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**Desensitization of the Dopamine D1 and D2 Receptor Heterooligomer Mediated
Calcium Signal by Agonist Occupancy of Either Receptor**

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ABBREVIATIONS

GPCR, G protein coupled receptor; GRK, G protein coupled receptor kinase; HA, hemagglutinin; HEK, human embryonic kidney; MEM, Minimum essential medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H89, N-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; KN93, N-[2-[[[3-(4'-Chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4'-methoxybenzenesulfonamide phosphate salt; D4476, 4-(4-(2,3-Dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-2-yl)benzamide; TBB, 4,5,6,7-Tetrabromo-2-azabenzimidazole; dbcAMP, dibutyryl-cAMP; PMA, phorbol-12-myristate-13 acetate; ADP β S, Adenosine-5'-0-(2-thiodiphosphate) trilithium salt; EGTA, Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; SKF81297, (\pm)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA; SERCA, sarcoplasmic endoplasmic Ca²⁺ ATPase; PKA, protein kinase A; PKC, protein kinase C; Bis, bisindolylmaleimide I; AFU, absolute fluorescence units; SQ22536, 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine; PTX, pertussis toxin.

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ABSTRACT

When dopamine D1 and D2 receptors were coactivated in D1-D2 receptor heterooligomeric complexes, a novel phospholipase C mediated calcium signal was generated. In this report, desensitization of this Gq/11-mediated calcium signal was demonstrated by pretreatment with dopamine or with D1 selective agonist SKF81297 or D2 selective agonist quinpirole. Desensitization of the calcium signal mediated by D1-D2 receptor heterooligomers was initiated by agonist occupancy of either receptor subtype even though the signal was generated only by occupancy of both receptors. The efficacy, potency and rate of calcium signal desensitization by agonist occupancy of the D1 receptor ($t_{1/2}$ ~1 minute) was far greater than by the D2 receptor ($t_{1/2}$ ~10 minutes). Desensitization of the calcium signal was not mediated by depletion of calcium stores or internalization of the heterooligomer and was not decreased by inhibiting second messenger activated kinases. The involvement of G protein coupled receptor kinases 2 or 3, not 5 or 6, in the desensitization of the calcium signal was shown, occurring through a phosphorylation independent mechanism. Inhibiting Gi protein function associated with D2 receptors increased D1 receptor mediated desensitization of the calcium signal, suggesting that cross-talk between the signals mediated by the activation of different G proteins controlled the efficacy of calcium signal desensitization. Together, these results demonstrated the desensitization of a signal mediated only by heterooligomerization of two G protein coupled receptors that was initiated by agonist occupancy of either receptor within the heterooligomer, albeit with differences in desensitization profiles observed.

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Dopamine receptors are members of the G protein coupled receptor (GPCR) superfamily and play important roles in neuronal transmission and signal transduction. The five dopamine receptors, like most GPCRs, have been demonstrated to exist as homooligomers (George et al., 1998; Lee et al., 2000; O'Dowd et al., 2005) and heterooligomers (So et al., 2005). Heterooligomerization may account for some of the observed synergism between D1 and D2 receptors since D1 and D2 receptors have been demonstrated to be coexpressed in neurons (Aizman et al., 2000; Maltais et al.; Surmeier et al., 1992). Our observations of D1 and D2 receptors occurring within the same signaling complexes were determined by coimmunoprecipitation from rat brain (Lee et al., 2004). In heterologous cells coexpressing both receptors, the existence of heterooligomers was established by fluorescence resonance energy transfer (So et al., 2005), cotrafficking studies (So et al., 2005) and visualization of D1-D2 heterooligomers in live cells (O'Dowd et al., 2005). Of greater physiological significance was the finding that activation of a novel phospholipase C- mediated calcium signal was observed only when both D1 and D2 receptors within heterooligomeric complexes were coactivated and were not observed when D1 and D2 receptors were expressed alone (Lee et al., 2004). These heterooligomeric complexes were observed in brain, resulting in novel activation of Gq/11 proteins (Rashid et al., 2007). The generation of this unique signal only by heterooligomerization between these two receptors provides an example of how diversity of receptor function can be achieved from a limited number of receptors and represents the first example within the rhodopsin family of GPCRs to elicit such an effect. The discovery of this dopamine mediated calcium signal is important as it implicates a direct link between dopamine receptors, generally considered to function largely by modulating

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adenylyl cyclase activation, to calcium generation. Since these D1-D2 receptor complexes are linked to calcium signaling, it is of major significance then to determine how this signal is regulated.

An important component of GPCR signaling is the termination of the signal by receptor desensitization. This process has been described to occur *in vivo* and is an important process by which neurons regulate and turn off signaling (Gainetdinov et al., 2004). All of the research on receptor desensitization, however, has been performed on signals generated by receptor homooligomers. Whether the mechanisms established for homooligomers are similar for heterooligomers remain to be elucidated.

In this study, we investigated the desensitization of the calcium signal associated with agonist activation of D1-D2 receptor heterooligomers. We provide evidence for rapid desensitization elicited by pretreatment with dopamine or with selective D1 or D2 receptor agonists. The efficacy, potency and rate of signal desensitization differed between agonists that selectively occupied the D1 or D2 receptors or both receptors simultaneously. A role was shown for G protein coupled receptor kinases (GRK) 2 or 3, but GRK5, GRK6, receptor internalization or any other second messenger activated kinases were not involved in the desensitization of the signal.

MATERIALS AND METHODS

Measurement of the Calcium Signal— Stable cell lines coexpressing the amino terminus HA epitope-tagged human D1 receptor and amino terminus FLAG epitope-tagged human D2 receptor were created in HEK293TSA cells utilizing the pBudCE 4.1 vector (Invitrogen, Carlsbad, CA) and was characterized previously (Lee et al., 2004). All cell

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culture and transfection reagents were obtained from Invitrogen. HEK293TSA cells were maintained as monolayer cultures at 37 °C in advanced minimum essential medium (MEM) supplemented with 6% fetal bovine serum, 300 µg/ml zeocin (Invitrogen) and antibiotics. Calcium mobilization assays were carried out using a FLEXstation multiwell plate fluorometer (Molecular Devices, Sunnyvale, CA). Stably transfected cells were seeded in black microtiter plates at a density of 100,000 cells/well and grown for 24 hours. The cells were then loaded with 2 µM Fluo-4AM indicator dye (Invitrogen) in advanced minimum essential medium supplemented with 2.5 mM probenecid (Sigma Aldrich, Oakville, ONT, CA) for 1 hour and subsequently washed twice with Hanks' balanced salt solution (HBSS) without sodium bicarbonate and phenol red and supplemented with 20 mM HEPES (Invitrogen). Baseline fluorescence values were measured for 15 seconds, and changes in fluorescence corresponding to alterations in intracellular calcium levels upon the addition of agonists (indicated on each calcium tracing by a downward arrow) thereafter were recorded. Fluorescence values were collected at 3-second intervals for 100 s. For calculation of concentration response curves, the difference between maximum and minimum fluorescence values for each agonist concentration was determined and analyzed using Prism software (GraphPad, San Diego, CA). For desensitization studies, cells were pretreated with agonists in serum free advanced MEM for the time indicated and washed off with HBSS prior to calcium measurement. For inhibitor studies, cells were pretreated with 10 µM H89 (Sigma), 500 nM bisindolylmaleimide I, 10 µM KN93, 1 µM D4476 or 1 µM TBB (Calbiochem, San Diego, CA) 1 hour prior to agonist pretreatment. To block receptor internalization, 250 µg/ml concanavalin A (Calbiochem) was coadministered with agonists tested. For protein

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kinase activator studies, cells were pretreated with 3 mM dibutyryl-cAMP (dbcAMP) (Calbiochem) for 15 minutes or with 1 μ M phorbol-12-myristate-13-acetate (PMA) (Calbiochem) for 30 minutes prior to calcium measurement. SKF81297, dopamine, quinpirole, SCH 23390, raclopride, ADP β S, EGTA, SQ22536 and pertussis toxin were purchased from Sigma. For studies involving G protein coupled receptor kinases, transient transfections of cDNA encoding GRK2, GRK3, GRK5 or GRK6 in the mammalian expression vector pcDNA3 (Invitrogen) were performed with Lipofectamine (Invitrogen). The GRK constructs were a kind gift from Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA). YM254890 was kindly provided by Astellas Pharma Inc (Tokyo, Japan).

Small Interfering RNA (siRNA) Silencing of Gene Expression— Chemically synthesized double-stranded siRNA duplexes (with 3' dTdT overhangs) were purchased from Qiagen Incorporation (Mississauga, Ontario) for GRK2 (5'-AAGAAGUACGAGAAGCUGAG-3') and GRK3 (5'-AAGCAAGCUGUAGAACACGUA-3') and were validated elsewhere (Violin et al., 2006). A nonsilencing RNA duplex (5'-UUCUCCGAACGUGUCACGU-3') was used as a control for all siRNA experiments. HEK-293TSA cells were transfected with Lipofectamine 2000 (Invitrogen), according to manufacturers' instructions.

Silencing was quantified by immunoblotting.

Cell Surface Immunofluorometry- Cells stably expressing D1 and D2 receptors were plated in 96-well clear bottomed plates (Corning Glassworks, Corning, NY) at a confluence of 150,000 cells per well 24 hours prior to the experiment. Cells were treated for 30 minutes with agonist, fixed by 4% paraformaldehyde, blocked with 4% bovine serum albumin in PBS, incubated with 1:200 dilution of anti-HA (Roche) or 1:1000

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dilution of anti-FLAG (Invitrogen) antibodies for 1 hour, washed with PBS, and then labeled with a secondary antibody conjugated with fluorescein isothiocyanate (Invitrogen). Fluorescence was detected with a spectrophotometer (Cytofluor 4400; Applied Biosystems, Foster City, CA).

[³H]cAMP accumulation- [³H]cAMP accumulation in cells stably expressing D1 receptors were determined as described previously (Hasbi et al., 1998). Briefly, cells were seeded in 24-well plates at a density of 300,000 cells per well and incubated overnight in a culture medium supplemented with 0.6 μ Ci [³H]adenine (final volume, 300 μ l). Cells were then washed once with DMEM/20 mM HEPES, pretreated with 1 μ M dopamine for 30 minutes and washed off. [³H]cAMP accumulation was measured in the presence of 1 mM isobutylmethylxanthine and 10 μ M dopamine for 5 minutes at 37°C. The reaction was stopped by addition of 250 μ L 5% trichloroacetic acid and the separation of [³H]cAMP was carried out by chromatography.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting- The procedures used for protein gel electrophoresis and immunoblotting were identical to those described previously (So et al., 2005). Rabbit anti-GRK2, -GRK3, -GRK5 and -GRK6 antibodies were obtained (Santa Cruz Biotechnology Inc. Santa Cruz, CA) and used at a 1:200 dilution.

