Reversal of dopamine inhibition of dopaminergic neurons of the ventral tegmental area is mediated by protein kinase C

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Running Title: PKC mediates D2 receptor desensitization in the VTA

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Non-Standard abbreviations:; pDAergic: putative dopaminergic; DAergic: dopaminergic; VTA: ventral tegmental area; LTP: long term potentiation; NMDA: N-methyl-D-aspartate; AMPA: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; DA: dopamine; cPKC: conventional PKC

Abstract:

Adaptation of putative dopaminergic (pDA) neurons in the ventral tegmental area (VTA) to drugs of abuse may alter information processing related to reward and reinforcement and is a key factor in the development of addiction. We have demonstrated that prolonged increases in the concentration of dopamine (DA) result in a time-dependent decrease in sensitivity of pDA neurons to DA, which we termed dopamine inhibition reversal (DIR). In the present study, we used extracellular recordings to examine factors mediating DIR. A 40 min administration of DA (2.5-10 μM), but not the DA D2 receptor agonist quinpirole (50-200 nM), resulted in inhibition of neuronal firing followed by DIR. In the presence of 100 nM cocaine, inhibition followed by DIR was seen with much lower DA concentrations. Reversal of quinpirole inhibition could be induced by an activator of protein kinase C, but not of protein kinase A. Inhibitors of protein kinase C or phospholipase C blocked the development of DIR. Disruption of intracellular calcium release also prevented DIR. Reduction of extracellular calcium or inhibition of store-operated calcium entry blocked DIR, but the L-type calcium channel blocker nifedipine did not. DIR was age-dependent and not seen in pDA VTA neurons from rat pups younger than 15 days postnatal. Our data indicate that DIR is mediated by protein kinase C, and implicate a conventional protein kinase C. This characterization of DIR gives insight into the regulation of autoinhibition of pDA VTA neurons, and the resulting long-term alteration in information processing related to reward and reinforcement.

Key Words: Dopamine, Protein kinase C, ventral tegmental area, desensitization, D2 receptor, D5 receptor

Introduction:

Changes in dopamine neurotransmission in the VTA and its targets have been related to the salience of stimuli, and may play a role in information processing related to reward and reinforcement (Mirenowicz and Schultz 1996; Schultz 2010). Increases in dopaminergic neurotransmission are associated with most drugs of abuse (Di Chiara and Imperato 1988; Imperato and Di Chiara 1986; Imperato et al. 1986) and may be a key event in reward and reinforcement (Wise 1996). Dendritic DA release in response to increased activity of mesencephalic DA neurons (Cragg et al. 1997) may affect the excitability of dopaminergic neurons of the VTA. Elevated dopamine can produce long-term changes in neurotransmission; for example, elevated dopamine can increase glutamatergic receptor expression in prefrontal cortex (Gao and Wolf 2008; Sun et al. 2008).

Of the 5 main classes of DA receptors, the dopaminergic neurons of the VTA possess the "D1-like" D5 receptors (Ciliax et al. 2000; Khan et al. 2000) and the "D2-like" receptors D2 (Sesack et al. 1994), D3 (Stanwood et al. 2000) and D4 (Defagot et al. 1997), with D2 predominating. The D1 receptors appear to be on presynaptic glutamatergic terminals projecting to the region rather than on the DA neurons themselves (Caille et al. 1996).

Putative dopaminergic (pDA) neurons of the VTA are spontaneously active *in vivo* and *in vitro*, and spontaneous firing is inhibited by the action of DA at D2 autoreceptors on the cell bodies and dendrites of these neurons (Lacey et al. 1987). Stimulation of D2 receptors on mesencephalic pDA neurons results in activation of G protein-coupled inwardly rectifying potassium channels by Gβγ without the involvement of cAMP or adenylyl cyclase (Kim et al. 1995). Inhibition of firing of pDA VTA neurons by elevation of extracellular dopamine has been demonstrated by numerous laboratories (Lacey et al. 1987; Brodie and Dunwiddie 1990); we recently demonstrated that elevation of dopamine for an extended period of time results in a time- and concentration-dependent decrease in the magnitude of DA-mediated inhibition, a phenomenon that we termed "dopamine

inhibition reversal" (Nimitvilai and Brodie 2010). This desensitization of the dopamine D2 receptor is dependent on concurrent activation of both D2- and D1-like receptors, requires 10-40 min to develop, and persists for up to 90 min (Nimitvilai and Brodie 2010). Therefore, it is neither due purely to homologous desensitization (dependent only upon D2 activation) nor to heterologous desensitization (dependent only upon activation of a non-D2 receptor). As drugs of abuse are known to increase dopaminergic neurotransmission (by increasing the firing rate of dopamine neurons or by blocking reuptake of dopamine at the terminal) (Di Chiara and Imperato 1988), it is possible that dopamine inhibition reversal could result from exposure to abused substances. In order to better understand this phenomenon, we investigated some of the second messenger mechanisms that might contribute to the expression of dopamine inhibition reversal. For these studies, extracellular recording from pDA VTA neurons in brain slices was used to avoid disrupting the intracellular milieu while monitoring spontaneous firing of pDA VTA neurons for long continuous time periods.

