G protein-coupled receptor kinase-2 confers isoform-specific calcium sensitivity to dopamine D₂ receptor desensitization

Richard Ågren¹ | Kristoffer Sahlholm¹²

¹Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden
²Department of Integrative Medical Biology, Wallenberg Centre for Molecular Medicine, Umeå University, Umeå, Sweden

Abstract
The dopamine D₂ receptor (D₂R) functions as an autoreceptor on dopaminergic cell bodies and terminals and as a postsynaptic receptor on a variety of neurons in the central nervous system. As a result of alternative splicing, the D₂R is expressed as two isoforms: long (D₂LR) and short (D₂SR) differing by a stretch of 29 residues in the third intracellular loop, with D₂SR being the predominant presynaptic isoform. Recent reports described a Ca²⁺ sensitivity of the desensitization time course of potassium currents elicited via D₂SR, but not via D₂LR, when either isoform was selectively expressed in dopaminergic neurons. Here, we aimed to study the mechanism behind this subtype-specific Ca²⁺ sensitivity. Thus, we measured the desensitization of potassium channel responses evoked by D₂LR and D₂SR using two-electrode voltage clamp in Xenopus oocytes in the absence and presence of different amounts of β-arrestin2 and G protein-coupled receptor kinase-2 (GRK2), both of which are known to play important roles in D₂R desensitization in native cells. We found that co-expression of both GRK2 and β-arrestin2 was necessary for reconstitution of the Ca²⁺ sensitivity of D₂SR desensitization, while D₂LR did not display Ca²⁺ sensitivity under these conditions. The effect of Ca²⁺ chelation by BAPTA-AM to slow the rate of D₂SR desensitization was mimicked by the GRK2 inhibitor, Cmpd101, and by the kinase-inactivating GRK2 mutation, K220R, but not by the PKC inhibitor, Gö6976, nor by the calmodulin antagonist, KN-93. Thus, Ca²⁺-sensitive desensitization of D₂SR appears to be mediated via a GRK2 phosphorylation-dependent mechanism.

KEYWORDS
beta-arrestin, dopamine receptor isoforms, kinase inhibitors, voltage clamp, Xenopus oocytes

Abbreviations: BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N',N''-tetraacetic acid; D₂R, dopamine D₂ receptor; DA, dopamine; EGTA, ethylene glycol-bis(2-aminoethyl ether)-N,N',N''-tetraacetic acid; GIRK, G protein-coupled inward rectifier potassium channel; GRK2, G protein-coupled receptor kinase-2; Kir, inward rectifier potassium channel; β-arr2, β-arrestin-2.
INTRODUCTION

The dopamine D₂ receptor (D₂R) couples to inhibitory Gαi/o proteins, in addition to signaling via arrestin-dependent pathways, and transmits intracellular signals that ultimately modulate motor activity, cognition and memory, and endocrine functions. Accordingly, D₂R is an important target for treatment of psychiatric, neurological, and endocrine disease. Due to alternative splicing, D₂R is expressed as two distinct isoforms, short (D₂SR) and long (D₂LR), differing by 29 amino acid residues in the third intracellular loop. D₂SR is the predominant isoform expressed in dopamine (DA) neurons of the midbrain, whereas postsynaptic D₂Rs in medium spiny neurons of the striatum are mainly made up of the long isoform. In agreement with this expression pattern, studies of D₂R−/− mice (lacking the exon responsible for the 29-residue insertion) suggested that D₂SR is the main isoform responsible for DA autoreceptor functions. The alternative splicing of D₂R is conserved between rodents and humans, suggesting an important physiological role. However, DA binding affinity and G protein-dependent signaling are similar between D₂SR and D₂LR. Furthermore, while D₂SR predominates in DA neurons, both isoforms can be detected in this cell population, and either is capable of restoring basal presynaptic and postsynaptic D₂R functions when exogenously expressed in D₂R knockout animals. Nevertheless, pharmacological challenge experiments have revealed differences between animals expressing exclusively D₂SR or D₂LR.}

Recent electrophysiological investigations reported that homologous desensitization of D₂R activation-induced G protein-coupled inward rectifier potassium (Kir3; also known as GIRK) channel responses in DA neurons occurs at a faster rate in neurons exclusively expressing D₂SR compared to corresponding neurons expressing only D₂LR. Moreover, replacing the slow-acting Ca²⁺ chelator, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), with the faster-acting Ca²⁺ chelator, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA), in the intracellular buffer reduced the rate of D₂R desensitization such as to resemble that of D₂LR. In contrast, the rate of D₂LR desensitization was found to be insensitive to the type of Ca²⁺ chelator used. Interestingly, cocaine treatment reduced the Ca²⁺-dependent component of agonist-induced desensitization in DA neurons exclusively expressing D₂SR, suggesting that this process can be dynamically regulated in response to altered DA tone.