RESULTS

To study calcium signal desensitization, a stable cell line coexpressing D1 and D2 receptors (Lee et al., 2004) (D1=2071 \pm 60 fmol/mg; D2= 2449 \pm 90 fmol/mg) was pretreated for 30 minutes with either dopamine, the D1 receptor selective agonist

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SKF81297 or the D2 receptor selective agonist quinpirole. The ability of 1 μ M dopamine, SKF81297 and quinpirole to activate the calcium signal is shown (Fig. 1A). The addition of dopamine stimulated a robust calcium signal which peaked within seconds of agonist activation and declined in less than 2 minutes. SKF81297 elicited a calcium signal which also peaked within seconds of agonist activation but achieved a peak value 50% of that elicited by dopamine. The addition of quinpirole did not elicit a calcium signal. Coadministration of SKF81297 and quinpirole generated a signal of the same magnitude as dopamine (data not shown). The calcium signal was Gq/11-protein linked, as the addition of 10 nM Gq/11 inhibitor YM254890 (Takasaki et al., 2004) completely abolished the dopamine activated signal (Fig. 1B). The coactivation of D1 and D2 receptors mobilized calcium release from intracellular calcium stores since the signal was maintained in the presence of 250 μ M calcium chelator EGTA (Fig. 1C). When cells were pretreated for 30 minutes with 1 μ M dopamine and then washed off, the signal activated subsequently by 10 μ M dopamine was attenuated compared to control (62.3 \pm 3.7% of control, n=4, p<0.05) with similar kinetics of the calcium signal compared to control (Fig. 1D). To characterize this effect further, cells pretreated with 1 μ M dopamine were activated with increasing concentrations of dopamine from 10⁻¹¹ to 10⁻⁵ M. No significant change in the EC₅₀ compared to control was observed in this concentration response experiment (control EC₅₀= 34.0 \pm 4.1 nM, n=4; treated EC₅₀= 39.4 \pm 8.3 nM, n=4, p>0.05) (Fig. 1E) with a 30% decrease in maximal activation. To determine if desensitization of the D1-D2 receptor mediated signal could be achieved by heterologous desensitization resulting from the activation of another Gq/11-linked receptor, the endogenously expressed purinergic P2Y1 receptors were activated with

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agonist ADP β S at 1 μ M for 30 minutes in D1-D2 receptor expressing cells (Fig. 1F). The P2Y1 receptor calcium signal activated by 10 μ M ADP β S was significantly desensitized by pretreatment with ADP β S but no attenuation of the D1-D2 receptor mediated signal was observed following stimulation of P2Y1R, suggesting that the desensitization of the D1-D2 receptor complex occurred specifically by a homologous mechanism.

Since D1 and D2 receptor homooligomers have been previously demonstrated to desensitize in response to their receptor selective agonists (Lamey et al., 2002; Ng et al., 1997), we examined the effect of selective D1 or D2 receptor agonists on calcium signal desensitization (Fig. 2). Pretreatment for 30 minutes with 1 μ M SKF81297 displayed desensitization that was significantly greater than that elicited by dopamine pretreatment ($37.1 \pm 7.1\%$ of control, $n=4$, $p < 0.05$) (Fig. 2A). Activating the signal after SKF81297 pretreatment with increasing concentrations of dopamine demonstrated no change in the EC_{50} of the signal compared to control (control $EC_{50} = 34.0 \pm 4.1$ nM, $n=4$; treated $EC_{50} = 33.3 \pm 11.3$ nM, $n=4$, $p > 0.05$) with a 60% decrease in maximal activation (Fig. 2B). Pretreatment with 1 μ M quinpirole for 30 minutes elicited desensitization that was less than that obtained with dopamine ($78.1 \pm 3.5\%$, $n=4$, $p < 0.05$) (Fig. 2C). Activating the signal after quinpirole pretreatment with increasing concentrations of dopamine demonstrated no change in the EC_{50} of the signal compared to control (control $EC_{50} = 34.0 \pm 4.1$ nM, $n=4$; treated $EC_{50} = 38.0 \pm 13.2$ nM, $n=4$, $p > 0.05$) with a 20% decrease in maximal activation (Fig. 2D). Persistent occupancy of the ligand binding pocket by dopamine, SKF81297 and quinpirole was not a concern since an increase in the EC_{50} value would have been expected with continued ligand occupancy but this was not observed. Since SKF81297 can act as a partial agonist for D2 receptors within the D1-D2

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heterooligomeric complex (Rashid et al., 2007), the desensitization may be mediated by occupancy of both receptors. To prevent SKF81297 occupancy of the D2 receptor, 1 μM raclopride, a D2 selective antagonist, was added with SKF81297 pretreatment for 30 minutes (Fig. 2E). No significant difference in the extent of desensitization was observed. However, SKF81297-mediated desensitization was abolished by 10 μM of the D1 antagonist SCH 23390 (Fig. 2E). This suggests that the desensitization elicited by SKF81297 was through the selective occupancy of the D1 receptor. To determine if dopamine is activating both receptors, D1-D2 expressing cells were cotreated with equivalent amounts of either SCH 23390 or raclopride. 50% inhibition of dopamine-mediated desensitization was observed upon cotreatment with either antagonist (Fig. 2F).

Since the occupancy of either receptor within the heterooligomer mediated different extents of signal desensitization, we queried if this may also have an effect on the potency to desensitize the signal (Fig. 3A). Cells were pretreated with increasing concentrations of dopamine, SKF81297 or quinpirole, from 10^{-11} to 10^{-5} M for 30 minutes, washed and activated with 10 μM dopamine. The D1 receptor selective agonist SKF81297, through selective occupation of the D1 receptor, was very potent in desensitizing the signal, with an EC_{50} of 29.8 ± 8.4 nM (n=5). Dopamine, which occupied both receptors, was less potent with an EC_{50} of 293.6 ± 83.2 nM (n=5). Quinpirole, which occupied the D2 receptor, was the least potent of the agonists tested with an EC_{50} of 1041 ± 160.8 nM (n=5). The rank order of potency correlated with that for the extent of desensitization. Since the extent and potency of desensitization depended on the selective occupancy of either receptor, we investigated whether this correlation was also observed for the rate of signal desensitization (Fig. 3B). Cells were pretreated with 1 μM

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dopamine, SKF81297 or quinpirole from 5 to 60 minutes, washed and activated with 10 μ M dopamine. SKF81297 desensitized the signal quickly with a $t_{1/2}$ of 0.94 ± 0.1 minute ($n=4$). Dopamine desensitized the signal slower than that elicited by SKF81297 with a $t_{1/2}$ of 4.9 ± 1.1 minutes ($n=4$). Quinpirole desensitized the signal the slowest of the agonists tested with a $t_{1/2}$ of 9.5 ± 2.5 minutes ($n=4$).

In order to determine the mechanism by which the calcium signal desensitized, we tested potential mediators of this effect. First, since depletion of intracellular calcium stores by prolonged agonist treatment had been previously demonstrated to be a mediator of desensitization of some Gq/11 protein coupled receptors (Yu and Hinkle, 1997), we queried if the amount of intracellular calcium remaining was a limiting factor contributing to lowered calcium responses by quantifying intracellular calcium release after agonist pretreatment. Sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (1 μ M) was added in the presence of the extracellular calcium chelator EGTA to quantify intracellular calcium levels in SERCA-controlled stores after treatment with various agonists and compared to control levels. The amount of calcium released was not significantly changed by pretreatment with any of the agonists tested compared to control, suggesting no calcium store depletion has occurred (dopamine, then thapsigargin= $98.5 \pm 6.2\%$ of control; SKF81297, then thapsigargin= $108.7 \pm 11.9\%$ of control; quinpirole, then thapsigargin= $108.3 \pm 3.5\%$ of control). To verify this result, P2Y1 receptors, which utilized Gq/11 protein and phospholipase C as means to generate calcium release through intracellular calcium stores (Schachter et al., 1996), were activated after dopaminergic agonist treatment in the presence of EGTA. No significant difference was observed suggesting that calcium stores were not significantly depleted

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after agonist treatment (dopamine, then $ADP\beta S=91.9\pm 4.0\%$ of control; SKF81297, then $ADP\beta S=104.6\pm 5.3\%$ of control; quinpirole, then $ADP\beta S=92.9\pm 3.9\%$ of control). Activation of P2Y1 receptors after dopaminergic agonist treatment was also performed in the absence of EGTA and no significant differences were observed (data not shown). Since the calcium stores were not depleted, these results suggested that calcium signal desensitization may occur at the level of the receptor complex, either from the loss of receptor complexes from the cell surface or alteration of receptor complex function.

To evaluate the role of internalization in the desensitization mechanism, cells coexpressing D1 and D2 receptors were cotreated with 250 $\mu\text{g/ml}$ concanavalin A (con A) to keep the receptor complex at the cell surface, along with the chosen agonists for 30 minutes (Fig. 4). Cotreatment of agonists and con A significantly attenuated agonist-induced receptor internalization of the D1 (Fig. 4A) and D2 receptors (Fig. 4B) in cells coexpressing both receptors as determined by quantitative cell surface immunofluorescence. Significantly attenuating receptor internalization did not result in a concomitant decrease in the extent of signal desensitization by the agonists tested but instead a small, but significant, increase was observed (Fig. 4C).

Two of the major protein kinase families that have been implicated in desensitization of GPCRs are protein kinase A (PKA) and protein kinase C (PKC). The involvement of these protein kinases in D1 and D2 receptor mediated desensitization when these receptors are expressed alone is well documented (Jiang and Sibley, 1999; Namkung and Sibley, 2004). Therefore, we tested if the inhibition of these kinases by PKA and PKC inhibitors, 10 μM H89 and 500 nM bisindolylmaleimide I (Bis), respectively, attenuated desensitization mediated by dopamine (Fig. 5A), SKF81297 (Fig.

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5B) and quinpirole (Fig. 5C). In all cases, neither the addition of the PKA nor PKC inhibitor decreased the extent of desensitization. Pretreatment with the broad spectrum PKC inhibitor staurosporine alone or with Bis also did not inhibit signal desensitization (data not shown). Interestingly, inhibiting PKA activity increased desensitization mediated by all agonists tested. Representative calcium tracings for the effect of PKA and PKC inhibitors on dopamine mediated desensitization are shown in figure 5D and 5E. To test if desensitization of the D1-D2 receptor mediated signal can occur by heterologous mechanisms involving PKA and PKC, their respective activators, 3 mM dibutyryl cAMP (dbcAMP) and 1 μ M phorbol-12-myristate-13-acetate (PMA), were utilized and were observed to decrease the calcium signal. The effects of these activators were attenuated significantly by H89 and Bis, respectively (Fig. 5F). This data suggest that these kinases did not have a direct role in homologous desensitization but may be able to indirectly desensitize the signal by heterologous activation of PKA and PKC.

G protein coupled receptor kinases (GRKs) have been implicated in the desensitization of D1 (Lamey et al., 2002) and D2 receptors (Ito et al., 1999) when expressed alone. Therefore, to explore these potential interactions, GRK2, 3, 5 and 6, reported to be endogenously expressed in HEK293T cells (Iwata et al., 2005), were transiently transfected into D1-D2 receptor expressing cells. This strategy was previously utilized successfully to determine the effect of GRKs on the desensitization of dopamine receptors (Kim et al., 2001). Increased expression of GRK2 and GRK3 increased desensitization significantly with all agonists tested (Fig. 6A and 6B), whereas increasing the expression of GRK5 and GRK6 did not (Fig. 6C and 6D), suggesting a role for GRK2 and 3 in signal desensitization. Increased expression of GRKs in transfected cells was

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confirmed by western blot (Fig. 6E). A representative calcium tracing for the effect of increased GRK2 expression on dopamine mediated desensitization is demonstrated in Figure 6F.

To confirm the role of GRK2 and 3, we silenced endogenous GRKs with siRNA as described previously (Violin et al., 2006). Transfection of siRNA to silence GRK2 in D1-D2 receptor expressing cells resulted in a significant decrease in the desensitization mediated by all agonists (Fig. 7A). Similarly, transfection of siRNA to silence GRK3 also significantly decreased the desensitization of the calcium signal mediated by all agonists (Fig. 7B). Decreased expression of these GRKs in transfected cells was confirmed by western blot (Fig. 7C).

To elucidate a potential mechanism by which GRKs mediate desensitization, D1-D2 receptor expressing cells were pretreated for 30 minutes with 200 μ M of the general GRK inhibitor zinc chloride, which inhibits the ability of GRKs to phosphorylate receptors (Benovic et al., 1987). Pretreatment with zinc chloride did not inhibit the desensitization of the calcium signal elicited by dopamine, SKF81297 or quinpirole pretreatment (Fig. 8A) but was successful in inhibiting the desensitization of the cAMP signal generated by the activation of the D1 receptor stably expressed in HEK cells (2071 \pm 60 fmol/mg) by a 30 minute dopamine pretreatment (Fig. 8B). Interestingly, desensitization was increased slightly but significantly by zinc pretreatment. To verify this result, the catalytic inert GRK2 mutant GRK2-K220R was expressed in D1-D2 expressing cells and was found to also not be able to inhibit desensitization (data not shown).

