signaling by internalization of the receptors.

PKC phosphorylation sites are not conserved among the other $\alpha$-adrenergic receptor subtypes, and differences in receptor desensitization and internalization between $\alpha_{1B}$-AR and $\alpha_{1A}$-AR (with modest internalization of $\alpha_{1A}$-AR as compared with $\alpha_{1B}$-AR) are mainly related to receptor subtype-dependent differences in $\beta$-arrestin binding (36). A recent study (44) describes that differences in the $\alpha$-AR subtype internalization account for activation of different, subtype-specific downstream $G_q$/PCR effectors. As revealed by our data (Fig. 5), receptor internalization (with a time course of $\geq 15$ min) does not contribute to the acute phase of $\alpha_{1B}$-AR desensitization (see also Fig. 2), which was almost complete within 60 s of agonist application. In addition, we were able to elicit DAG signals upon repetitive agonist application (Fig. 2), excluding a significant reduction in the number of receptor molecules on the cell surface. Furthermore, the fast recovery from desensitization (Fig. 5) suggests that direct modification of the receptor protein instead of receptor endocytosis is the underlying mechanism of receptor desensitization.

It was not the aim of the present study to investigate $\alpha_{1A}$-AR desensitization, but previous studies showed significant differences in the extent of phosphorylation between $\alpha_{1A}$-AR and $\alpha_{1B}$-AR (34). $\alpha_{1A}$-ARs are phosphorylated to a lesser extent as compared with $\alpha_{1B}$-ARs and display only minor desensitization. These data are in line with our observation that overexpression of the wild-type PKC$\delta$, but not of the inactive mutant PKC$\delta$ DN, rapidly terminated DAG signaling during $\alpha_{1A}$-AR stimulation (Fig. 7). Furthermore, these experiments demonstrate the efficiency of PKC$\delta$ overexpression. Apparently, overexpression of the wild-type PKC$\delta$ augments $\alpha_{1A}$-AR desensitization. However, the contribution of different PKC isoforms to endogenous $\alpha_{1A}$-AR signaling is evident from our experiments shown in Figs. 6 and 7. Although staurosporine abrogates desensitization of both $\alpha_{1A}$ and $\alpha_{1B}$ receptors, a contribution of cPKCs to $\alpha_{1A}$-AR desensitization can be excluded. Inhibition of cPKCs with Gö6976 abolishes acute desensitization of $\alpha_{1B}$ but not of $\alpha_{1A}$ receptors (Figs. 6 and 7), indicating receptor-specific regulation by different PKC isoforms.

Moreover, our experiments investigating the kinetics of $\alpha_{1B}$/$\alpha_{1A}$-CT chimera-induced desensitization indicate that the origin of the $\alpha$-AR subtype C terminus determines the time course and kinetics of receptor desensitization. These data are in line with a previous study (34) on chimeric $\alpha_{1A}$ and $\alpha_{1B}$ receptors, carrying the mutual C-terminal region of each receptor subtype. Chimeric $\alpha_{1A}$-AR displayed marked basal and agonist-induced phosphorylation (in contrast to the wild-type $\alpha_{1A}$-AR), whereas the opposite was observed in chimeric $\alpha_{1B}$-AR (34). The rapid decline of the $\alpha_{1B}$/\$\alpha_{1A}$-CT-induced DAG signal upon overexpression of WT PKC$\delta$ supports the idea that the C ter-

by activating different branches of the signaling pathway downstream of $G_\alpha_q$ (4, 5), or by connecting signaling enzymes to their substrates in specific microdomains (8).

The aspect of receptor species-dependent desensitization that determines temporal aspects of downstream G protein signaling has not been elucidated so far. In our study, we investigated the receptor-specific desensitization of $\alpha_{1B}$ and $M_1$ receptors as paradigmatic $G_\alpha_q$-coupled receptors (37, 38).

We provide evidence that receptor-specific desensitization shapes the kinetics of $G_q$ signaling, resulting in receptor species-dependent modulation of effectors. By using a variety of FRET-based biosensors, we demonstrated receptor species-dependent differences in desensitization on all levels of downstream $G_q$PCR signaling, including G protein activation (Fig. 1), PIP$_2$ depletion upon activation of PLC (Fig. 4), and DAG formation (Fig. 2).