Here, we used two-electrode voltage clamp in Xenopus oocytes co-expressing D₂LR or D₂SR with Kir3 channels and β-arrestin-2 (β-ar2; also known as arrestin-3) to investigate homologous desensitization of the two D₂R isoforms and its sensitivity to Ca²⁺. The role of G protein-coupled receptor kinase-2 (GRK2), which is known to regulate DA autoreceptor function in vivo, in isoform-specific D₂R desensitization was also investigated. Our expression system utilizes cRNA microinjection of individual oocytes, thus providing stoichiometric control and enabling us to titrate the relative amounts of β-ar2 and GRK2 co-expressed with D₂R. We present evidence that GRK2 co-expression increases β-ar2-dependent desensitization of Kir3 responses evoked via both D₂SR and D₂LR in a dosage-dependent manner and reconstitutes the isoform-specific Ca²⁺ dependence of D₂SR desensitization previously reported from DA neurons. In addition, we find that this subtype-specific effect is abolished by an inhibitor of GRK2 kinase activity and by a kinase-inactivating mutation of GRK2.

MATERIALS AND METHODS

Molecular biology

Wildtype human D₂SR and D₂LR (DRD2), regulator of G protein signaling-4 (RGS4), Kir3.1 (KCNJ3) and Kir3.4 (KCNJ5), β-ar2(ARRB2), GRK2 (ADRBK1), and melanin-concentrating hormone 2 receptor (MCHR2; GenScript, Piscataway, NJ) cDNA were in pXOOM (provided by Dr Søren-Peter Olesen, University of Copenhagen, Denmark). GRK2 mutants and the truncated β-adrenergic receptor kinase C-terminus (βARK-ct) containing the last 195 C-terminal residues of GRK2 were prepared by Genscript and the resulting constructs were sequenced in their entirety. Plasmids were linearized using the proper restriction enzymes (D₂SR, D₂LR, MCHR2, RGS4, Kir3.1, and Kir3.4; XhoI, β-ar2, GRK2, and βARK-ct; XbaI), followed by in vitro transcription using the T7 mMessage mMachine kit (Ambion, Austin, TX, USA). cRNA concentration and purity were determined by spectrophotometry. RGS4 was included since it accelerates the G protein cycle and thus makes receptor desensitization have a more rapid impact on Kir3 current amplitudes. RGS4 is expressed in DA neurons.

Oocyte preparation

Oocytes from female African clawed toads, Xenopus laevis (NASCO, Fort Atkinson, WI, USA), were surgically isolated under tricaine (MS-222) anesthesia as described previously. The procedure conforms to Directive 2010/63/EU and has been approved by the Swedish National Board for Laboratory Animals and the Animal Welfare Ethical Committee in Stockholm (approval number N245/15).
and 686-2021). Following one day of incubation at 12°C, individual oocytes were injected with 50 nl containing 0.2 ng D2R or D2L cRNA, 1.4 ng of RGS4, and 40 pg of each of Kir3.1 and Kir3.4 cRNA using the Nanoinject II (Drummond Scientific, Broomall, PA, USA). When applicable, 0.6–9.7 ng of β-arr2 and 0.07–4.2 ng of GRK2 cRNA were injected. For experiments with MCH2R, 0.2 ng was injected per oocyte.

### 2.3 | Electrophysiology

Injected cells were cultured for 6 days at 12°C in a modified Barth’s solution (MBS; in mM: 88 NaCl, 1 KCl, 2.4 NaHCO3, 15 HEPES, 0.33 Ca(NO3)2, 0.41 CaCl2, 0.92 MgSO4, 2.5 sodium pyruvate, supplemented with 25 U/ml penicillin and 25 µg/ml streptomycin, adjusted to pH 7.6 with NaOH). Electrophysiological recordings were performed using the parallel eight-channel, semi-automated, two-electrode voltage-clamp OpusXpress 6000A (Molecular Devices, San José, CA, USA). Continuous perfusion was maintained at 3.5 ml/min. Data were acquired at membrane potentials of −80 mV and sampled at 156 Hz using the OpusXpress 1.1.0.42 (Molecular Devices) software. Before and after recordings, oocyte stability was assessed at a holding potential of −40 mV. To increase the inward rectifier potassium channel current at negative potentials, a high-K+ extracellular buffer was used (in mM: 64 NaCl, 25 KCl, 0.8 MgCl2, 0.4 CaCl2, 15 HEPES, 1 ascorbic acid, adjusted to pH 7.4 with NaOH), yielding a K+ reversal potential of about −40 mV. When Ca2+ chelators were used (see below), CaCl2 was omitted from the buffer. Ascorbic acid was included to prevent the spontaneous oxidation of DA.

### 2.4 | Ligands

DA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and prepared fresh in recording buffer containing 1 mM ascorbic acid. Melanin-concentrating hormone (MCH) was obtained from Bachem AG (Bubendorf, Switzerland), EGTA-AM (Merck Millipore, Darmstadt, Germany), BAPTA-AM (Tocris Bioscience, Bristol, UK), Cmpd101 (Hello Bio, Princeton, NJ), and Gö6976 (BioNordika, Stockholm, Sweden) were dissolved in dimethyl sulfoxide (DMSO). KN93 (Sigma-Aldrich) was dissolved in water. Oocytes were preincubated at 12°C for >2 h under control conditions or in the respective ligands, dissolved in MBS solution. For BAPTA-AM and EGTA-AM, the MBS solution was nominally Ca2+-free. Efficient Ca2+ chelation was verified by the absence of Gq- mediated intracellular Ca2+ release. Preincubation of cells in 100 µM BAPTA-AM (blue trace) or 100 µM EGTA-AM (purple trace) abolished the currents. Data are displayed as mean ± SEM, normalized to the maximum current amplitude recorded in the dataset.