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Ca²⁺-calmodulin kinase II (CAMKII) and casein kinases I and II, other second messenger activated kinases that could potentially mediate desensitization, were tested for their involvement in the desensitization of the calcium signal. Cells were pretreated with 10 μ M CAMKII inhibitor KN-93, 1 μ M casein kinase I inhibitor D4476 and 1 μ M casein kinase II inhibitor TBB prior to agonist pretreatment. The calcium signal associated with the activation of the D1-D2 heterooligomer with dopamine and activation of the P2Y1 receptor with ADP β S were both significantly attenuated by pretreatment with KN93, suggesting that this effect relates to the modulatory role CAMKII plays on the IP3 receptor, which had been shown previously (Aromolaran and Blatter, 2005) (D1R+D2R+dopamine= 79.6 \pm 6.9 AFU \times 10³, D1R+D2R+dopamine+KN93= 51.2 \pm 5.3 AFU \times 10³, n=5. p<0.05. P2Y1R+ADP β S= 87.2 \pm 6.9 AFU \times 10³, P2Y1R+ADP β S+KN93= 45.2 \pm 6.5 AFU \times 10³, n=5. p<0.05.). Treatment with KN-93 did not attenuate D1-D2 signal desensitization associated with dopamine, SKF81297 and quinpirole pretreatment (Fig. 9A). Surprisingly, inhibiting CAMKII activity slightly, but significantly, increased the extent of desensitization in all cases. Since casein kinases are involved in desensitization of Gq/11 protein linked receptors (Budd et al., 2000), cells were pretreated with 1 μ M casein kinase I inhibitor D4476 (Fig. 9B) or 1 μ M casein kinase II inhibitor TBB (Fig. 9C). In both cases, the extent of desensitization was not decreased but was slightly, but significantly, increased.

Since the D1 and D2 receptors can couple to Gs and Gi proteins, (Lee et al., 2004), the involvement of the activation of these proteins in the desensitization of the heterooligomer was investigated. The adenylyl cyclase inhibitor, SQ22536, was used to inhibit Gs protein mediated signaling activated by the D1 receptor and pertussis toxin

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(PTX) was used to selectively inhibit Gi protein mediated signaling activated by the D2 receptor. Cells pretreated with 10 μ M SQ22536 demonstrated no change in the extent of desensitization by any agonist tested (Fig. 10A). Treatment with 250 ng/ml PTX, however, selectively increased desensitization by dopamine and SKF81297, but not that mediated by quinpirole or ADP β S (Fig. 10B).

DISCUSSION

Coactivation of D1 and D2 receptors within a D1-D2 heterooligomer generated a phospholipase C linked calcium signal (Lee et al., 2004). In this report, we determined that the desensitization of this signal could be elicited by agonist occupancy of either receptor within the complex and occurred under a minute or up to ten minutes for the agonists utilized. This signal was homologously desensitized by pretreatment with dopamine or by the selective D1 agonist, SKF81297, or the selective D2 agonist, quinpirole, with the efficacy, rate and potency of desensitization varying among the three agonists tested. Desensitization occurred independent of calcium storage capacity, exogenous calcium entry or receptor internalization, suggesting modification of the heterooligomer itself allowed for signal desensitization. The inhibition of the second messenger activated kinases PKA, PKC, CAMKII or casein kinases I and II did not decrease signal desensitization. A role for GRK2 and 3, not GRK5 or 6, was established that was independent of their catalytic activity. Gi protein activity associated with the D2 receptor controlled the efficacy of the desensitization mediated by the D1 receptor within the D1-D2 complex.

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Desensitization of the calcium signal occurred when either receptor within the D1-D2 complex was occupied by selective agonists. Characteristics of desensitization elicited were different, however, as SKF81297 treatment resulted in a more robust, more efficacious and faster rate of desensitization compared to that following quinpirole treatment, with dopamine displaying a response intermediate of the two. This difference may be attributed to differential agonist induced conformations and the selectivity of these agonists for D1 and D2 receptors. Targeting either the D1 or D2 receptor within the D1-D2 complex resulted in desensitization characteristics reminiscent of the receptor when expressed alone. D1 homooligomers desensitized quickly, within minutes (Gardner et al., 2001; Lamey et al., 2002). D2 homooligomers, on the other hand, desensitized very slowly, requiring prolonged agonist treatment (Ng et al., 1997; Zhang et al., 1994). For the D1-D2 complex, when the D1 receptor was selectively targeted, the desensitization of the calcium signal occurred in less than a minute whereas, when the D2 receptor was selectively targeted, desensitization took approximately 10 minutes. Interestingly, the desensitization induced by dopamine, which targets both D1 and D2 receptors, displayed an intermediate profile between the two, taking place in five minutes. This intermediate profile may be a distinct feature of dopamine induced conformations or the result of its coactivation of D1 and D2 receptors. The activation of the D2 receptor within the heterooligomer may act as a braking mechanism to decrease the efficacy, rate and efficiency of desensitization mediated by agonist occupancy of the D1 receptor.

This study also demonstrated that signal desensitization does not correlate with the efficacy of signal activation. Quinpirole desensitized the signal but did not independently activate calcium signaling. Desensitization mediated by dopamine or

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SKF81297 were both greater than that mediated by quinpirole, suggesting that the activation of the Gq-mediated signal may dictate the extent of desensitization. However, the observation that dopamine, which caused maximal activation of the signal, was not as efficacious in causing desensitization as SKF81297, which only partially activated the signal, suggests that there are factors beyond signal activation that mediates the efficacy of calcium signal desensitization. One potential factor may be the ability of each agonist to initiate protein kinase-mediated phosphorylation of the heterooligomer. For homooligomers, variable desensitization of its signal can stem from the ability of agonists to mediate differential phosphorylation of the receptor (Zhang et al., 1998). The lack of correlation between the ability of agonists to activate and their ability to desensitize receptor signaling has been reported for agonists within homooligomers (Barak et al., 2006) and this report is the first demonstration that this is also observed with GPCR heterooligomers.

The observation that preventing agonist mediated internalization of the complex did not decrease the extent of signal desensitization suggests that desensitization of the calcium signal has already occurred prior to recruitment of the complex into vesicles by endocytic machinery. We showed that a temporal dissociation of the processes of agonist mediated desensitization and internalization existed for D1 receptor homooligomers (Ng et al., 1995). Instead of mediating desensitization, internalization may allow resensitization of receptors to occur through interactions with phosphatases in endosomes (Lefkowitz et al., 1998). This is a possibility since attenuating the agonist induced internalization of the heterooligomer increased signal desensitization, suggesting a loss of the resensitization process. Another possibility to consider is whether the D1-D2 receptor

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complex breaks apart during the desensitization process prior to receptor internalization, thus disrupting and turning off the signal.

The involvement of GRK2 and GRK3 in signal desensitization was confirmed by both increasing and decreasing the expression of these proteins in D1-D2 co-expressing cells. Inhibiting GRK mediated phosphorylation of receptors, however, did not inhibit desensitization of the calcium signal. This suggests that, in addition to phosphorylating receptors, GRKs may also mediate signal desensitization by phosphorylation independent mechanisms. For instance, GRK2 and GRK3 may sequester Gq/11 proteins, which interact with the RGS domain on these GRKs (Iwata et al., 2005), thereby eliciting signal desensitization by a phosphorylation independent mechanism. GRKs can also desensitize the receptor without phosphorylation by sequestering G $\beta\gamma$ subunits and competing against PLC β binding to activated forms of G α_q proteins (Tobin, 2002).

Since both D1 and D2 receptors contain motifs in their intracellular domains specific for phosphorylation by a variety of kinases, their potential roles in the desensitization of the calcium signal was investigated. However, the ineffectiveness of their respective inhibitors to decrease homologous desensitization suggests that these kinases play no direct role in mediating homologous desensitization of the D1-D2 receptor complex. However, a potential modulatory role of some of these kinases is suggested by the ability of their respective inhibitors to increase (instead of decrease) calcium signal desensitization. We postulate that these kinases may alter the resensitization of the calcium signal. It has been demonstrated that blocking PKA and CAMKII phosphorylation of GPCRs inhibited receptor resensitization and increased desensitization (Hishinuma and Ogura, 2000; Tran et al., 2007). Despite the fact that

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inhibiting PKA and PKC activities did not affect homologous desensitization, these kinases are involved in heterologous desensitization of this signal. However, the activation of P2Y1 receptors was not able to heterologously desensitize this signal, suggesting that specific PKC isoforms that are not activated by P2Y1 receptors may be involved in this effect.

Desensitization is mediated by receptors coupled to a Gq/11 signaling pathway within the heterooligomeric complex and not by activation of Gs or Gi proteins. Inhibiting adenylyl cyclase activity did not decrease the desensitization mediated by dopamine or SKF81297, both of which activate adenylyl cyclases through the D1 receptor. Inhibiting the activation of Gi proteins by the D2 receptor did not decrease the desensitization mediated by quinpirole. However, inhibiting the ability of the D2 receptor to activate Gi proteins did result in increased desensitization mediated by agonists selectively targeting the D1 receptor. This suggests that downstream Gi protein signaling activated by D2 receptors may decrease desensitization resulting from D1 receptor activation. The activation of Gi proteins, and not Gs proteins, could accelerate the rate of resensitization of the Gq/11 coupled receptor and thus allow for decreased desensitization (Werry et al., 2003). It is not clear if the Gi proteins activated are associated with D2 receptors within D2 homooligomers or within D1-D2 heterooligomers.

This study demonstrates, for the first time, desensitization of a signal generated by a GPCR heterooligomer. These findings suggest different signaling regulation could result from selectively targeting either receptor within the complex, leading to differential development of tolerance and neuronal adaptation. Furthermore, our report showing differences in the effect of D1 or D2 receptor selective agonists on the calcium signal

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mediated by the D1-D2 receptor heterooligomer may have relevance for clinical conditions where these selective agonists are used therapeutically. Continued investigation of this unique signal may yield a better understanding of behavioral and central nervous system disorders mediated by dopamine such as drug addiction and schizophrenia. For instance, there is a large body of evidence suggesting that dysfunction of calcium signaling may be associated with the etiology of schizophrenia (Lidow, 2003). Therefore, the elucidation of the control and termination of this signal may be integral to the understanding of normal physiology as well as disorders linked to dopamine activated signaling systems.

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FOOTNOTES

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FIGURE LEGEND

Fig. 1. The calcium signal associated with the coactivation of D1 and D2 receptors in cells stably expressing both receptors is desensitized by prior treatment with dopamine for 30 minutes. Data were expressed in absolute fluorescence units (AFU). A) The calcium signal from the addition of 1 μ M dopamine, SKF81297 or quinpirole in cells stably expressing both receptors (n=5). B) Activation of the calcium signal by 1 μ M dopamine is inhibited by 10 nM Gq/11 inhibitor YM254890 (n=4). C) Activation of the calcium signal by 1 μ M dopamine is not abolished by 250 μ M calcium chelator EGTA (n=4). D) The calcium signal activated by 10 μ M dopamine (control) and following pretreatment with 1 μ M dopamine for 30 minutes. E) Effect of increasing concentrations of dopamine on the activation of the calcium signal without (control) and after 1 μ M dopamine treatment for 30 minutes (n=4). Arrows indicate EC₅₀ values. F) Desensitization of the calcium signal associated with dopamine activation does not occur following the activation of the purinergic P2Y1 receptor with the selective agonist ADP β S (n=5). Activation of the P2Y1 receptor by 10 μ M ADP β S without (Bar 1) or after 30 minute pretreatment with 1 μ M ADP β S (Bar 2). Activation of the calcium signal by 10 μ M dopamine (Bar 3) or after 30 minute pretreatment with 1 μ M ADP β S (Bar 4) (*, p<0.05).