Methods:

Animals: Fischer 344 (F344; adult rats, 4-6 weeks old, 90 - 150 g; or rat pups, 12-18 days old) used in these studies were obtained from Harlan Sprague-Dawley (Indianapolis, IN). All rats were treated in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and all experimental methods were approved by the Animal Care Committee of the University of Illinois at Chicago.

Preparation of brain slices: Brain slices containing the ventral tegmental area (VTA) were prepared from the subject animals as previously described (Brodie et al. 1999a). Briefly, following brief isoflurane anesthesia and rapid removal of the brain, the tissue was blocked coronally to contain the VTA and substantia nigra; the cerebral cortices and a portion of the dorsal mesencephalon were removed. The tissue block was mounted in the vibratome and submerged in chilled cutting solution to cut coronal sections (400 μm thick). An individual slice was placed onto a mesh platform in the recording chamber and was totally submerged in aCSF maintained at a flow rate of 2 ml/min; the temperature in the recording chamber was kept at 35º C. The composition of the aCSF in these experiments was (in mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.24, $CaCl_2$ 2.4, $MgSO_4$ 1.3, $NaHCO_3$ 26, glucose 11. In some experiments, a low calcium buffer was used; composition of this solution was (in mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.24, $CaCl_2$ 0.24, $MgSO_4$ 1.3, $NaHCO_3$ 26, glucose 11, adjusted to pH 7.4 with $MgSO_4$. The composition of the cutting solution was (in mM): KCl 2.5, $CaCl_2$ 2.4, $MgSO_4$ 1.3, $NaHCO_3$ 26, glucose 11, and sucrose 220. Both solutions were saturated with 95% O_2/ 5% CO_2 (pH=7.4). Equilibration time of at least one hour was allowed after placement of tissue in the recording chamber before electrodes were placed in the tissue.

Cell identification: The VTA was clearly visible in the fresh tissue as a grey area medial to the darker substantia nigra, and separated from the nigra by white matter. Recording electrodes were placed in the VTA under visual control. pDAergic neurons have been shown to have distinctive electrophysiological characteristics (Grace and Bunney 1984; Lacey et al. 1989). Only those neurons

which were anatomically located within the VTA and which conformed to the criteria for pDAergic neurons established in the literature and in this laboratory (Lacey et al. 1989; Mueller and Brodie 1989) were studied. These criteria include broad action potentials (2.5 msec or greater, measured as the width of the bi- or tri-phasic waveform at the baseline), slow spontaneous firing rate (0.5 - 5 Hz), and a regular interspike interval. Cells were not tested with opiate agonists as has been done by other groups to further characterize and categorize VTA neurons (Margolis et al. 2006). It should be noted that some neurons with the characteristics we used to identify DA VTA neurons may not, in fact, be DA-containing (Margolis et al. 2006). For this reason, we refer to the neurons recorded in this study as putative dopaminergic (pDA) VTA neurons.

Additional characterization, such as determining the projection target of our cells of study (Margolis et al. 2008) would have been difficult as we have used extracellular recording to insure high quality, long duration recordings. The long-duration, low frequency action potentials which characterized the cells from which we recorded are associated with DA-sensitive, DA-containing neurons projecting to the nucleus accumbens, and DA sensitivity also is associated with DA VTA neurons projecting to prefrontal cortex (Margolis et al. 2008). One consequence of differential initial sensitivity to dopamine inhibition among groups of neurons projecting to different brain areas (Margolis et al. 2008; Lammel et al. 2008) would be different amounts of dopamine inhibition reversal (Nimitvilai and Brodie 2010), resulting in a greater relative change in neurons more sensitive to dopamine inhibition.

Drug Administration: In most experiments, drugs were added to the aCSF by means of a calibrated infusion pump from stock solutions 100 to 1000 times the desired final concentrations. The addition of drug solutions to the aCSF was performed in such a way as to permit the drug solution to mix completely with aCSF before this mixture reached the recording chamber. Final concentrations were calculated from aCSF flow rate, pump infusion rate and concentration of drug

stock solution. The small volume chamber (about 300µl) used in these studies permitted the rapid application and washout of drug solutions. Typically drugs reach equilibrium in the tissue after 2 to 3 minutes of application.

In some experiments, drugs were added to the microelectrode filling solution (0.9% NaCl) at a concentration about 10 times greater than that which would have been used in the extracellular medium. To allow time for the drug to diffuse from the pipette to the cell, the effects of bath-applied drugs were tested no less than 20 min after initiating the recording; this pipette-application method has produced comparable results to the administration of drugs through the extracellular medium in the cases in which both methods were tested (data not shown), with the advantage of more localized application and reduced expense. One disadvantage of this method is that the exact concentration of drug received by the neurons from which we recorded is unknown.