TMEM16A channels19,20 following MCH application to MCH2R-expressing oocytes (see Figure 1).

### 2.5 | Data analysis

Electrophysiology recordings were initially screened for inclusion in Clampfit 10.6 (Molecular Devices). Inclusion criteria were peaked agonist responses (characteristic of RGS4 expression; Ref. [15]) and stable holding currents at −40 mV before and after the −80 mV step. Holding currents after the −80 mV step were considered stable when they did not exceed the initial (before −80 mV) holding current and when they were not reduced by more than 15% of the maximal peak agonist response. In experiments where the response was evoked by 10 nM DA (a submaximally effective concentration), recordings with up to 38% reduction in holding current, relative to the peak agonist response, were accepted for inclusion. Recordings were peak normalized and time-averaged using Matrix Laboratory 2018b (MathWorks, Natick, MA, USA). Data points were represented as mean ± SEM. Current amplitudes were compared using the Mann–Whitney U-test, two-tailed, unpaired Student’s t-test, or one-way ANOVA with Dunnett’s test for multiple comparisons, as appropriate. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Data sets with n > 10 were tested for normality using the Shapiro–Wilk test and the Mann–Whitney U-test was
used in place of Student's *t*-test when normality could not be assumed.

3 | RESULTS

3.1 | β-arr2-mediated desensitization of D2R

Homologous desensitization of D_{28}R and D_{21}R was investigated by monitoring the Kir3 currents elicited by application of 1 µM DA for 420 s to oocytes co-expressing D_{28}R or D_{21}R with Kir3 channels, RGS4, and varying amounts of β-arr2. Co-expression of β-arr2 markedly increased the rate of Kir3 response decay following D_{28}R and D_{21}R activation by 1 µM DA, as measured by the residual response after 415 s of DA application (Figure 2A,B). The effect of Ca^{2+} chelation on D_{28}R desensitization was studied by preincubating oocytes co-injected with D_{28}R, RGS4, Kir3 subunits, and an intermediate effective amount of β-arr2 cRNA (1.9 ng/oocyte) with the membrane-permeable BAPTA-acetoxymethyl ester (AM), which is hydrolyzed to yield BAPTA in the cell cytoplasm. Instead of injecting BAPTA into the oocytes, we chose this ester prodrug approach in order to avoid unnecessary damage to the cell membrane. The pretreatment abolished Ca^{2+}-activated Cl^- currents normally elicited by activation of the G_{q/11}-coupled receptor, MCH_{2}R (Figure 1; Ref. [22]). However, there was no significant effect of BAPTA-AM pretreatment on D_{28}R desensitization rate, as measured by the remaining agonist-induced current at the end of the 415-s interval (Figure 2A,B). The peak currents decreased with increasing amounts of co-expressed β-arr2 (Figure 2C).

3.2 | GRK2 enhancement of β-arr2-mediated D_{2}R desensitization

GRK2 is known to increase β-arr2 recruitment to D_{2}R and to regulate DA autoreceptor function in vivo. We thus aimed to investigate the effect of GRK2 co-expression on β-arr2-mediated D_{2}R desensitization in order to address the putative role of this kinase in D_{2}R isoform-specific properties and its putative role in Ca^{2+}-sensitive desensitization.

First, D_{28}R and D_{21}R desensitization in the presence of β-arr2 was evaluated in oocytes co-injected with a range of different amounts of GRK2 cRNA. The amount of β-arr2 cRNA injected into each oocyte (1.9 ng) was selected to induce robust, but not complete, D_{2}R desensitization during the 415 s of DA application (see Figure 2). The rate and extent of desensitization of both D_{28}R- and D_{21}R-evoked Kir3 currents increased in a dosage-dependent manner with the amount of co-injected GRK2 cRNA (Figure 3A,B). In addition, greater amounts of co-injected GRK2 tended to decrease peak current amplitudes evoked by DA through both receptor isoforms (Figure 3C).