Fig. 2. The calcium signal associated with the coactivation of D1 and D2 receptors in cells stably expressing both receptors is desensitized by prior treatment with selective D1 or D2 agonists for 30 minutes. Data were expressed in absolute fluorescence units (AFU). A) The calcium signal activated by 10 μ M dopamine (control) and following

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pretreatment with 1 μM SKF81297 for 30 minutes (n=3). B) Effect of increasing concentrations of dopamine on the activation of the calcium signal without (control) and after 1 μM SKF81297 treatment for 30 minutes (n=4). Arrows indicate EC_{50} values. C) The calcium signal activated by 10 μM dopamine (control) and following pretreatment with 1 μM quinpirole for 30 minutes (n=4). D) Effect of increasing concentrations of dopamine on the activation of the calcium signal without (control) and after 1 μM quinpirole treatment for 30 minutes (n=4). Arrows indicate EC_{50} values. E) Desensitization of the calcium signal elicited by pretreatment with 1 μM SKF81297 for 30 minutes without or with pretreatment with 10 μM SCH 23390 or 1 μM raclopride (n=4). F) Desensitization of the calcium signal elicited by pretreatment with 1 μM dopamine for 30 minutes without or with pretreatment with 1 μM SCH 23390 or 1 μM raclopride (n=4).

Fig. 3: The efficacy, potency and rate of desensitization of the D1-D2 activated calcium signal varied among the agonists tested. Data were represented as the percent of peak fluorescence of the calcium signal from 10^{-11} M dopamine treated cells \pm SEM. A) Effect of increasing concentrations of agonists used during pretreatment on the desensitization of the calcium signal. Cells were pretreated with increasing concentrations of dopamine, SKF81297 or quinpirole, from 10^{-11} to 10^{-5} M for 30 minutes, washed and activated with 10 μM dopamine. The rank order of drug potency mediating desensitization was SKF81297>dopamine>quinpirole. Arrows indicate EC_{50} values (n=5). (Right Panel) Representative calcium tracing of the signal activated by 10 μM dopamine following pretreatment with increasing concentrations of dopamine for 30 minutes is shown. B)

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Effect of increasing pretreatment times of agonists on calcium signal desensitization. Cells were pretreated with 1 μ M dopamine, SKF81297 or quinpirole from 5 to 60 minutes, washed and activated with 10 μ M dopamine. The rank order of the drug effect mediating the rate of desensitization was SKF81297>dopamine>quinpirole. Arrows indicate $t_{1/2}$ values in minutes (n=5). (Right Panel) Representative calcium tracing of the signal activated by 10 μ M dopamine without pretreatment (t=0 minutes) and following pretreatment with 1 μ M dopamine from 5 to 30 minutes is shown.

Fig. 4. Decreasing D1 and D2 receptor internalization in cells coexpressing both receptors with cotreatment of concanavalin A (Con A) and dopaminergic agonists enhanced agonist-mediated D1-D2 activated calcium signal desensitization. Internalization of the D1 receptor (A) or D2 receptor (B) in cells coexpressing D1 and D2 receptors treated for 30 minutes with 1 μ M dopamine, SKF81297 or quinpirole in the absence (black bars) and presence (white bars) of 250 μ g/ml Con A (n=4-6). Percentage internalization represents the loss of fluorescence from the cell surface of intact cells after agonist treatment compared with vehicle treated controls C) Desensitization of the calcium signal after 30 minute pretreatment with 1 μ M dopamine, SKF81297 or quinpirole in the absence (black bars) and presence (white bars) of 250 μ g/ml of Con A (n=10). (Right Panel) Representative calcium tracing for the effect of concanavalin A on dopamine-mediated signal desensitization is shown. Data were represented as the percent of peak fluorescence of the calcium signal generated by 10 μ M dopamine in vehicle pretreated cells \pm SEM. (*, p<0.05).

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Fig. 5. Desensitization of the D1-D2 activated calcium signal was not decreased by inhibiting protein kinase A or protein kinase C. Desensitization of the calcium signal in cells pretreated with 1 μ M dopamine (A), SKF81297 (B) quinpirole (C) without pretreatment of cells with inhibitors (black bar), with 1 hour pretreatment with 10 μ M H89 (white bar) or 500 nM Bis (grey bar) (n=5-7). Representative calcium tracings for the effect of inhibiting PKA (D) and PKC (E) on dopamine mediated desensitization of the calcium signal are shown. (F) The calcium signal generated by 10 μ M dopamine activation after the addition of 3 mM PKA activator dbcAMP or 1 μ M PKC activator PMA (black bars). These effects are significantly decreased by cotreatment with 10 μ M PKA inhibitor H89 or 500 nM PKC inhibitor Bis (white bars). Data were represented as the percent of peak fluorescence of the calcium signal generated by 10 μ M dopamine in vehicle pretreated cells \pm SEM. (*, p<0.05).

Fig. 6: Increased expression of G protein coupled receptor kinases (GRK) 2 or 3, not 5 or 6, increased agonist-mediated desensitization of the D1-D2 activated calcium signal. Desensitization of the calcium signal after 30 minute pretreatment with 1 μ M dopamine, SKF81297 or quinpirole without (black bars) or with (white bars) increased expression of GRK2 (A), GRK3 (B), GRK5 (C) or GRK6 (D) (n=6). E) Immunoblot demonstrating increased expression of GRK2, GRK3, GRK5 or GRK6 in D1-D2 expressing cells transiently transfected with GRK constructs. Data were represented as the percent of peak fluorescence of the calcium signal generated by 10 μ M dopamine in vehicle pretreated cells \pm SEM. (*, p<0.05). F) Representative calcium tracing for the effect of increased GRK2 expression on dopamine-mediated signal desensitization is shown.

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Fig. 7: Decreased expression of G protein coupled receptor kinases 2 or 3 decreased agonist mediated desensitization of the D1-D2 activated calcium signal. Desensitization of the calcium signal after 30 minute pretreatment with 1 μ M dopamine, SKF81297 or quinpirole without (black bars) or with (white bars) decreased expression of GRK2 (A) or GRK3 (B) (n=3-4). In each case, representative calcium tracings for dopamine mediated signal desensitization are shown on the right panel. Data were represented as the percent of peak fluorescence of the calcium signal generated by 10 μ M dopamine in vehicle pretreated cells \pm SEM. (*, p<0.05). C) Immunoblot demonstrating decreased expression of GRK2 and GRK3. Average silencing for these experiments was 65-70% of basal levels.

Fig. 8: The inhibition of the catalytic domain of G protein coupled receptor kinases did not inhibit agonist mediated desensitization of the D1-D2 activated calcium signal. A) The calcium signal activated by 10 μ M dopamine after 30 minute pretreatment with 1 μ M dopamine, SKF81297 or quinpirole without (black bar) or with (white bar) 200 μ M zinc chloride for 30 minutes (n=6). (Lower Panel) Representative calcium tracing for the effect of zinc chloride on dopamine-mediated signal desensitization is shown. Data were represented as the percent of peak fluorescence of the calcium signal from vehicle treated cells \pm SEM. B) [3 H]cAMP accumulation of the D1 receptor expressed alone pretreated with 1 μ M dopamine for 30 minutes without (black bar) or with 200 μ M zinc chloride (white bar). Data were represented as the percent of peak fluorescence of the calcium signal generated by 10 μ M dopamine in vehicle pretreated cells \pm SEM. (*, p<0.05).

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Fig. 9: The inhibition of Ca²⁺-calmodulin dependent kinase II (CAMKII) and casein kinases slightly increased agonist mediated desensitization of the D1-D2 activated calcium signal. Desensitization of the calcium signal after 30 minute pretreatment with 1 μ M dopamine, SKF81297 or quinpirole without (black bars) or with (white bars) 10 μ M KN93 (A), 1 μ M D4476 (B) or 1 μ M TBB (C) pretreatment for 1 hour (n=5). In each case, representative calcium tracings for dopamine mediated signal desensitization are shown on the right panel. Data were represented as the percent of peak fluorescence of the calcium signal generated by 10 μ M dopamine in vehicle pretreated cells \pm SEM. (*, p<0.05).

Fig. 10: Dopamine D1 receptor mediated desensitization is increased by pertussis toxin pretreatment but no effect on signal desensitization is observed with the adenylyl cyclase inhibitor SQ22536 (n=4-5). A) Desensitization of the calcium signal after 30 minute pretreatment with 1 μ M dopamine, SKF81297 or quinpirole without (black bars) or with (white bars) 10 μ M SQ22536 (A) or overnight pretreatment with 250 ng/ml PTX (B). In each case, representative calcium tracings for dopamine-mediated signal desensitization are shown on the right panel. Data were represented as the percent of peak fluorescence of the calcium signal generated by 10 μ M dopamine in vehicle pretreated cells \pm SEM. (*,p<0.05).