Several lines of evidence support the idea that receptor-specific desensitization reflects intrinsic receptor properties rather than being related to insufficient receptor-G protein coupling or insufficient effector activation. First, receptor-specific desensitization of $\alpha_{1B}$ and $M_1$ receptors was not abolished upon decreasing or increasing their respective expression levels (Fig. 2) and was present at saturating and moderate agonist concentrations at a given receptor expression level (compare 100 nM versus 1 $\mu$M responses in Fig. 1). Second, as yielded by the concentration-response curves for $\alpha_{1B}$-AR- and $M_1$-R-induced $G_q$ activation (Fig. 1), the agonist concentrations used in the present study induced full activation of G proteins and downstream $G_\alpha_q$ components. Third, similar temporal aspects of $\alpha_{1B}$ and $M_1$ receptor signaling were evident in CHO cells stably expressing GRK to $\alpha_{1B}$-AR and $M_1$ receptors, a contribution of cPKCs to $\alpha_{1A}$-AR desensitization (see Fig. 6) does not contribute to the acute phase of $\alpha_{1B}$-AR desensitization (see also Fig. 2), which was almost complete within 60 s of agonist application. In addition, we were able to elicit DAG signals upon repetitive agonist application (Fig. 2), excluding a significant reduction in the number of receptor molecules on the cell surface. Furthermore, the fast recovery from desensitization (Fig. 5) suggests that direct modification of the receptor protein instead of receptor endocytosis is the underlying mechanism of receptor desensitization.

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minus represents a transferable element that confers PKCδ sensitivity of $\alpha_{1A}$-AR to $\alpha_{1B}$-AR. In contrast, overexpression of WT PKCδ failed to modulate $\alpha_{1B}$-AR-induced DAG signals (Fig. 6). However, the inhibition of $\alpha_{1B}$-AR desensitization with BAPTA and G66976 favors the idea that cPKCs rather than nPKCs contribute to receptor phosphorylation and desensitization. The most striking result of the present study is the fact that receptor desensitization did not abate, but instead fine-tuned the effector response downstream of $G_{q\alpha}$. We measured K$^+$ currents through KCNQ1/KCNE1 channels ($I_{ks}$) as a sensitive effector system downstream of $G_{q\alpha}$ activation and related receptor species-dependent desensitization to different time courses of $I_{ks}$ modulation. In our study, the time course of $I_{ks}$ modulation upon activation of $\alpha_{1B}$-AR (Fig. 3A) is characterized by an inhibitory component related to PIP$_2$-induced current inhibition and subsequent $I_{ks}$ facilitation that is due to phosphorylation of the channel protein. Although the rapid desensitization of the $\alpha_{1B}$-AR limits PIP$_2$ depletion and diminishes $I_{ks}$ inhibition, the activation of PKC facilitates $I_{ks}$ amplitude at the same time, resulting in net reduction of the inhibitory component to about 15% as compared with the initial current amplitude (Fig. 3C). Such a biphasic time course of $I_{ks}$ regulation upon activation of $G_q$PCRs has been described in previous studies (19, 20, 27), but to present a novel finding, we provide detailed insights into the contribution of different PKC isoforms to channel regulation. By using pharmacological tools (Fig. 6), we were able to dissect the contribution of different PKC isoforms to $\alpha_{1B}$ receptor desensitization (mediated by Ca$^{2+}$-dependent PKC isoforms) and $I_{ks}$ facilitation (PKCδ). Pharmacological inhibition of all PKC isoforms eliminates $\alpha_{1B}$-AR desensitization and $I_{ks}$ facilitation, thus augmenting the inhibitory current component related to PIP$_2$ depletion.

In contrast to the $\alpha_{1B}$-induced biphasic time course of $I_{ks}$ modulation, stimulation of muscarinic receptors induced sustained PIP$_2$ depletion (Fig. 2), resulting in net inhibition of $I_{ks}$ of about 30% (Fig. 3), but no PKC-induced $I_{ks}$ facilitation. As indicated by Fig. 4, $M_1$-R-induced PIP$_2$ depletion was sustained during agonist exposure, in line with the fact that $M_1$ receptors reveal no desensitization in the presence of acetylcholine.

To summarize, receptor-specific desensitization is a mechanism of effector modulation by restricting the spatiotemporal activation of downstream $G_q$ signaling components. Receptor-dependent differences in desensitization account for different $I_{ks}$ modulation by $\alpha_{1B}$-AR and $M_1$-R by controlling the duration of PIP$_2$ depletion and the recruitment of different PKC isoforms.