3.3 | D_{25}R desensitization is Ca^{2+}-dependent in the presence of GRK2

Next, we proceeded to assess the Ca^{2+} sensitivity of desensitization at the two D_{2}R isoforms in the presence of GRK2 by preincubating oocytes co-injected with cRNA encoding D_{25}R or D_{21}R, Kir3 subunits, RGS4, 1.9 ng β-arr2, and 0.5 ng GRK2 with either BAPTA-AM and EGTA-AM. Similarly to BAPTA-AM, EGTA-AM is converted into EGTA in the cell cytoplasm and efficiently chelated Ca^{2+} under the conditions used, as verified by the absence of MCH-evoked Ca^{2+}-activated Cl^- currents in oocytes expressing MCH_{2}R (Figure 1). Under control conditions, DA-evoked Kir3 currents elicited via D_{25}R desensitized nearly completely (98% on average) during the 415-s interval and pre-incubation in EGTA-AM (100 µM) was virtually without effect on the extent of desensitization. BAPTA-AM, however, significantly reduced desensitization such that the terminal Kir3 current was on average 86% less than its initial value (Figure 4A,B). In contrast, when D_{21}R was expressed instead of D_{25}R, neither EGTA-AM- nor BAPTA-AM-preincubated cells demonstrated any significant differences in desensitization compared to untreated control cells (Figure 4A,B). Desensitization of D_{25}R-mediated Kir3 responses was sensitive to BAPTA-AM treatment also when the receptor was activated by a lower, submaximally effective concentration of DA (10 nM; Figure 5).

Pre-incubation with the GRK2 inhibitor, Cmpd101 (100 µM; Pack et al.), reduced the desensitization of D_{25}R to levels similar to those observed with BAPTA-AM, whereas no effect of Cmpd101 was observed at D_{21}R (Figure 4A,B). Peak Kir3 current amplitudes were increased for D_{25}R-expressing oocytes incubated in BAPTA-AM, compared to untreated oocytes (Figure 4C). Similarly, when WT GRK2 was replaced with a kinase-dead GRK2 mutant, K220R, or with βARK-ct; a truncated protein lacking the kinase domain but retaining the C-terminal, Gβγ-binding Pleckstrin Homology domain of this kinase, D_{25}R desensitization was insensitive to BAPTA-AM (Figure 6). The fast desensitization of receptor-evoked Kir3 currents imparted by co-expression of WT GRK2 in the absence of β-arr2 was also unaffected by preincubation with BAPTA-AM, nor was it modified by expression of GRK2 K220R or βARK-ct in place of WT GRK2 (Figure 7). Finally, we attempted
to investigate whether the constitutive activity of D$_{28}$R was modulated by coexpression of β-arr2 and GRK2. However, the response of the baseline (i.e., agonist-independent) current to haloperidol (1 µM), which is an inverse agonist at the D$_2$R, was negligible and did not differ significantly between oocytes expressing D$_{28}$R, RGS4, and Kir3 subunits alone, or in the presence of β-arr2 and GRK2 (Figure 8).
Desensitization of $D_2S$R-mediated Kir3 responses is not modulated by PKC or calmodulin

$Ca^{2+}$-dependent isoforms of protein kinase-C (PKC) have previously been proposed to modulate $D_2$R desensitization. In addition, PKC modulates GRK2 activity via phosphorylation of a serine residue, S29, such as to increase GRK2 catalytic activity. To investigate whether BAPTA-AM-induced $Ca^{2+}$ chelation reduces $D_2S$R desensitization due to decreased PKC-mediated GRK2 phosphorylation, we co-expressed a PKC-insensitive GRK2-S29A mutant in oocytes together with $D_2S$R, RGS4, Kir3 subunits, and $\beta$-arr2 and assessed the $Ca^{2+}$ sensitivity of desensitization (Figure 9). The extent of desensitization observed with this mutant was similar to that observed in control oocytes expressing WT GRK2. Furthermore, preincubation in BAPTA-AM significantly reduced the rate of desensitization of the response to 1 $\mu$M DA, again similar to control oocytes (Figure 9; cf. Figure 4).

Previously, effects of $Ca^{2+}$-dependent PKC subtypes $\alpha$ and $\beta_1$ have been studied using Gö6976, an inhibitor specific to these isozymes. However, preincubation of oocytes with Gö6976 (100 $\mu$M) did not affect the extent of desensitization of DA responses evoked via $D_2S$R (Figure 9). Preincubation with 100 $\mu$M KN93, a $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) inhibitor acting at calmodulin, was also without significant effect (Figure 9).