DA hydrochloride, quinpirole, and most of the salts used to prepare the extracellular media were purchased from Sigma (St. Louis, MO). Forskolin, 8-bromo cyclic AMP, phorbol 12-myristate 13-acetate (PMA), bisindolylmaleimide-I (BIS-I), Gö6976 (5,6,7,13-tetrahydro-13-methyl-5-oxo-12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrile), PKC β inhibitor (3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione), nifedipine, caffeine, SKF96365 (1-[2-(4-methoxy-phenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1H-imidazole hydrochloride), thapsigargin, chelerythrine chloride, ryanodine, 2-aminoethoxydiphenyl borate (2-APB), and U73122 were purchased from Tocris (Ellisville, MO). (-) Cocaine HCl was purchased from Research Biochemicals, Inc, Natick, MA.

Extracellular recording: Extracellular recording was chosen for these studies as this method permits the recordings to be of long duration and allows us to assess the effects of extended exposure (>30 minutes) to drugs. The limitation of only measuring spontaneous action potential frequency (rather than membrane potential or other electrophysiological parameters) is counterbalanced by the advantage of being able to determine the time course of drug actions and

interactions. Extracellular recording electrodes were made from 1.5 mm diameter glass tubing with filament and were filled with 0.9% NaCl. Tip resistance of the microelectrodes ranged from 2 – 4 MΩ. A Fintronics amplifier was used in conjunction with an IBM-PC-based data acquisition system (ADInstruments Inc., Colorado Springs, CO). Offline analysis was used to calculate, display and store the frequency of firing in 1-minute intervals. Additional software was used to calculate the firing rate over 5-second intervals. Firing rate was determined before and during drug application. Firing rate was calculated over 1-minute intervals prior to administration of drugs and during the drug effect; peak drug-induced changes in firing rate were expressed as the percentage change from the control firing rate according to the formula ((FRD - FRC) / FRC) X 100, where FRD is the firing rate during the peak drug effect and FRC is the control firing rate. The change in firing rate thus is expressed as a percentage of the initial firing rate, which controls for small changes in firing rate that may occur over time. This formula was used to calculate both excitatory and inhibitory drug effects. Peak excitation produced by the drug (e.g., DA) was defined as the peak increase in firing rate over pre-drug baseline. Inhibition was defined as the lowest firing rate below the pre-drug baseline. Inhibition reversal was identified as a statistically significant reduction in the inhibition.

Data collection: For comparison of the time course of effects on firing rate, the data were normalized and averaged. Firing rates over one minute intervals were calculated and normalized to the one-minute interval immediately prior to DA administration. These normalized data were averaged by synchronizing the data to the DA administration period, and graphs of the averaged data were made.

Statistical analysis: Averaged numerical values were expressed as the mean ± the standard error of the mean (S.E.M.). Mean response graphs are shown as relative change in firing rate, to normalize the response to the inhibition observed in the first five minute interval; mean percentage inhibition as a function of baseline firing rate is indicated in the text. The differences among firing rates during the long drug administration intervals in these studies was assessed with repeated

measures ANOVA; degrees of freedom and statistical error terms are shown as subscripts to F in the text (Kenakin 1987). Statistical analyses were performed with OriginPro 8.5 (OriginLab Corp. Northampton, MA.).

Results:

VTA neuron characteristics

A total of 285 VTA neurons were examined. Their firing rate in normal extracellular medium ranged from 0.61 to 4.56 Hz, with a mean of 1.85 ± 0.05 Hz. All neurons had regular firing rates and were inhibited by DA. Sensitivity to DA (0.5 – 5 μM) was initially assessed by administering the agonist for five minutes, and then washing it out until the baseline firing rate recovered; quinpirole (25-200 nM) was administered for 5 min, and the concentration was increased if inhibition greater than 50% was not achieved. The concentrations of agonist were adjusted for each neuron so that inhibition exceeded 50%, as inhibition that was less than 50% was not reliably reversed (Nimitvilai and Brodie 2010). This method of adjusting the concentration of dopaminergic agonist controlled for differences in sensitivity between neurons, but also sometimes resulted in the mean concentrations of dopamine or quinpirole slightly differing between groups. Overall, for pDA VTA neurons from adult rats (not including cells tested with DA in the presence of cocaine), the concentration of dopamine used was 6.2 ± 0.3 μM (n=92) which produced a mean change in firing rate of -68.1 ± 1.3 %; the concentration of quinpirole used was 52.6 ± 2.2 nM (n=25) which produced a mean change in firing rate of -73.6 ± 2.2 %. For the rat pups used in these experiments, 7.6 ±0.6 μM (n=66) dopamine was used to produce a change in firing rate of -71.0 ± 1.9%. There were no significant differences in the concentration of dopaminergic agonist or in the percentage inhibition between the groups (One-way ANOVA, $p > 0.05$). In most experiments, as blockers of the dopamine transporter were not used, there is normally sufficient dopamine release in brain slices to cause complete cessation of spontaneous firing in the presence of dopamine transporter blockers (Brodie and Dunwiddie 1990). In the absence of DA transporter blockers, dopamine produces inhibitory effects at concentrations ranging from 0.5 to 100 μM, although in dissociated DA VTA neurons, concentrations as low as 50 nM can completely inhibit spontaneous action potential firing (Brodie et al. 1999b). Cells which did not return to at least 70% of their pre-DA firing rate during this washout were not used. One benefit

of the extracellular recording method used in these studies is that long duration recordings can be made reliably; the average recording duration was 71.5 ± 0.7 minutes, with a range of 55 to 90 minutes.