Experimental Procedures

**Molecular Biology and Cell Culture**—To generate fusion proteins of $\alpha$-ARs, the cDNA fragments indicated below were generated by PCR and inserted into linearized pcDNA3 (Invitrogen) using the In-Fusion cloning method (Clontech) following the manufacturer’s instructions. To generate YFP-labeled versions of the human $\alpha_{1B}$-AR (NM_000679), yellow fluorescent protein (YFP) was amplified by PCR and either attached to the C terminus of the receptor or inserted between amino acids (aa) 253 and 254. The latter results in a receptor that carries YFP in its third intracellular loop, which is not expected to interfere with phosphorylation of the C terminus. The constructs were termed $\alpha_{1B}$-AR-YFP or $\alpha_{1B}$-AR-YFP-IL3, respectively. To generate a chimeric $\alpha_{1A}/\alpha_{1B}$-AR, a cDNA fragment encoding for aa 1–351 of human $\alpha_{1B}$-AR was directly fused to a fragment encoding for the C terminus (aa 330–466) of human $\alpha_{1A}$-AR (NM_000680). This construct was termed $\alpha_{1B}/\alpha_{1A}$-CT chimera. All constructs were verified by DNA sequencing. The aa positions refer to entries P35348 ($\alpha_{1A}$-AR) and P35368 ($\alpha_{1B}$-AR) of the UniProt database.

All experiments were performed using either CHO cells stably expressing the subunits KCNQ1/KCNE1 underlying $I_{ks}$ (45) or native HEK 293 cells. CHO cells were grown in Iscove’s modified Dulbecco’s medium containing fetal bovine serum (10%), HT supplement, non-essential amino acids, and 400 $\mu$g/ml G-418. HEK 293 cells were grown in DMEM medium, supplemented with glutamine (1%) and fetal calf serum (10%). Both cell lines were cultured with penicillin/streptomycin using standard cell culture conditions. All cell culture media and supplements were purchased from Gibco. Both cell lines were transiently transfected with cDNAs encoding for $G_q$PCRs and the following FRET biosensors (amount in $\mu$g per 3-cm culture dish). To monitor the $G_q$ protein cycle, we used $\alpha_1$-ARs (0.5) or $M_1$-R (0.5), Go-$\alpha_q$-YFP (1.0), Go-$\beta_2$-Cerulean (0.5), Gy2 (0.2), and GPCR kinase 2 (GRK2) (0.5) (17). To monitor receptor-arrestin interactions, we used $\alpha_{1B}$-AR-YFP or $M_1$-R-YFP (0.6), Turbo-arrestin3 (0.6), and GRK2 (0.8) (24). To monitor the breakdown of PIP$_2$ as a measure for PLC activation, we used receptor species as indicated (0.5) and a PIP$_2$ biosensor (0.5).
that is based on CFP- and YFP-labeled PH domains of PLCδ1 (22). To monitor the production of the second messenger DAG, we used receptor species as indicated (0.5) and the biosensor DAGR (0.5), which reports conformational changes of a CFP/YFP-labeled DAG-binding domain of protein kinase C (PKCδ2) (18). DAGR was kindly provided by Dr. Alexandra Newton (Addgene plasmid number 14865). For some experiments, cells were cotransfected with 0.5 μg of a plasmid encoding for WT PKCδ or PKCδ DN (29), kindly provided by Dr. Bernard Weinstein via Addgene (WT, plasmid number 16386, and DN, plasmid number 16389). Functionality of WT PKCδ and PKCδ DN was confirmed in experiments on KCNQ1/KCNE1-expressing CHO cells measuring IKS modulation during α1b receptor stimulation (see supplemental Fig. S4). Both cell lines were transfected using either polyethyleneimine as described in Ref. 22 or Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Prior to experiments, cells were seeded on sterile, poly-L-lysine-coated glass coverslips and analyzed 24 h (cells expressing DAGR) or 48 h after transfections.