4 | DISCUSSION

This study reproduced and extended on previous findings by Gantz et al. of $Ca^{2+}$-dependent desensitization of $D_2S$R but not $D_2L$R-mediated Kir3 responses. Whereas the original observations were made in native DA neurons, the present investigation employed a heterologous expression system, which allowed us to identify some key aspects of this isofrom-specific feature. Notably, whereas $\beta$-arr2 and GRK2 expression increased the rate of desensitization of DA-evoked Kir3 response in a dosage-dependent manner for both $D_2S$R and $D_2L$R, $Ca^{2+}$-dependent desensitization was observed only for $D_2S$R and required co-expression of both $\beta$-arr2 and GRK2.
Supporting a role for GRK2-mediated phosphorylation in Ca^2+ dependent D_{2S}R desensitization, the GRK2 kinase inhibitor, Cmpd101, reduced D_{2S}R desensitization to levels similar to those observed in oocytes pretreated with BAPTA-AM. In further agreement, in oocytes expressing β-arr2 together with the kinase-dead GRK2 mutant, K220R,^{26} or the C-terminal GRK2 domain responsible for Gβγ sequestration,^{14} desensitization of Kir3 currents evoked by D_{2S}R was insensitive to BAPTA-AM pretreatment. While GRK2 co-expression clearly increased the rates of β-arr2-mediated desensitization of both D_{2S}R and D_{2L}R, Cmpd101 had no significant effect on desensitization rate in D_{2L}R-expressing cells, suggesting that the enhancement of the rate of D_{2L}R desensitization observed upon co-expression of GRK2 with β-arr2, as compared to β-arr2 alone, may not be dependent on GRK2 kinase activity. This is in agreement with observations from mammalian cells suggesting that GRK2-mediated enhancement of D_{2S}R β-arr2 recruitment does not require receptor phosphorylation^{36,37} although other recent studies have come to different conclusions.^{38} In oocytes expressing D_{2S}R and WT GRK2, but lacking β-arr2, desensitization of DA-evoked Kir3 currents was also unaffected by Cmpd101, again in agreement with earlier reports that the rapid desensitization of Kir3 currents mediated by GRK2 is independent of its kinase activity but rather due to Gβγ sequestration.^{27}

The phosphorylation site targeted by GRK2 to mediate the Ca^2+ -sensitive desensitization of D_{2S}R remains unknown. Although D_{2S}R contains all the phosphorylatable residues found in D_{2L}R, the distinct behavior of the two isoforms could putatively be explained by
FIGURE 5  BAPTA-AM pretreatment reduces desensitization of D_{2S}R responses elicited by 10 nM DA. (A) Peak-normalized and averaged traces of D_{2S}R-mediated Kir3 current responses to 10 nM DA in oocytes injected with 1.9 ng β-arr2 and 0.5 ng GRK2 K220R cRNA. (B) Residual Kir3 responses (fraction of initial peak response) after 415 s of DA (10 nM) application, under control conditions (white bars) and following preincubation in BAPTA-AM (100 µM; blue bars). (C) Peak DA-evoked currents for the conditions shown in A. BAPTA-AM pretreatment significantly reduced the extent of desensitization (**p = .006; unpaired, two-tailed Student’s t-test), but did not significantly affect peak current amplitudes (p > .05; unpaired, two-tailed Student’s t-test). Data are displayed as mean ± SEM. In the bar graphs, superimposed dots represent individual data points. The number of individual experiments is shown under each bar.

FIGURE 6  BAPTA-AM has no significant effect on desensitization of D_{2S}R responses when kinase-dead GRK2 K220R mutant or βARK-ct is expressed in place of WT GRK2. (A) Peak-normalized and averaged traces of D_{2S}R-evoked Kir3 currents in oocytes injected with 1.9 ng β-arr2 and 0.5 ng GRK2 K220R cRNA (left panel), and D_{2S}R-evoked Kir3 currents in oocytes injected with 1.9 ng β-arr2 and 0.5 ng βARK-ct cRNA (right panel). (B) Residual Kir3 responses (fraction of initial peak response) after 415 s of DA (1 µM) application, under control conditions (white bars) and following preincubation in BAPTA-AM (100 µM; blue bars). (C) Peak DA-evoked currents for the conditions shown in A. BAPTA-AM pretreatment did not significantly alter the extent of desensitization, nor peak current amplitudes, neither in cells co-expressing GRK2 K220R nor βARK-ct (p > .05; unpaired, two-tailed Student’s t-test). Data are displayed as mean ± SEM. In the bar graphs, superimposed dots represent individual data points. The number of individual experiments is shown under each bar.
conformational differences in the third intracellular loop which would make phosphorylation of a critical residue unfavorable in D2S. Alternatively, a distinct protein related to D2R desensitization, such as β-arr2 itself, could become phosphorylated by GRK2 such as to increase β-arr2 affinity for D2S, but not D2L. Indeed, arrestins have been described to be phosphorylated both by GRKs and by other kinases. Finally, another possibility is that only D2S is able to elicit a downstream Ca\(^{2+}\) response which in turn regulates GRK2 activity. In *Xenopus* oocytes, β-arr2-mediated desensitization of Kir3 currents evoked by GPCRs (including D2R) has been reported not be associated with receptor internalization, suggesting that the desensitization observed here is due to functional uncoupling of the receptor from its G protein.

Due to their differential Ca\(^{2+}\) binding kinetics, EGTA and BAPTA have been used to estimate the proximity of Ca\(^{2+}\) release to its site of action. Thus, signaling events sensitive to BAPTA but not to EGTA are considered to take place within Ca\(^{2+}\) nanodomains, whereas processes sensitive to both chelators have been postulated to reside within larger Ca\(^{2+}\) microdomains. Thus, the present results, and those of Ref. [9], may suggest a spatially restricted nanodomain-type coupling between the Ca\(^{2+}\) release site and the putative Ca\(^{2+}\) binding site relevant for D2R isoform-specific desensitization. However, the exact mechanism behind the Ca\(^{2+}\) sensitivity of GRK2-enhanced desensitization remains elusive. Alanine substitution of the GRK2 S29, a residue phosphorylated by Ca\(^{2+}\)-sensitive PKC isoforms, to enhance GRK2 activity, did not affect the rate of D2S desensitization and did not prevent BAPTA-AM from decreasing the rate of desensitization. Neither did pharmacological inhibition of PKC and calmodulin by incubation with Gö6976 and KN93, respectively, affect D2S desensitization. Thus, the higher
sensitivity of the short isoform to PKC-mediated desensitization, as previously reported, does not appear to underlie the isoform-specific Ca²⁺ sensitivity observed here.