In the course of performing the experiments described below, we used a number of pharmacological agents, delivered either via the extracellular medium or via the recording pipette, and these agents were applied for 15 to 20 min before the 40 min administration of either quinpirole or dopamine. In separate experiments, we tested whether these agents alone altered the firing rate of pDA VTA neurons. The results of these control experiments are shown in Table 1; the firing rates shown in the table are the pre-drug baseline, the firing rate at a time point equivalent to the 5 min time point of DA agonist administration, and the firing rate at a time point equivalent to the 40 min time point at the end of DA agonist administration. In brief, in the concentrations applied in the experiments described below, most of the agents did not significantly alter the firing rate of the neurons tested between the equivalents of the 5 min time point and the 40 min time point (One-way repeated measures ANOVA, $p > 0.05$ for all groups). Two exceptions to this are BIS-I, which produced a small but significant decrease in firing with time and thapsigargin, which produced a small but significant increase in firing with time (One-way repeated measures ANOVA, $p < 0.05$).

Dopamine inhibition reversal occurs at low DA concentrations in cocaine

Reversal of dopamine inhibition with time occurs with dopamine but not with quinpirole, as previously reported (Nimitvilai and Brodie 2010). We extended this observation to determine if the same phenomenon occurred in the presence of a low concentration of cocaine (100 nM) (Figure 1). Figures 1A-1D illustrate data from single neurons, and the pooled data in Figure 1E are presented normalized to the firing rate at the 5 min time point for clarity. A concentration of dopamine was chosen that inhibited the firing rate by 60-80%, and this concentration was applied in the superfusate for 40 min. As in our previous study, DA (6.1 ± 1.4 μM; n=7) produced 80% inhibition at 5 min, and

at 40 min there was significant reduction in this inhibition, illustrated as a relative increase in firing rate of 31.7 ± 3.8 % compared to the 5 min time point, which is indicative of DA inhibition reversal (Figure 1A and 1E) (One-way repeated measures ANOVA, $F_{(7,42)} = 34.42$, $P < 0.01$). In the presence of 100 nM cocaine, only 0.88 ± 0.25 μM DA was needed to achieve 77.9 ± 5.1% inhibition at 5 min, which showed a similar reversal trajectory over time to that of higher dopamine, so that at 40 min, there was a relative increase in firing rate of 32.7 ± 13.6 % (Figure 1B and 1E) (n=8; One-way repeated measures ANOVA, $F_{(7,49)}=19.61$, $P < 0.01$). In the absence of cocaine, 0.88 μM dopamine produced 9.29 ± 3.0% inhibition at 5 min, which by the end of 40 min had a relative decrease of 6.4 ± 3.0 % (Figure 1C and 1E), which was statistically different from the 5 min time point (n=8; One-way repeated measures ANOVA, $F_{(7,70)}= 4.61$, $P < 0.01$). In an additional experiment, 100 nM cocaine alone (Figure 1D) was added to the superfusate for a total of 55 min, a time period equivalent to the 15 min pre-incubation period plus the 40 min period during which DA would have been administered (as in Figure 1B). Cocaine alone produced a small but significant decrease in firing rate over the course of the last 40 min of the administration (n=8; One-way repeated measures ANOVA, $F_{(11,77)}= 5.93$, $P < 0.01$). The results of these experiments indicate that cocaine not only potentiates the inhibitory effect of dopamine (Brodie and Dunwiddie 1990), but increases the likelihood that low dopamine concentrations can produce desensitization of pDA VTA neurons to dopamine.

Activators of PKC but not of PKA reverse quinpirole inhibition of firing

We have demonstrated previously that quinpirole-induced inhibition was sustained over a 40 min application period and did not reverse except in the presence of a D1/D5 agonist (Nimitvilai and Brodie 2010). As D1/D5 and D2 receptors have been linked to the activation and inhibition of the adenylyl cyclase second messenger system, we tested whether quinpirole inhibition was reversed in the presence of forskolin (2.5 μM) or 8-bromo-cAMP (100 μM). As there is evidence of