Figure 1

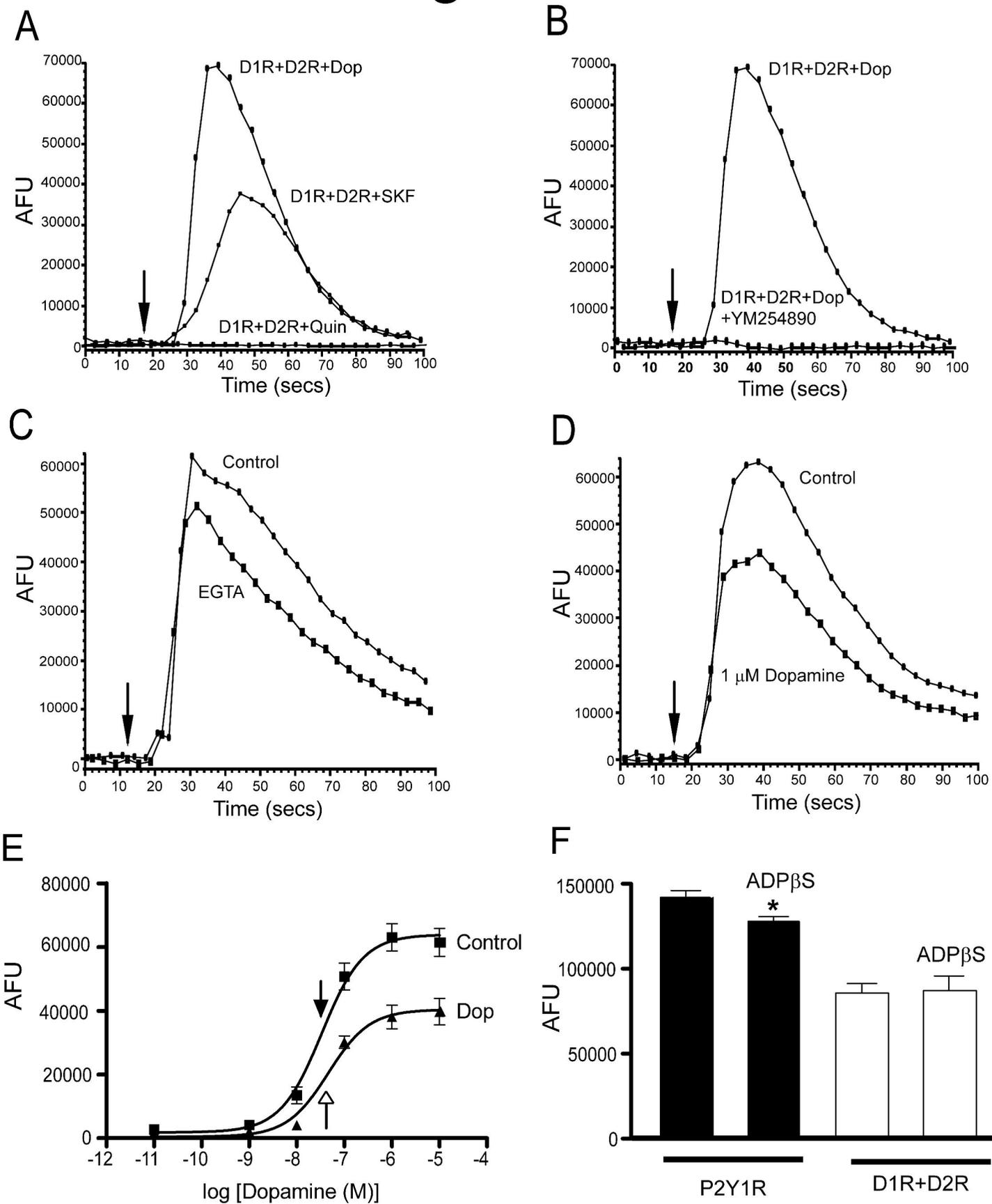
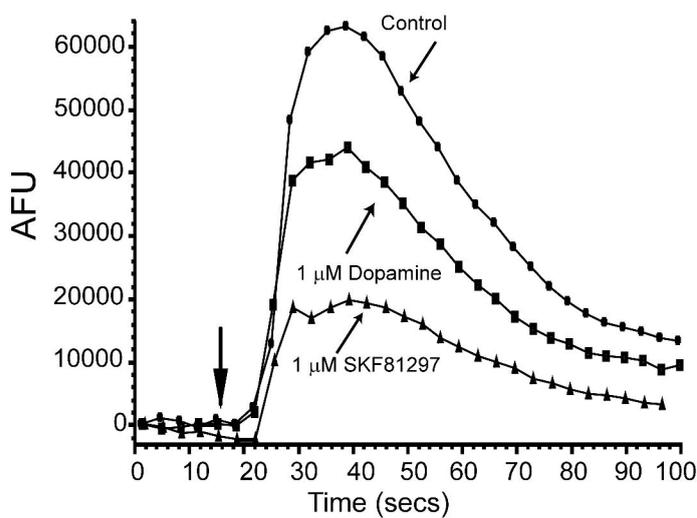
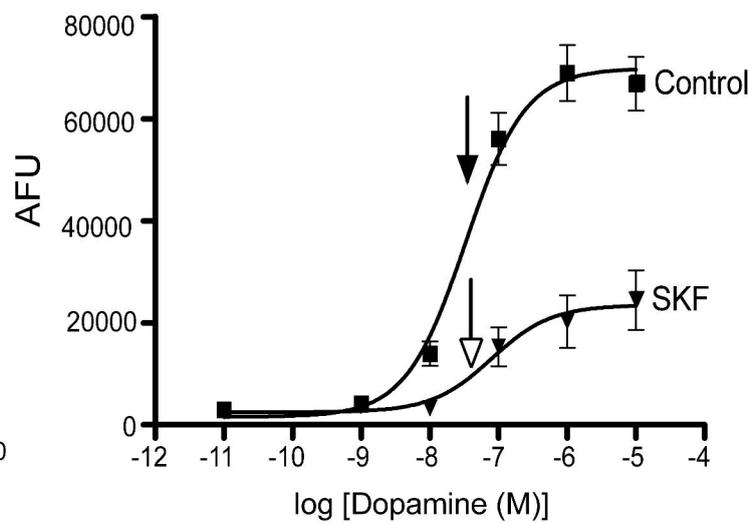


Figure 2

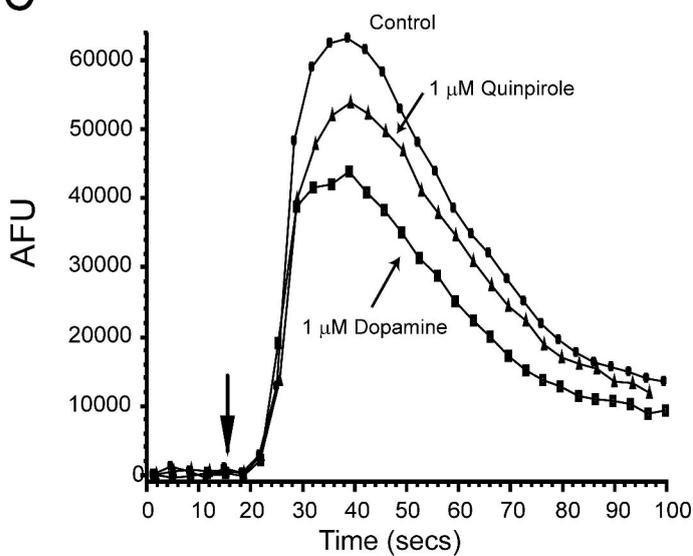
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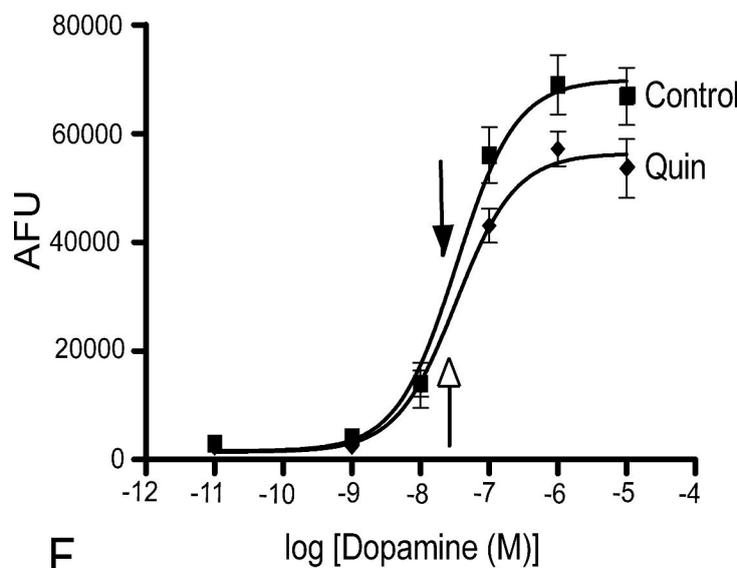
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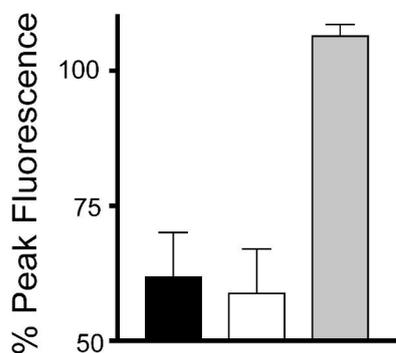
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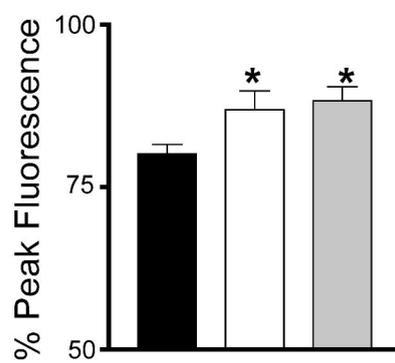


E



SKF81297	+	+	+
SCH23390	-	-	+
Raclopride	-	+	-

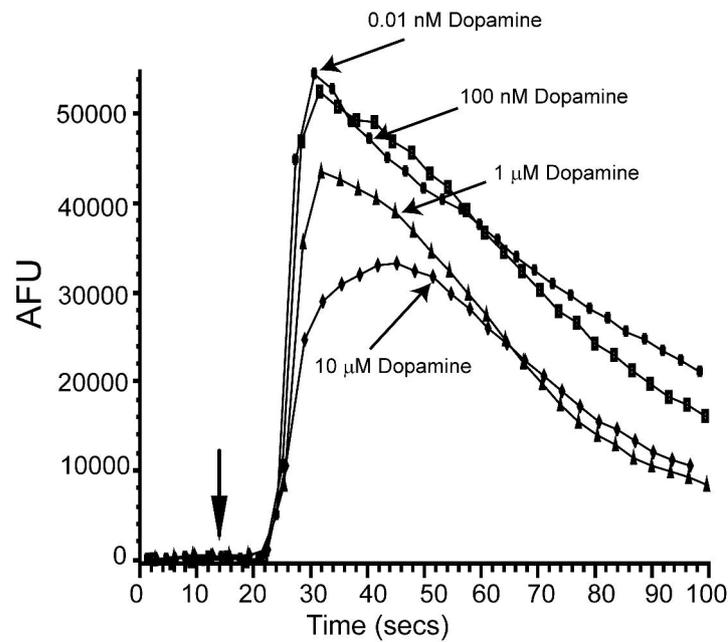
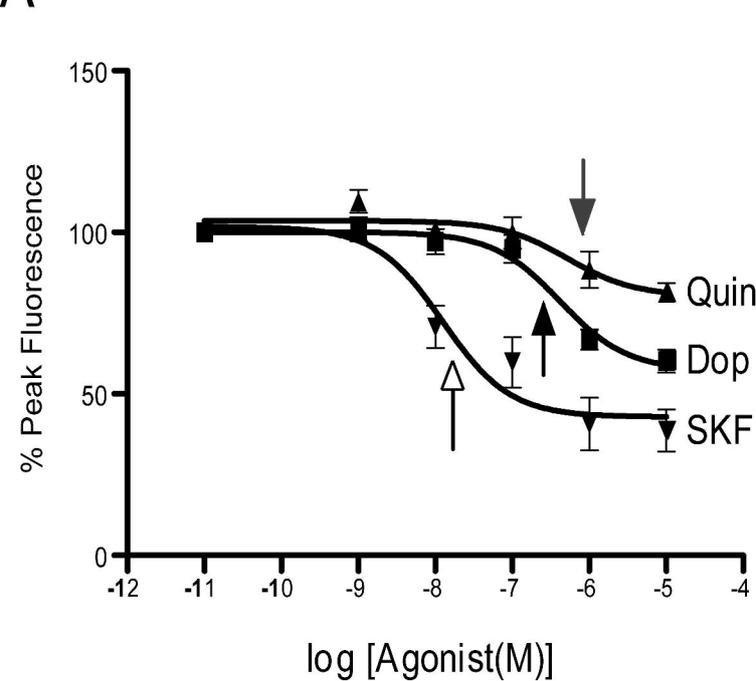
F