GRKs are crucial for regulation of GPCR signaling and associated physiology, and changes in GRK expression is relevant to the pathogenesis of a range of conditions, including substance abuse, heart failure, and pain. Interestingly, selective deletion of GRK2 in D₂R-expressing neurons in mice decreased acute cocaine-induced DA release and hyperlocomotion as well as sensitization of DA release to repeated cocaine administration. While a previous investigation reported that the closely related GRK3 did not affect β-arr2-mediated desensitization of D₂R-evoked Kir3 currents in Xenopus oocytes, the present findings are in agreements with reports from other expression systems, as well as native tissue, supporting a role for GRK2 in potentiating β-arr2 recruitment to D₂R. The discrepancy between the present study and that of Celver et al. may be due to the different GRK isoforms used and the fact that we tested several different amounts of GRK2 cRNA combined with an intermediately effective amount of β-arr2 cRNA, thus increasing the sensitivity of our assay to detect GRK-mediated enhancement of desensitization. Conversely, another report found that GRK co-expression was essential for β-arr2-mediated desensitization of Kir3 currents elicited by the delta opioid receptor and β2 adrenergic receptor. These differences might reflect the differential dependence of β-arr2 recruitment on phosphorylation at various GPCRs. In this context, it is interesting to note that, compared to other members of the arrestin family, β-arr2 is the least selective, showing high affinity for a
wide range of GPCRs and also displaying a lower preference for phosphorylated over unphosphorylated receptors, which would be in agreement with the prominent β-arr2-mediated desensitization of D2R-mediated Kir3 responses reported here and in previous work, even in the absence of GRK2.

The *Xenopus* oocytes used in the present investigation constitute a heterologous expression system and as such, come with several limitations. DA neurons, where D2R autoreceptors are natively expressed, contain numerous proteins interacting with- and regulating D2R signaling, and it is likely that many of these interactors are not present in oocytes. This fact might, for example, explain differences in the rates of desensitization observed here as compared to other studies. Despite this, the oocytes provide minimal interference with Kir3 current measurements thanks to their low expression of endogenous ion channels and receptors, which facilitates the study of exogenous proteins. Due to their large size, oocytes can be injected individually with a defined stoichiometry of several different cRNAs. Moreover, they provide a cellular background free of detectable GRKs and arrestins.

The last two factors were decisive in the present investigation, where we studied the dosage-dependence both of β-arr2-mediated desensitization at D2L and D2S, and of its GRK2-mediated enhancement, by co-injection of different amounts of β-arr2 and GRK2 cRNA.

In summary, our results suggest that desensitization of D2S, but not D2L, is Ca2+-dependent in the simultaneous presence of GRK2 and β-arr2. This Ca2+ sensitivity further requires kinase activity of GRK2, since it was blocked by the GRK2-specific kinase inhibitor, Cmpd101, and was absent in cells expressing the kinase-dead mutant, K220R, in place of WT GRK2. Thus, the plasticity-dependent change in Ca2+ sensitivity of D2S autoreceptors previously observed in DA neurons may be a result of altered expression or function of GRK2 in these neurons.

**ACKNOWLEDGMENTS**

This study was supported by grants from Stiftelsen Lars Hiertas Minne (grant number FO2016-0434; to KS and FO2020-0289; to RÅ), Åhlén-stiftelsen (grant number 2018-02980; to KS) and Magnus Bergvalls stiftelse (grant number 2018-02980; to KS and 2020-04055; to RÅ). KS is currently a fellow at the Wallenberg Center for Molecular Medicine at Umeå University. RÅ is funded by Region Stockholm. RÅ and KS participate in the European COST Action CA18133 (ERNEST).

**DISCLOSURES**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**AUTHOR CONTRIBUTIONS**

Richard Ågren and Kristoffer Sahlholm designed research; Richard Ågren and Kristoffer Sahlholm analyzed data; Richard Ågren performed research; Richard Ågren and Kristoffer Sahlholm wrote the paper.