physiological functions of protein kinase C in pDA VTA neurons (Liu et al. 2003), we also tested whether quinpirole-induced inhibition was reversed in the presence of an activator of protein kinase C, PMA (phorbol 12-myristate 13-acetate, 100 nM). The results of these experiments are shown in Figure 2; Figures 2A-2D illustrate data from single neurons, and the pooled data in Figure 2E are presented normalized to the effect of quinpirole from each group at the 5 min time point for clarity. Similar to our previous study (Nimitvilai and Brodie 2010), administration of quinpirole for 40 min (n=7) inhibited the firing of pDA VTA neurons and showed no reduction of that inhibition for the duration of the 40 min application (Figure 2A and 2E). Comparison of inhibition at 5 min intervals over the 40 min period of drug exposure was normalized to the value measured at the first 5 min time point and showed that there was a significant decrease in firing rate over the duration of quinpirole application (repeated measures ANOVA $F_{(7,42)}=16.68$, $P < 0.01$). Since dopamine receptors are coupled to adenylyl cyclase, we tested the effect of forskolin and 8-bromo-cAMP on quinpirole-mediated inhibition of firing. Administration of quinpirole (n = 7) with 2.5 μM forskolin produced a similar pattern of inhibition as control (Figures 2B and 2E) and comparison of firing rates at 5 min intervals showed a significant decrease in firing rate over the 40 min period of drug exposure (repeated measures ANOVA $F_{(7,42)}=2.58$, $P < 0.05$). Similarly, co-administration of 8-bromo-cAMP (100 μM) and quinpirole (n=6) produced similar inhibition (Figure 2C and 2E); again, comparison of the inhibition at 5 min intervals indicated a significant decrease in firing rate from the 5 min time point (repeated measures ANOVA, $F_{(7,35)}=7.04$, $P < 0.01$).

Since pDA VTA neurons are regulated by protein kinase C (Liu et al. 2003; Luu and Malenka 2008), we investigated if activation of PKC alters the effect of quinpirole. In the presence of the PKC activator phorbol 12-myristate, 13-acetate (PMA, 100 nM), quinpirole (n= 5) (Figures 2D and 2E) inhibition declined significantly over time after 10 min, reaching a firing rate 20% greater than the firing rate at the 5 min time point by the end of the 40-min period of drug exposure (repeated measures ANOVA, $F_{(7,28)}=7.18$, $P < 0.001$) and a Tukey post-hoc comparison indicated a

significant difference between rates measured at 10 min versus 30, 35 or 40 min ($P < 0.05$). PMA alone had no significant effect on firing (Table 1), so the reversal of quinpirole inhibition was not due simply to an additive effect of PMA and quinpirole. These results suggest that PKC, but not PKA, antagonizes the inhibitory effect of quinpirole on pDA VTA neuron firing.

PKC inhibitors prevent reversal of DA inhibition of firing

To confirm the importance of PKC in dopaminergic regulation of pDA VTA firing, we co-administered DA with PKC inhibitors and measured firing rates every 5 min, normalizing the results to firing measured after the first 5-min of drug exposure (Figure 3). The general PKC inhibitors bisindolylmaleimide I (BIS-I,) and chelerythrine, and the conventional PKC inhibitor Gö6976 prevented the reversal of DA inhibition of firing. In the presence of BIS-I, chelerythrine or Gö6976, reversal of dopamine induced inhibition was not observed. The inhibition produced by dopamine in the presence of each PKC inhibitor at 5-min intervals was compared using a one-way repeated measures ANOVA. There was no significant change produced by dopamine when BIS-I (repeated measures, $F_{(7,42)}=0.64$, $P > 0.05$) or chelerythrine (repeated measures, $F_{(7,49)}=1.35$, $P > 0.05$) was present in the superfusate. Coapplication of dopamine and Gö6976 produced a significant decrease in firing rate over the time course (repeated measures ANOVA, $F_{(7,28)}=11.23$, $P < 0.01$). In the presence of PKCβ inhibitor (3-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione, 1 μM; n=7), however, dopamine did produce a significant reversal of dopamine induced inhibition over the duration of drug administration (repeated measures ANOVA, $F_{(7,35)}=8.01$, $P < 0.01$) These results implicate the involvement of a conventional isoform of PKC in mediating DA inhibition reversal, but not PKCβ.

Dopamine inhibition reversal: Dependence on phospholipase C

The classical mechanism for activation of PKC is through the phospholipase C (PLC) pathway. In addition, concomitant activation of either D1-D2 or D5-D2 heterooligomers have been demonstrated to stimulate Gq and PLC (Lee et al. 2004; So et al. 2005; Rashid et al. 2007). For these reasons, we examined the action of a PLC inhibitor, U73122, on DA inhibition reversal. In the presence of U73122 (2 μM), there was no reduction of DA inhibition (n = 8) over the 40 min application period (One-way repeated measures ANOVA, $F_{(7,49)} = 1.55$; $P > 0.05$), indicating a blockade of dopamine inhibition reversal (Figure 4).

Dopamine inhibition reversal: Dependence on calcium

Since Gö6976 blocked reversal of DA inhibition, it is likely that a conventional PKC mediates this effect. PKCα, PKCβ, and PKCγ comprise the conventional PKC subfamily, and among all other PKC isozymes, these isozymes are unique in that they can be activated by calcium (Akita et al., 1990). Therefore, we investigated if reversal of DA inhibition was calcium-dependent. Activation of PLC triggers the release of calcium from intracellular stores via stimulation of inositol 1,4,5-triphosphate (IP3) receptors. To determine the importance of calcium release from intracellular stores to DIR, the development of DIR was assessed in the presence of IP3 receptor inhibitor 2-aminothoxydiphenyl borate (2-APB) (10 μM). With 2-APB, no reversal of dopamine inhibition (n = 5) was observed (One-way repeated measures ANOVA, $F_{(7,28)} = 0.26$; $P > 0.05$) (Figure 5A). We also tested whether ryanodine receptors were involved in DIR. Ryanodine acts at ryanodine receptors as either an agonist or antagonist, depending on the concentration, and induces persistent inactivation of the ryanodine receptor at concentrations greater than 100 nM (Zimanyi et al. 1992; Buck et al. 1992). In the presence of ryanodine (10 μM), DA inhibition in firing rate did not reverse but the inhibition increased over the 40 min time course (n = 6) (One-way repeated measures ANOVA, $F_{(7,35)} = 4.43$; $P < 0.01$) (Figure 5B).