Fluorescence Microscopy and Imaging—All experiments were performed using single cells at ambient temperature. Fluorescence was recorded using an inverted microscope (Zeiss Axiosvert 200, Carl Zeiss AG, Göttingen, Germany) equipped with a Zeiss oil immersion objective (100×/1.4), a Polychrome V illumination source, and a photodiode-based dual emission photometry system suitable for CFP/YFP FRET (TILL Photonics/FEI GmbH, Munich, Germany). For FRET measurements, single cells were excited at 435-nm wavelength with light pulses of variable duration (10–50 ms) at a frequency of 5 Hz to minimize photobleaching. Corresponding emitted fluorescence from CFP (F480 or FCFP) or from YFP (F535 or FYFP) was acquired simultaneously, and FRET was defined as ratio FYFP/FCFP. Fluorescent signals were recorded and digitized using a commercial hardware/software package (EPC10 amplifier with an integrated D/A board and Patchmaster software, HEKA, HEKA Elektronik, Lambrecht/Pfalz, Germany). Details on optical filters and beam splitters of the setup are given in Ref. 22. The individual FRET traces for obtaining concentration-response curves were normalized to the maximal response of the G protein biosensor at saturating agonist concentrations (FRET/FRET10max), denoted as FRET/FRETmax. All other traces were normalized to the initial ratio value before agonist application (FRET/FRET0). For receptor internalization experiments, YFP was excited at 500 nm, and fluorescence images were acquired with a Zeiss AxioCam MRm epifluorescence camera and corresponding AxioVision software. After application of phenylephrine, consecutive pictures were taken after the incubation times as indicated.

Current Measurement—Membrane currents were measured using whole-cell patch clamp technique. Pipettes were fabricated from borosilicate glass and filled with the solution listed below (direct current resistance, 4–6 MΩhms). Currents were measured by means of a patch clamp amplifier (LM/EPC 7, List Electronics, Darmstadt, Germany). Signals were filtered (corner frequency, 1 kHz), digitally sampled at 1 kHz, and stored on a computer equipped with a hardware/software package (ISO2, MFK, Frankfurt/Main, Germany) for voltage control and data acquisition. Experiments were performed at ambient temperature (23–26 °C). For combined patch clamp and FRET measurements, standard patch clamp equipment was attached to the optical setup. Application of different solutions was performed by means of a custom-made solenoidal-operated flow system. Whole-cell IKS was routinely measured during depolarizing pulses (to +60 mV, duration 5 s, applied every 20 s) from a holding potential of −80 mV (see also supplemental Fig. S5).

Solution and Chemicals—For whole-cell measurements of membrane currents an extracellular solution of the following composition was used (in mM): 137 NaCl; 5.4 KCl; 0.5 CaCl2; 1.0 MgCl2; 10.0 Hepes/NaOH, pH 7.4. The standard pipette solution contained (in mM): 100 potassium aspartate; 40 KCl; 5.0 NaCl; 2.0 MgCl2; 5.0 Na2ATP; 2.0 EGTA; 0.025 GTP; 20.0 HEPES/KOH, pH 7. Where indicated, EGTA was replaced by 5 mM BAPTA. Standard chemicals were from Merck (Darmstadt, Germany), BAPTA, EGTA, HEPES, Na2ATP, phenylephrine, sodium salt hydrate (GTP), and ACh were from Sigma-Aldrich (Taufkirchen, Germany). Staurosporine and Gö6976 were purchased from Tocris Bioscience (Bristol, UK).

Statistical Analysis—All data are presented as individual observations or summarized data (mean ± S.E. of n cells). Student’s t test was used to compare the means between two groups. p values less than 0.05 were considered statistically significant.

Author Contributions—M.-C. K., L. P., and A. R. participated in research design. M.-C. K., D. V., and C. M. conducted experiments and performed data analysis. M.-C. K., L. P., and A. R. wrote the manuscript or contributed to the manuscript.