**ORCID**

Kristoffer Sahlholm https://orcid.org/0000-0001-6536-1972

**REFERENCES**

1. Beaulieu JM, Espinoza S, Gainetdinov RR. Dopamine receptors—JUPHAR Review 13. *Br J Pharmacol*. 2015;172:1-23.
2. Khan ZU, Mrzljak L, Gutierrez A, de la Calle A, Goldman-Rakic PS. Prominence of the dopamine D2 short isoform in dopaminergic pathways. *Proc Natl Acad Sci USA*. 1998;95: 7731-7736.
3. Usiello A, Baik J-H, Rougé-Pont F, et al. Distinct functions of the two isoforms of dopamine D2 receptors. *Nature*. 2000;408:199-203.
4. Xu R, Hranilovic D, Fetsko LA, Bucan M, Wang Y. Dopamine D2S and D2L receptors may differentially contribute to the actions of antipsychotic and psychotic agents in mice. *Mol Psychiatry*. 2002;7:1075-1082.
5. Neve KA, Ford CP, Buck DC, Grandy DK, Neve RL, Phillips TJ. Normalizing dopamine D2 receptor-mediated responses in D2 null mutant mice by virus-mediated receptor restoration: comparing D2L and D2S. *Neuroscience*. 2013;248:479-487.
6. Dragicevic E, Poetschke C, Duda J, et al. Cav1.3 channels control D2-autoreceptor responses via NCS-1 in substantia nigra dopamine neurons. *Brain*. 2014;137:2287-2302.
7. Jonghe C, Tiberi M, Trudeau LE. Expression of D2 receptor isoforms in cultured neurons reveals equipotent autoreceptor function. *Neuropsychopharmacology*. 2006;50:595-605.
8. Radl D, Chiacchiaretta M, Lewis KG, Brami-Cherrier K, Arcuri L, Borrelli E. Differential regulation of striatal motor behavior and related cellular responses by dopamine D2L and D2S isoforms. *Proc Natl Acad Sci U S A*. 2018;115:198-203.
9. Gantz SC, Robinson BG, Buck DC, et al. Distinct regulation of dopamine D2S and D2L autoreceptor signaling by calcium. *eLife*. 2015;4:e09358.
10. Robinson BG, Condon AF, Radl D, Borrelli E, Williams JT, Neve KA. Cocaine-induced adaptation of dopamine D2S, but not D2L autoreceptors. *eLife*. 2017;6:e31924.
11. Agren R, Arhem P, Nilsson J, Sahlholm K. The beta-arrestin-biased dopamine D2 receptor ligand, UNC9994, is a partial agonist at G-protein-mediated potassium channel activation. *Int J Neuropsychopharmacol*. 2018;21:1102-1108.
12. Celver J, Sharma M, Thanawala V, Christopher Octeau J, Kovoor A. Arrestin-dependent but G-protein coupled receptor kinase-independent uncoupling of D2-dopamine receptors. *J Neurochem*. 2013;127:57-65.
13. Daigle TL, Ferris MJ, Gainetdinov RR, et al. Selective deletion of GRK2 alters psychostimulant-induced behaviors and dopamine neurotransmission. *Neuropsychopharmacology*. 2014;39:2450-2462.
14. Koch WJ, Hawes BE, Inglese J, Luttrell LM, Lefkowitz RJ. Cellular expression of the carboxyl terminus of a G
protein-coupled receptor kinase attenuates G beta-gamma-mediated signaling. J Biol Chem. 1994;269:6193-6197.

15. Doupnik CA, Davidson N, Lester HA, Kofuji P. RGS proteins reconstitute the rapid gating kinetics of Gβγ-activated inwardly rectifying K+ channels. Proc Natl Acad Sci. 1997;94:10461.

16. Hoekstra EJ, van Oerthel L, van der Linden AJ, et al. Lmx1a is an activator of Rgs4 and Grb10 and is responsible for the correct specification of rostral and medial mdDA neurons. Eur J Neurosci. 2013;37:23-32.

17. Sahlholm K, Barchad-Avitzur O, Marcellino D, et al. Agonist-specific voltage sensitivity at the dopamine D2S receptor—molecular determinants and relevance to therapeutic ligands. Neuropharmacology. 2011;61:937-949.

18. Ägren R, Sahlholm K. Voltage-dependent dopamine potency at D1-like dopamine receptors. Front Pharmacol. 2020;11:581151. https://doi.org/10.3389/fphar.2020.581151

19. Schroeder BC, Cheng T, Jan YN, Jan LY. Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. Cell. 2008;134:1019-1029.

20. Weiss S, Keren-Raifman T, Oz S, Ben Mocha A, Haase H, Dascal N. Modulation of distinct isoforms of L-type calcium channels by G(q)-coupled receptors in Xenopus oocytes: antagonistic effects of Gbetagamma and protein kinase C. Channels. 2012;6:426-437.

21. Tymianski M, Charlton MP, Carlen PL, Tator CH. Properties of nonprotective cell-permeant Ca2+ chelators: effects on [Ca2+]i and glutamate neurotoxicity in vitro. J Neurophysiol. 1994;72:1973-1992.

22. Hawes BE, Kil E, Green B, O'Neill K, Fried S, Graziano MP. The melanin-concentrating hormone receptor couples to multiple G proteins to activate diverse intracellular signaling pathways. Endocrinology. 2000;141:4524-4532.