Additional studies were performed to determine the involvement of intracellular calcium stores in the development of DA inhibition reversal. High concentrations of caffeine affect intracellular calcium stores by causing release of calcium via activation of the ryanodine receptor (Guerreiro et al. 2011). Thapsigargin causes depletion of calcium from intracellular stores by inhibiting the endoplasmic reticulum Ca^{2+}-ATPase (Bian et al. 1991). To determine the importance of intracellular calcium stores to DA inhibition reversal, caffeine (5 mM) was added to the extracellular medium at least one hour before recordings were performed. In the presence of 5 mM caffeine, there was a significant decrease in firing rate produced by DA over the 40 min administration period (n=5) (repeated measures ANOVA, $F_{(7,35)} = 9.74$, $P < 0.01$), indicating a blockade of DA inhibition reversal (Figure 6A). Likewise, there was no significant reversal of dopamine induced inhibition when thapsigargin was present over the 40 min application period (30 μM in the recording pipette, n=5) (One-way repeated measures ANOVA, $F_{(7,28)} = 1.76$, $P > 0.05$) (Figure 6B).

As there is evidence that calcium release from the intracellular store can trigger the influx of calcium from the extracellular medium (Meldolesi et al. 1992; Sah et al. 1993), we determined the contribution of dihydropyridine-sensitive Ca^{++} channels and store-operated calcium channels. In these experiments, the test drugs were added to the microelectrode filling solution instead of the external medium. The recordings in which drug was added to the micropipette were maintained for 20 min before dopamine was tested to permit sufficient time for the drug to diffuse from the pipette onto the target cells. When a 40 min application of DA (n=10) was tested with voltage-gated calcium channel blocker nifedipine (10 μM) present in the filling solution, there was a significant reduction in the magnitude of DA inhibition over the time course (repeated measures ANOVA, $F_{(7,56)} = 17.71$, $P <0.01$), indicating that nifedipine did not antagonize DA inhibition reversal (Figure 7). In contrast, when an inhibitor of store-operated calcium entry (SOCE) SKF96365 (100 μM) was included in the microelectrode filling solution, dopamine inhibition reversal was blocked (n=8, repeated measures

ANOVA, $F_{(7,49)} = 0.14$, $P > 0.05$) (Figure 7). Therefore, the reversal is dependent on calcium from SOCE, but not on voltage-gated dihydropyridine-sensitive calcium channels.

To reinforce these findings, the development of DIR was assessed in external medium containing a low calcium (0.24 mM) concentration. DA was added stepwise in increasing concentrations until inhibition of firing was 50% or greater compared with baseline. In low calcium medium, dopamine (n=10) inhibited firing and the magnitude of inhibition did not change significantly over 40 min (repeated measures ANOVA, $F_{(7,63)} = 0.64$, $P > 0.05$; Figure 8). This result supports the contention that DA inhibition reversal requires external calcium.

Dopamine inhibition reversal: Age-dependence

The results described above indicate that DA inhibition reversal is dependent upon PKC and both extracellular calcium concentration and intracellular calcium release. The dependence on calcium suggests that a conventional PKC mediates DA inhibition reversal. It has been demonstrated in the rat that different isoforms of PKC are present in different amounts during postnatal development. At birth, PKCα and PKCβ are present in brain and increase rapidly to reach stable levels at 14 days postnatal; in contrast, PKCγ is not present in brain at birth and is at very low levels for the first two weeks, and only reaches a stable level at three to four weeks postnatal (Hashimoto et al. 1988; Yoshida et al. 1988). We assessed DA inhibition reversal in pDA VTA neurons in brain slices of Fisher 344 rat pups of 12 to 18 days postnatal age. The results of these experiments are shown in Figure 9. At ages 12 (n = 10), 13 (n = 8), and 14 (n = 12) days postnatal, dopamine inhibition did not reverse for the duration of the 40 min DA exposure. A significant inhibition in firing rate was observed from 15 to 40 min time points compared to the 5 min time point in the pups of ages 12 (repeated measures ANOVA, $F_{(7,63)} = 4.05$, $P < 0.01$) and 14 (repeated measures ANOVA, $F_{(7,77)} = 5.74$, $P < 0.01$) days, but no significant change was observed in the pups of age 13 days (repeated measures ANOVA, $F_{(7,49)} = 1.23$, $P > 0.05$). At age 15 days, there was a small, but

significant decrease in dopamine inhibition over time (n = 17), and a Tukey post-hoc comparison indicated a significant difference between rates measured at 5 min versus 35 or 40 min (One-way repeated measures ANOVA, $F_{(7,112)} = 3.89$, $P < 0.01$). For ages 16 (n = 12) and 18 (n = 7) days, the firing rate was initially inhibited, followed by a significant decrease in dopamine-induced inhibition over the course of the 40 min application. There was a significant decrease in dopamine inhibition at the last three time points compared to the 5 or 10 min time point for the pups of ages 16 (One-way repeated measures ANOVA, $F_{(7,70)} = 7.62$, $P < 0.01$) and 18 (One-way repeated measures ANOVA, $F_{(7,42)} = 12.05$, $P < 0.01$) days.