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References
1. Berridge, M. J. (1987) Inositol trisphosphate and diacylglycerol: two interacting second messengers. Annu. Rev. Biochem. 56, 159–193
2. Hubbard, K. B., and Hepler, J. R. (2006) Cell signalling diversity of the Gq family of heterotrimeric G proteins. Cell. Signal. 18, 135–150
3. Cho, H., Kim, Y. A., Yoon, J.-Y., Lee, D., Kim, J. H., Lee, S. H., and Ho, W.-K. (2005) Low mobility of phosphatidylinositol 4,5-bisphosphate underlies receptor specificity of Gqα-mediated ion channel regulation in atrial myocytes. Proc. Natl. Acad. Sci. U.S.A. 102, 15241–15246
4. Dickson, E. J., Falkenburger, B. H., and Hille, B. (2013) Quantitative properties and receptor reserve of the IP3 and calcium branch of Gq-coupled receptor signaling. J. Gen. Physiol. 141, 521–535
5. Falkenburger, B. H., Dickson, E. J., and Hille, B. (2013) Quantitative properties and receptor reserve of the DAG and PKC branch of Gq-coupled receptor signaling. J. Gen. Physiol. 141, 537–555
6. Shirai, Y., and Saito, N. (2002) Activation mechanisms of protein kinase C: maturation, catalytic activation, and targeting. J. Biochem. 132, 663–668
7. Mochly-Rosen, D., Das, K., and Grimes, K. V. (2012) Protein kinase Cα, an elusive therapeutic target? Nat. Rev. Drug Discov. 11, 937–957
8. Hoque, M., Rentero, C., Cairns, R., Tebar, F., Enrich, C., and Grewal, T. (2014) Annexins: scaffolds modulating PKC localization and signaling. Cell. Signal. 26, 1213–1225
9. Steinberg, S. F. (2008) Structural basis of protein kinase C isoform function. Physiol. Rev. 88, 1341–1378
10. Gallegos, L. L., Kunkel, M. T., and Newton, A. C. (2006) Targeting protein kinase C activity reporter to discrete intracellular regions reveals spatio-
Desensitization Controls $I_{Ks}$ as an Effector of $G_q$PCRs