Dopamine	+	+	+
SCH23390	-	+	-
Raclopride	-	-	+

Figure 3

A



B

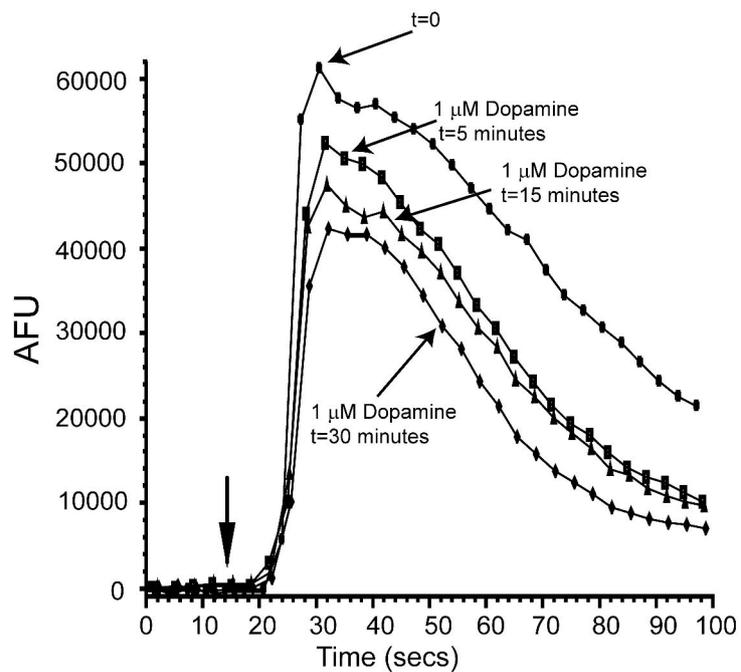
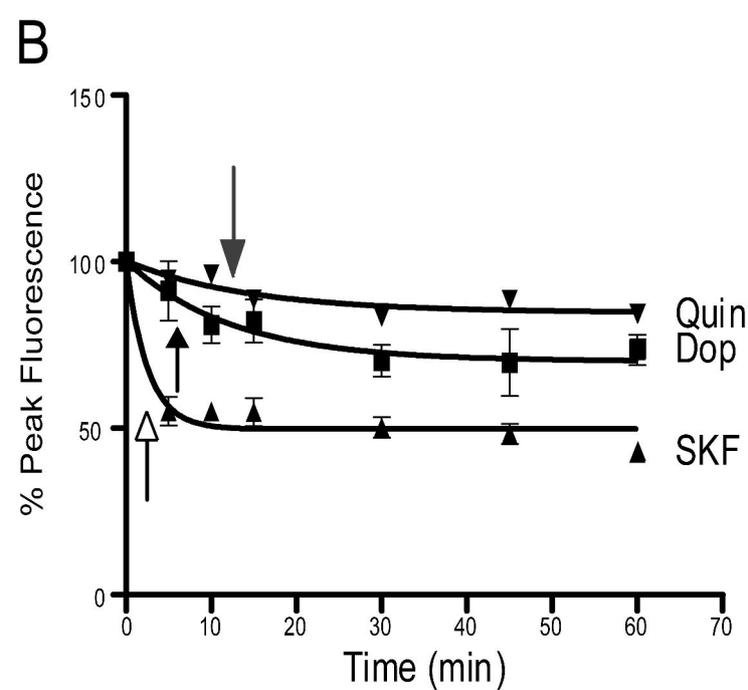


Figure 4

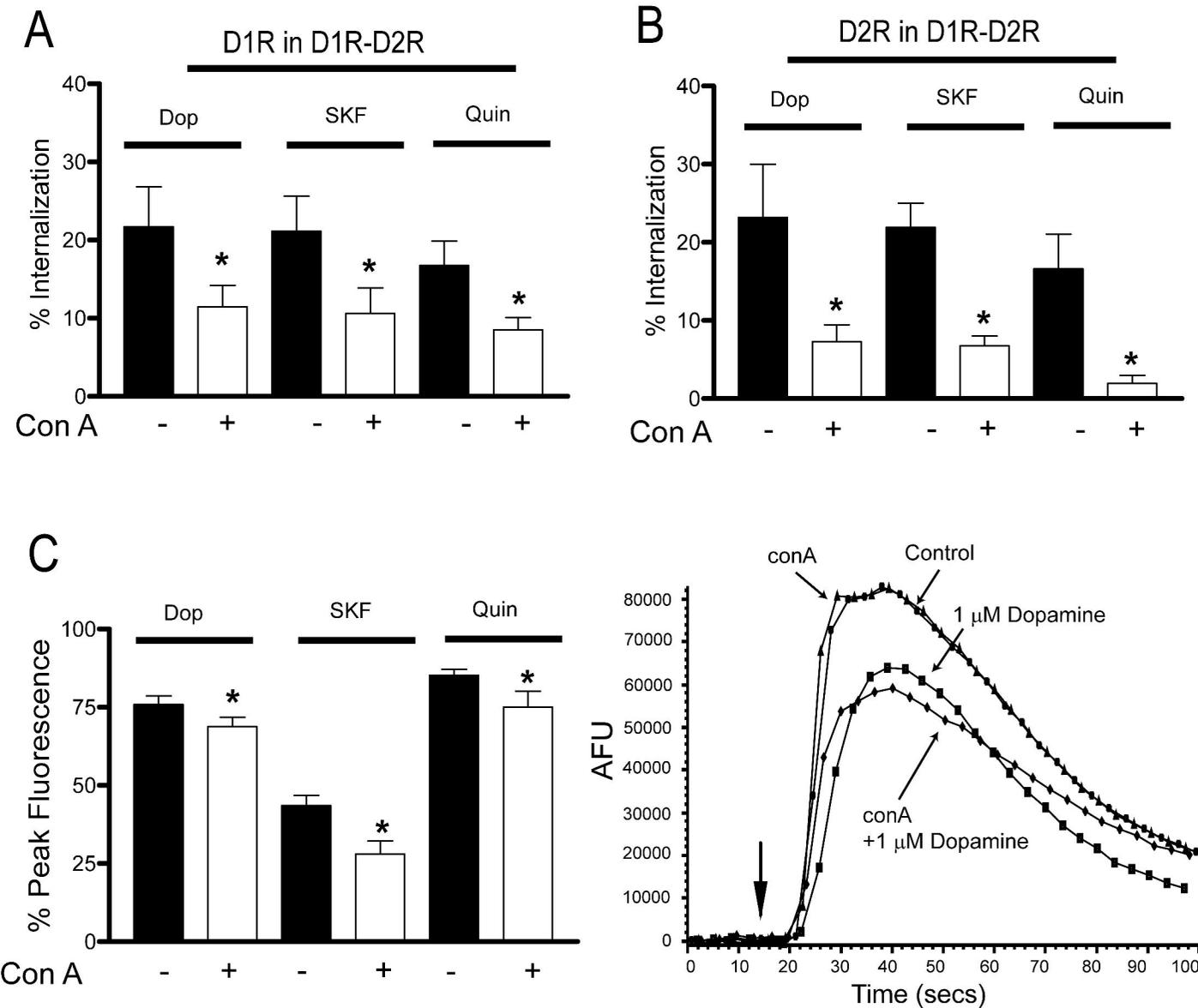


Figure 5

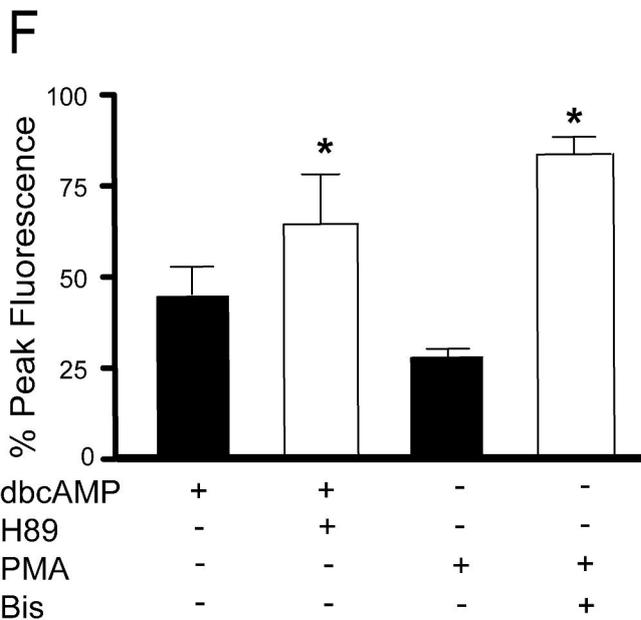
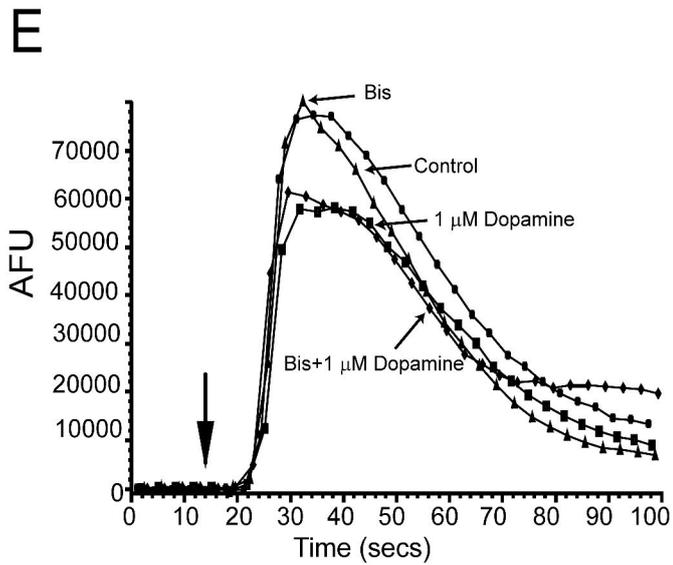
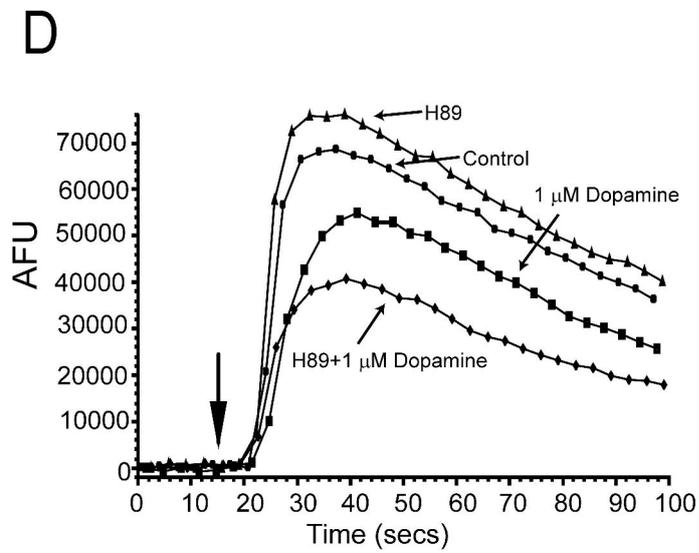
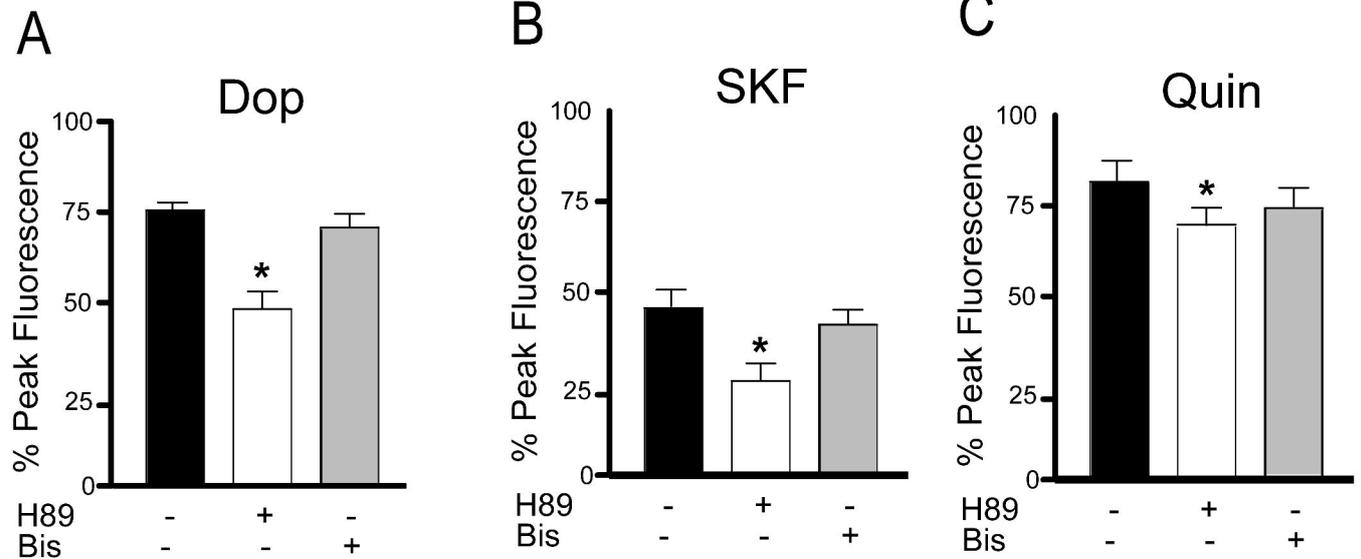