Discussion:

We have previously reported that extended exposure of pDA VTA neurons to inhibitory concentrations of DA results in a time-dependent and concentration-dependent decrease in response to DA, which required concurrent activation of D2-like and D1-like DA receptors (Nimitvilai and Brodie 2010). Recovery of the sensitivity of pDA VTA neurons after DIR can be up to 90 min (Nimitvilai and Brodie 2010). Because it requires concurrent stimulation of two different subtypes of DA receptor, it is neither homologous desensitization nor heterologous desensitization, so we termed this phenomenon "DA inhibition reversal". The results of the present study indicate that this DA inhibition reversal is mediated by PKC, and is also dependent on extracellular calcium concentration and intracellular calcium release, and is age-dependent. In light of the literature on PKC isoforms, the evidence suggests that PKCγ is the subtype of PKC that mediates DA inhibition reversal. Many additional studies might be required to conclusively identify this subtype of PKC, such as gene knock-out studies, but such studies would require availability of a rat knock-out of various PKC isoforms, or establishment of this phenomenon and replication of many of the present results in a mouse strain that would serve as a background for such knockouts. While the results in rat pups suggests age-dependence of the phenomenon, assessment of PKCγ at those ages, beyond what is

found in the literature (Hashimoto et al. 1988), would be suggestive, but not conclusive, as many proteins may show age-dependence (Brewer et al. 2007). More detailed studies in the future will be necessary to work out the full molecular mechanism of this phenomenon. The present report reveals important information on several factors influencing D2 desensitization in rats, and age-dependence of DA inhibition reversal, that is critical for understanding and exploring this phenomenon.

The results reported here indicate our initial efforts to characterize the mechanism of DA inhibition reversal. While there are decades of work linking the activation of dopaminergic receptors with adenylyl cyclase and protein kinase A, there is a relatively recent and growing literature on the actions of dopaminergic agonists not related to increases or decreases in cyclic AMP. Future studies will need to investigate the details of the interaction of protein kinase C and the D2 receptor, including whether protein kinase C initiates internalization of the D2 receptor. Interactions between protein kinase C and dopamine receptors in the literature has focused on heterooligomerization of D1-like and D2-like dopamine receptors (George and O'Dowd 2007). Co-activation of D1-D2 heterooligomers results in stimulation of phospholipase C activity, resulting in an increase in intracellular calcium concentration that is dependent on intracellular calcium stores but not on extracellular calcium (Rashid et al. 2007; So et al. 2009). Stimulation of heterooligomers of D5 and D2 receptors, on the other hand, increase intracellular calcium by mechanisms that require both extracellular calcium and calcium from intracellular stores (So et al. 2009). Increases in intracellular calcium evoked by stimulation of D5-D2 heteromeric receptors was not reduced by nifedipine but was blocked by the SOCE inhibitor SKF96365 (So et al. 2009), similar to our results with DIR. As evidence suggests that D5 receptors (Khan et al. 2000; Ciliax et al. 2000) and D2 receptors (Sesack et al. 1994) are present on dopaminergic neurons of the VTA, and we show here that DIR requires both intracellular and extracellular calcium, the data in the present paper are consistent with the possibility that there are functional D5-D2 heterooligomers on pDA VTA neurons. As intriguing as the possibility of D5-D2 heterooligomers in the VTA may be, other possibilities for interactions

between D2 receptors and protein kinase C may be responsible for DA inhibition reversal. For example, alpha-1 adrenergic (Nabekura et al. 1996) and neurotensin (Thibault et al. 2011) receptors can activate protein kinase C and are present on pDA VTA neurons, and neurotensin can cause desensitization of D2 receptors. While the agonist concentrations used in these experiments would not activate either receptor, there may be more complex interactions at the level of signal transduction that could account for our observations.

A role for PKC in desensitization of D2 receptors has been reported in many systems such as HEK293 cells, striatal and hippocampal neurons (Namkung and Sibley 2004; Bofill-Cardona et al. 2000; Thibault et al. 2011; Lee et al. 2004; So et al. 2005; Rashid et al. 2007). In experiments employing site directed mutagenesis, two PKC phosphorylation sites have been identified in the third intracellular loop of the D2 receptor. Activation of PKC in HEK293T cells caused phosphorylation of D2 receptors, resulting in desensitization and internalization of those receptors; activation of PKA had no effect on the phosphorylation state of D2 receptors (Namkung and Sibley 2004). Additional studies in the VTA will be needed to establish the mechanisms by which reduction in D2 function occurs during DA inhibition reversal, and whether known mechanisms, such as the involvement of beta-arrestin and dynamin (Thibault et al. 2011), contribute to the desensitization.