23. Urs NM, Gee SM, Pack TF, et al. Distinct cortical and striatal actions of a β-arrestin–biased dopamine D2 receptor ligand reveal unique antipsychotic-like properties. Proc Nat Acad Sci. 2016;113(50):E8178-E8186

24. Stepniewski TM, Mancini A, Ägren R, et al. Mechanistic insights into dopaminergic and serotonergic neurotransmission—concerted interactions with helices 5 and 6 drive the functional outcome. Chem Sci. 2021;12:10990-11003.

25. Pack TF, Orlen MI, Ray C, Peterson SM, Caron MG. The dopamine D2 receptor can directly recruit and activate GRK2 without G protein activation. J Biol Chem. 2018;293:6161-6171.

26. Kong G, Penn R, Benovic JL. A beta-adrenergic receptor kinase dominant negative mutant attenuates desensitization of the beta 2-adrenergic receptor. J Biol Chem. 1994;269:13084-13087.

27. Raveh A, Cooper A, Guy-David L, Reuvény E. Nonenzymatic rapid control of GIRK channel function by a G protein–coupled receptor kinase. Cell. 2010;143:750-760.

28. He D, Lasek AW. Anaplastic lymphoma kinase regulates internalization of the dopamine D2 receptor. Mol Pharmacol. 2019;97:123-131.

29. Namkung Y, Sibley DR. Protein kinase C mediates phosphorylation, desensitization, and trafficking of the D2 dopamine receptor. J Biol Chem. 2004;279:49533-49541.

30. Morris SJ, Van H II, Daigle M, Robillard L, Sajedi N, Albert PR. Differential desensitization of dopamine D2 receptor isoforms by protein kinase C: the importance of receptor phosphorylation and pseudosubstrate sites. Eur J Pharmacol. 2007;574:44-53.

32. Krasel C, Dammeier S, Winstel R, Brockmann J, Mischak H, Lohse MJ. Phosphorylation of GRK2 by protein kinase C abolishes its inhibition by calmodulin. J Biol Chem. 2001;276:1911-1915.

33. Martiny-Baron G, Kazarinetz MG, Mischak H, et al. Selective inhibition of protein kinase C isoforms by the indolocarbazole Go 6976. J Biol Chem. 1993;268:9194-9197.

34. Sumi M, Kiuchi K, Ishikawa T, et al. The newly synthesized selective Ca2+/-calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. Biochem Biophys Res Commun. 1991;181:968-975.

35. Wong MH, Samal AB, Lee M, et al. The KN-93 molecule inhibits calcium/calmodulin-dependent protein kinase II (CaMKII) activity by binding to Ca(2+)/CaM. J Mol Biol. 2019;431:1440-1459.

36. Namkung Y, Dipace C, Urizar E, Javitch JA, Sibley DR. G protein-coupled receptor kinase-2 constitutively regulates D2 dopamine receptor expression and signaling independently of receptor phosphorylation. J Biol Chem. 2009;284:34103-34115.

37. Sanchez-Soto M, Schildkan IA, Inbody L, Free RB, Sibley DR. G protein-coupled receptor kinase 2 can enhance beta-arrestin recruitment to the D2 dopamine receptor in the absence of receptor phosphorylation. FASEB J. 2020;34(S1):1. https://doi. org/10.1096/fasebj.2020.34.s1.03478

38. Mann A, Keen AC, Mark H, et al. New phosphosite-specific antibodies to unravel the role of GRK phosphorylation in dopamine D2 receptor regulation and signaling. Sci Rep. 2021;11:8288. https://doi.org/10.1038/s41598-021-87417-2

39. Delom F, Fessart D. Role of phosphorylation in the control of Clathrin-mediated internalization of GPCR. Int J Cell Biol. 2011;2011:246954.

40. Cassier E, Gallay N, Bourquard T, et al. Phosphorylation of beta-arrestin2 at Thr(383) by MEK underlies beta-arrestin-dependent activation of Erk1/2 by GPCRs. eLife. 2017;6:e23777. https://doi.org/10.7554/eLife.23777

41. Augustine GJ, Santamaria F, Tanaka K. Local calcium signaling in neurons. Neuron. 2003;40:331-346.

42. Rengo G, Lymeropoulos A, Leosco D, Koch WJ. GRK2 as a novel gene therapy target in heart failure. J Mol Cell Cardiol. 2011;50:785-792.

43. Abraham AD, Schattauer SS, Reichard KL, et al. Estrogen regulation of GRK2 inactivates kappa opioid receptor signaling mediating analgesia, but not aversion. J Neurosci. 2018;38:8031-8043.

44. Celver J, Vishnivetskiy SA, Chavkin C, Gurevich VV. Conservation of the phosphate-sensitive elements in the arrestin family of proteins. J Biol Chem. 2002;277:9043-9048.

45. Zhan X, Gimenez LE, Gurevich VV, Spiller BW. Crystal structure of arrestin-3 reveals the basis of the difference in receptor binding between two non-visual subtypes. J Mol Biol. 2011;406:467-478.

How to cite this article: Ägren R, Sahlholm K. G protein-coupled receptor kinase-2 confers isof orm-specific calcium sensitivity to dopamine D2 receptor desensitization. FASEB J. 2021;35:e22013. doi:10.1096/fj.202100704RR