Figure 6

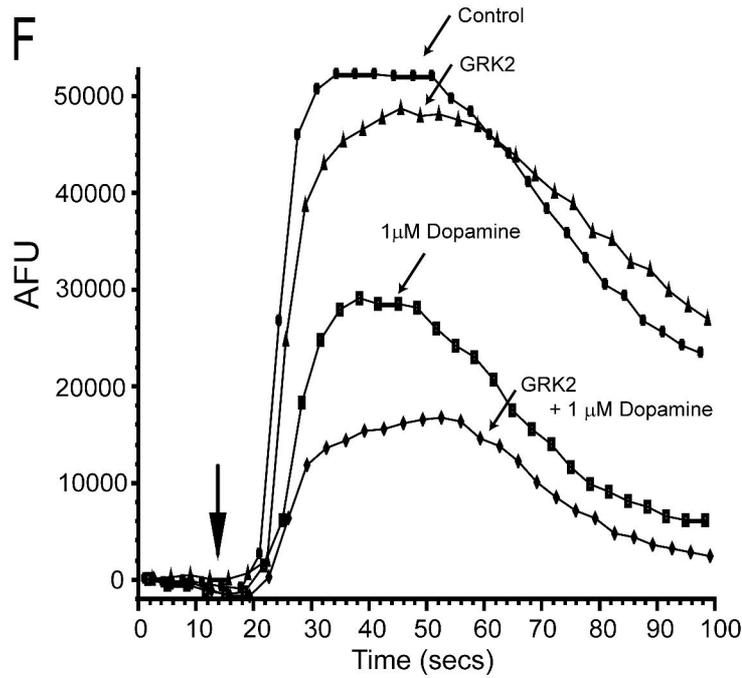
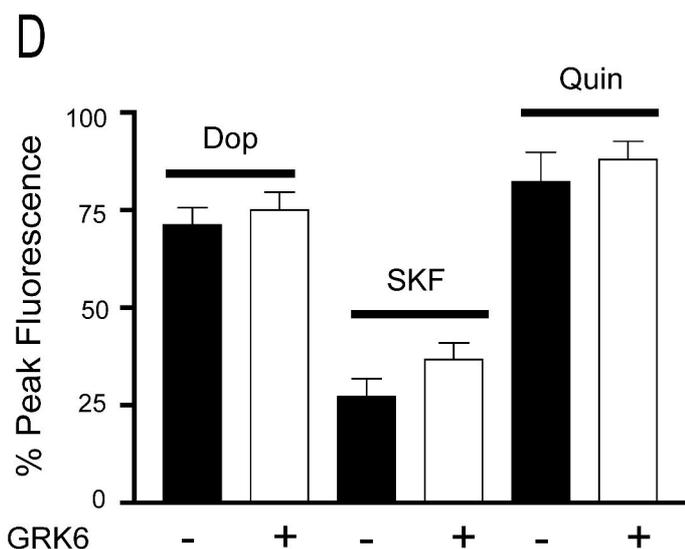
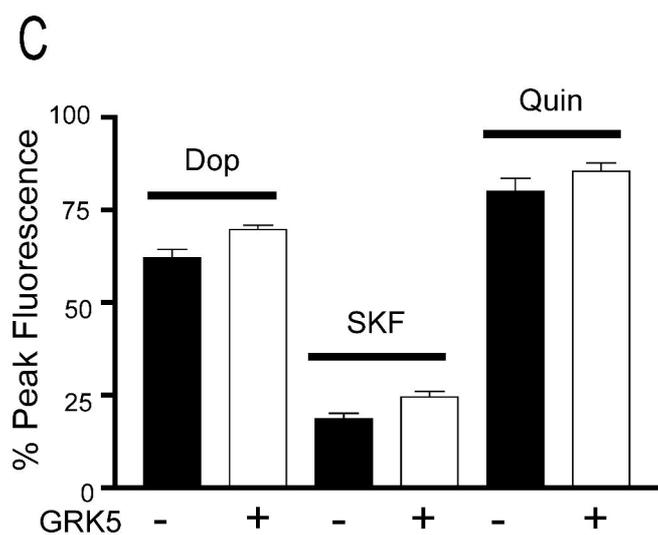
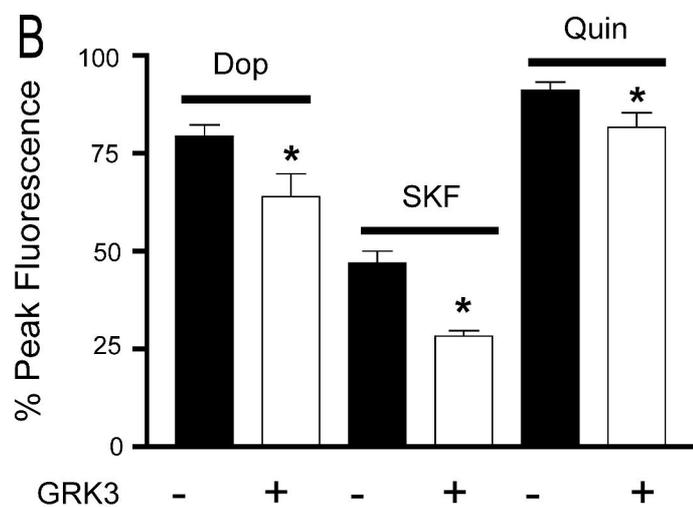
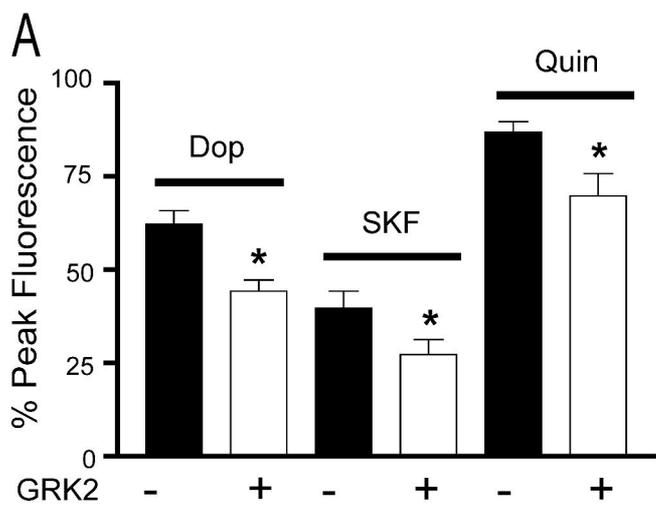
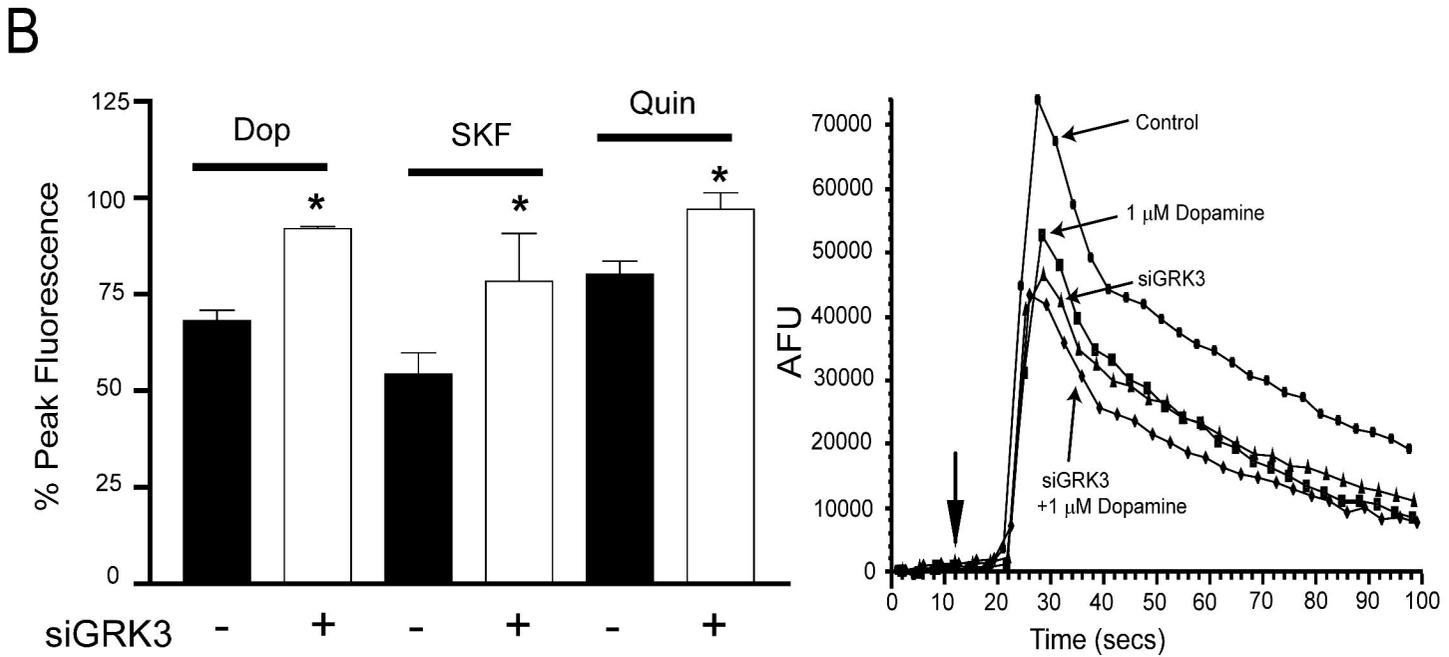
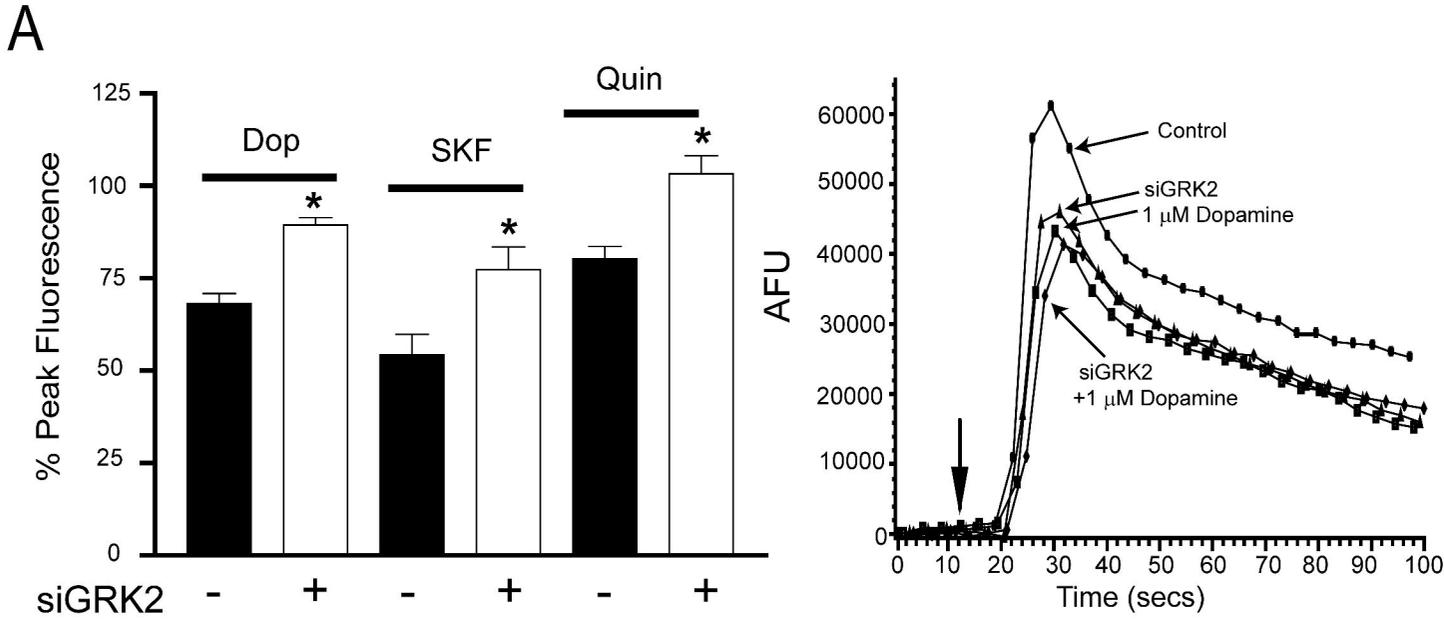