temporal differences in agonist-dependent signaling. J. Biol. Chem. 281, 30947–30956
11. Hui, X., Reither, G., Kaestner, L., and Lipp, P. (2014) Targeted activation of conventional and novel protein kinases through differential translocation patterns. Mol. Cell. Biol. 34, 2370–2381
12. Gamper, N., Reznikov, V., Yamada, Y., Yang, J., and Shapiro, M. S. (2004) Phosphatidylinositol 4,5-bisphosphate signals underlie receptor-specific $G_{q11}$-mediated modulation of N-type $Ca^{2+}$ channels. J. Neurosci. 24, 10980–10992
13. Matavel, A., and Lopes, C. M. (2009) PKC activation and PIP$_2$ depletion underlie biphasic regulation of $I_{Ks}$ by $G_{q}$-coupled receptors. J. Mol. Cell. Cardiol. 46, 704–712
14. Masuho, I., Ostrovskaya, O., Kramer, G. M., Jones, C. D., Xie, K., and Martemyanov, K. A. (2015) Distinct profiles of functional discrimination among G proteins determine the actions of G protein-coupled receptors. Sci. Signal. 8, ra123
15. Bodmann, E.-L., Wolters, V., and Bünemann, M. (2015) Dynamics of G protein effector interactions and their impact on timing and sensitivity of G protein-mediated signal transduction. Eur. J. Cell Biol. 94, 415–419
16. Chen, X., Egly, C., Riley, A. M., Li, W., Tewson, P., Hughes, T. E., Quinn, A. M., and Obukhov, A. G. (2014) PKC-dependent phosphorylation of the H1 histamine receptor modulates TRPC6 activity. Cells 3, 247–257
17. Falkenburger, B. H., Jensen, B. J., and Hille, B. (2010) Kinetics of M$_1$, muscarinic receptor and G protein signaling to phospholipase C in living cells. J. Gen. Physiol. 135, 81–97
18. Violin, J. D., Zhang, J., Tsien, R. Y., and Newton, A. C. (2003) A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. J. Cell Biol. 161, 899–909
19. Matavel, A., Medei, E., and Lopes, C. (2010) PKA and PKC partially rescue $I_{Ks}$ events in LQT1.
20. O-Uchi, J., Rice J. J., Ruwald, M. H., Parks, X. X., Ronzier, E., Moss, A. J., Zareba, W., and Lopes, C. M. (2015) Impaired $I_{Ks}$ channel activation by $Ca^{2+}$-dependent PKC shows correlation with emotion/arousal-triggered events in LQT1. J. Mol. Cell. Cardiol. 79, 203–211
21. van der Wal, J., Habets, R., Várnai, P., Balla, T., and Jalink, K. (2001) Monitoring agonist-induced phospholipase C activation in live cells by fluorescence resonance energy transfer. J. Biol. Chem. 276, 15337–15344
22. Hertel, F., Switalski, A., Mintert-Jancke, E., Karavassiliou, K., Bender, K., Pott, L., and Kienitz, M. C. (2011) A genetically encoded tool kit for manipulating and monitoring membrane phosphatidylinositol 4,5-bisphosphate in intact cells. PLoS One 6, e20855
23. Luttrell, L. M., and Gesty-Palmer, D. (2010) Beyond desensitization: physiological role of arrestin-dependent signaling. Pharmacol. Rev. 62, 305–330
24. Krasel, C., Bünnemann, M., Lorenz, K., and Lohse, M. J. (2005) $\beta$-Arrbin binding to the $\beta_1$-adrenergic receptor requires both receptor phosphorylation and receptor activation. J. Biol. Chem. 280, 9528–9535
25. Castillo-Badillo, J. A., Sánchez-Reyes, O. B., Alfonzo-Méndez, M. A., Romero-Avila, M. T., Reyes-Cruz, G., and García-Sáinz, J. A. (2015) $\alpha_{1b}$-adrenergic receptors differentially associate with Rab proteins during homologous and heterologous desensitization. PLoS One 10, e0121165
26. García-Sáinz, J. A., Romero-Avila, M. T., and Alcántara-Hernández, R. (2011) Mechanisms involved in $\alpha_{1b}$-adrenergic receptor desensitization. IUBMB Life 63, 811–815
27. O-Uchi, J., Sorenson, J., Jhun, B. S., Mishra, J., Hurst, S., Williams, K., Sheu, S.-S., and Lopes, C. M. B. (2015) Isoform-specific dynamic translocation of PKC by $\alpha_{1b}$-adrenergic receptor stimulation in live cells. Biochem. Biophys. Res. Commun. 465, 464–470
28. Martin-Gallon, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marmé, D., and Schächtele, C. (1993) Selective inhibition of protein kinase C isoforms by the indolocarbazole Gö6976. J. Biol. Chem. 268, 9194–9197
29. Soh, I.-W., and Weinstein, I. B. (2003) Roles of specific isoforms of protein kinase C in the transcriptional control of cyclin D1 and related genes. J. Biol. Chem. 278, 34709–34716
30. Yeatman, H. R., Lane, J. R., Choy, K. H. C., Lambert, N. A., Sexton, P. M., Christopoulos, A., and Canals, M. (2014) Allosteric modulation of M$_3$ muscarinic acetylcholine receptor internalization and subcellular trafficking. J. Biol. Chem. 289, 15856–15866
31. van Koppen, C. J., and Kaiser, B. (2003) Regulation of muscarinic acetylcholine receptor desensitization by synaptic activity in cultured hippocampal neurons. J. Neurochem. 103, 2268–2280
32. Lattion, A. L., Diviani, D., and Cotecchia, S. (1994) Truncation of the receptor carboxyl terminus impairs agonist-dependent phosphorylation and desensitization of the $\alpha_{1b}$-adrenergic receptor. J. Biol. Chem. 269, 22887–22893
33. García-Sáinz, J. A., Vázquez-Prado, J., and del Carmen Medina, L. (2000) $\alpha_1$-Adrenoceptors: function and phosphorylation. Eur. J. Pharmacol. 389, 1–12
34. Diviani, D., Lattion, A. L., Larbi, N., Kunapuli, P., Pronin, A., Benovic, J. L., and Cotecchia, S. (1996) Effect of different G protein-coupled receptors kinases on phosphorylation and desensitization of the $\alpha_{1b}$-adrenergic receptor. J. Biol. Chem. 271, 28712–28719
35. Stanisla, L., Abuin, L., Dey, J., and Cotecchia, S. (2008) Different internalization properties of the $\alpha_{1A}$ and $\alpha_{1b}$-adrenergic receptor subtypes: the potential role of receptor interaction with $\beta$-arrestins and AP$\beta$0. Mol. Pharmacol. 74, 562–573
36. Felder, C. C. (1995) Muscarinic acetylcholine receptors: signal transduction through multiple effectors. FASEB J. 9, 619–625
37. Violin, J. D., Dewire, S. M., Barnes, W. G., and Lefkowitz, R. J. (2006) G protein-coupled receptor kinase and $\beta$-arrestin-mediated desensitization of the angiotensin II type 1A receptor elucidated by diacylglycerol dynamics. J. Biol. Chem. 281, 36411–36419
38. García-Sáinz, J. A., Vázquez-Prado, J., and del Carmen Medina, L. (2000) $\alpha_1$-Adrenoceptors: function and phosphorylation. Eur. J. Pharmacol. 389, 1–12