Figure 7



C

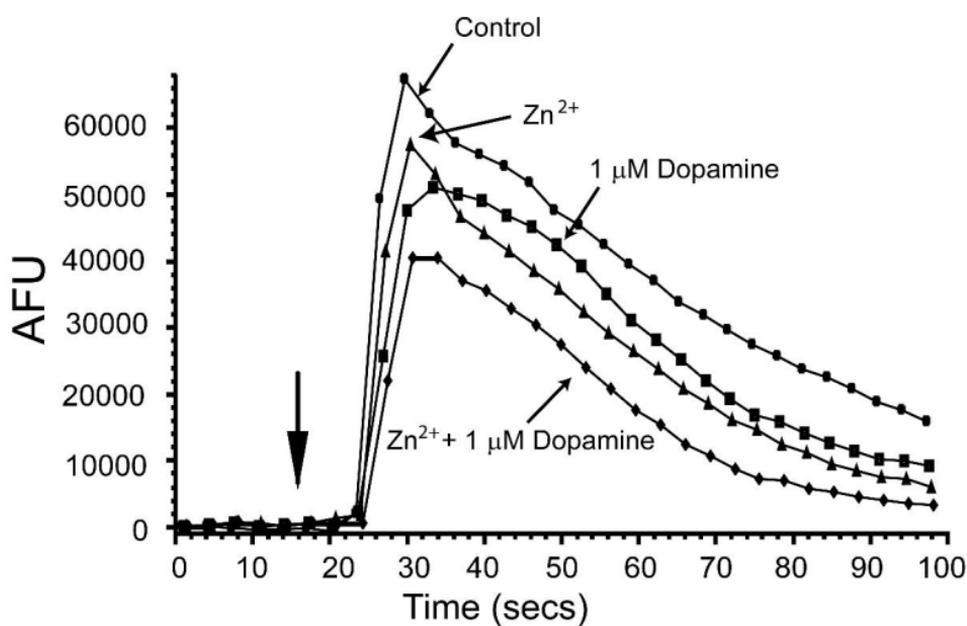
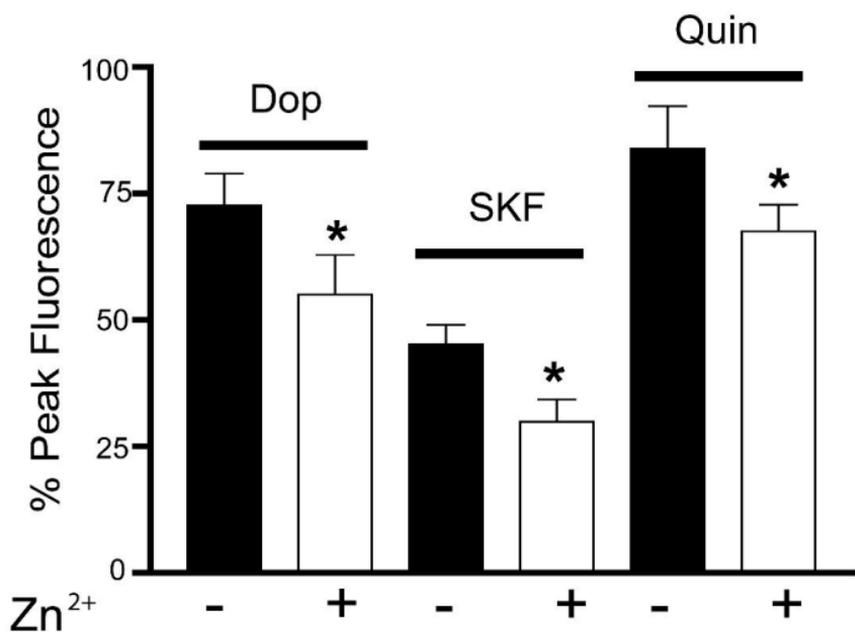
Control siRNA

IB:GRK2 

IB:GRK3 

Figure 8

A



B

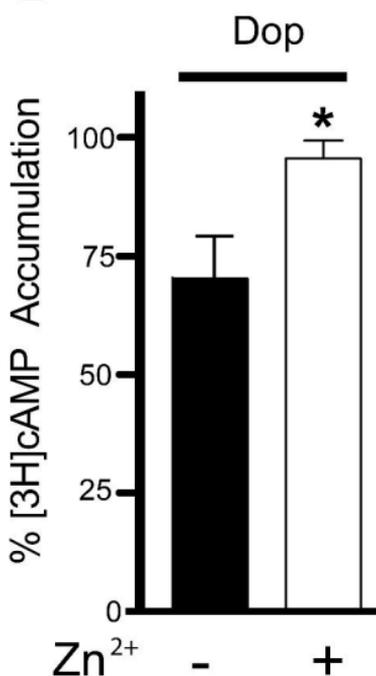


Figure 9

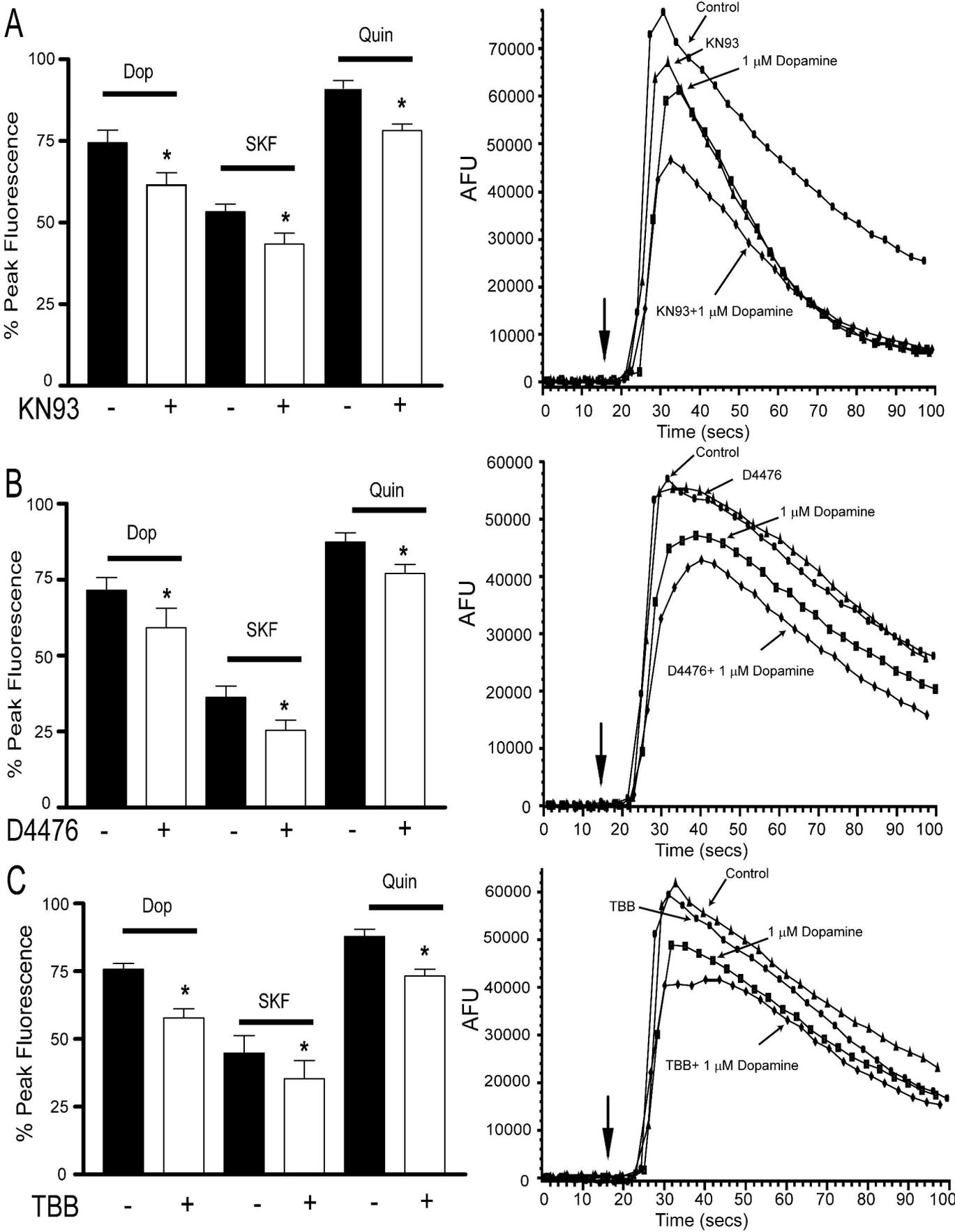
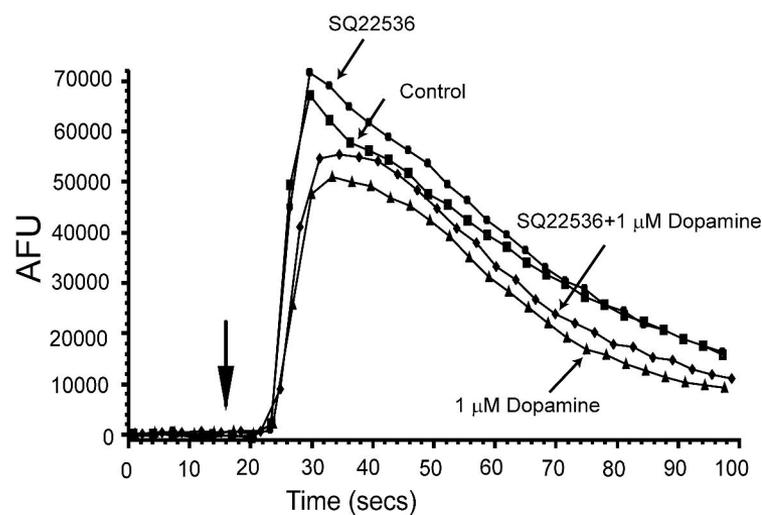
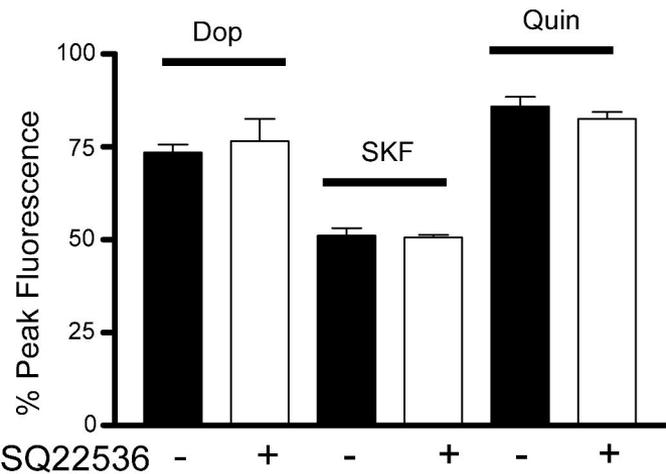


Figure 10

A



B