In general, in control experiments, the antagonists used in this study had no significant effects on their own over the period equivalent to the 40 min DA agonist application period (Table 1). One exception was BIS-I, which produced a small but significant change in firing rate. The reasons may be related to the spectrum of action of BIS-I, which can also antagonize PKCδ and PKCε at concentrations 10 fold higher than those that block conventional PKCs (Toullec et al. 1991), whereas Gö6976 has more selectivity for conventional PKC isoforms (Martiny-Baron et al. 1993). The other antagonist that altered the firing rate in a time-dependent manner was thapsigargin, which alters internal calcium release by interfering with the Ca^{2+} ATPase, producing an increase in cytosolic calcium (Treiman et al. 1998), which may be the cause of the significant increase in firing

rate. Ryanodine and 2-APB, on the other hand, had no significant time-dependent effect on firing rate, and neither of these drugs produces a rise in internal calcium (Sutko et al. 1997; Peppiatt et al. 2003). Our results in rat pups suggest that there is a minimum age at which DA inhibition reversal is expressed. While clearly seen at 16 days postnatal, it was not observed at ages of 14 days postnatal or younger (Figure 9). Information from the literature indicates that there are developmental differences in the amounts of different protein kinase C isoforms present in rat brain. For conventional PKC isoforms in whole brain, an early study was unable to detect the gamma isoform of protein kinase C (PKCγ) at ages younger than 2 weeks in Sprague-Dawley rats (Hashimoto et al. 1988). Another study showed that whole brain PKCγ levels increase later than those of other conventional PKCs, reaching less than 25% of the adult level by 14 days, whereas PKCα and PKCβ are nearly to their adult levels at 14 days (Yoshida et al. 1988). Examination of mRNA expression in rat brain indicate that different isoforms of PKC are expressed heterogeneously in various brain areas, and that there are changes (increases and decreases) between postnatal day 7 and postnatal day 21 of conventional PKCs in most brain regions that were examined (Minami et al. 2000). Additional studies will be required to determine whether the age-dependent expression of DA inhibition reversal in Fischer 344 rat pups is related to the developmental emergence of PKCγ, another PKC isoform, or other proteins integral to DIR.

When quinpirole was tested in the presence of forskolin or 8-bromo-cAMP, the mean concentrations of quinpirole required to produce greater than 50% inhibition were greater (72 and 60 nM, respectively) than those needed in the presence of PMA (35 nM) or under control conditions (40 nM) (Figure 2). We previously reported that much higher concentrations of quinpirole alone (3 μM) are needed to observe DIR, and this DIR is also blocked by D1 antagonists (Nimitvilai and Brodie 2010). In the case of forskolin and 8-bromo-cAMP in the present study, moderately higher concentrations of quinpirole may have been required due to an action of increased protein kinase A activity on D2 sensitivity. Desensitization of D1 receptors can be at least partly mediated by protein

kinase A phosphorylation of the D1 receptor (Jiang and Sibley 1999). However, in HEK293T cells protein kinase C, not protein kinase A, caused desensitization of D2 receptors (Namkung and Sibley 2004). Desensitization of D1-D2 heterooligomers can be produced by occupancy of with either D2 or D1 agonist alone (So et al. 2007; So et al. 2005). Detailed additional studies could determine whether D2 receptors in the VTA are regulated in a similar manner, or whether phosphorylation of the D2 receptor, D5 receptor, or both receptors, occurs via protein kinase A and can lead to decreased sensitivity of D2 receptors.

The literature suggests some involvement of calcium in interactions between D1-like and D2 receptors. Studies with heterooligomers of D1-like and D2 receptors indicate an involvement of PLC in mediating increases in intracellular calcium in HEK293 cells (Lee et al. 2004) and brain neurons (Lee et al. 2004; Rashid et al. 2007). We show here participation of PLC in mediating DA inhibition reversal in pDA VTA neurons. PLC was shown to be involved in D5-D2 heterooligomer-induced increases in intracellular calcium (So et al. 2009). In addition, it has been shown that neurotensin, which activates PLC via Gq (Grisshammer and Hermans 2001), can cause desensitization of D2 receptors (Thibault et al. 2011), and this is consistent with our observation that PLC is involved in mediation of DA inhibition reversal. Activation of PLC results in formation of IP3 and DAG from PIP2 , and can produce increases in PKC activity as well as increases in intracellular calcium concentration (Nishizuka 2001). Phosphorylation of D2 receptors by PKC is critical for the mechanism of desensitization of D2 receptors (Thibault et al. 2011). Co-activation of D5-D2 receptors on pDA VTA neurons likely results in activation of PKC and increase in intracellular calcium necessary for phosphorylation of D2 receptors that accounts for the decreased inhibition of firing that we observe during DA inhibition reversal. Future studies will be needed to determine whether the D5-D2 receptor and D2 homomeric receptors of pDA VTA neurons are internalized as a result of the action of PKC (Namkung and Sibley 2004), or if phosphorylation mediates desensitization through a novel mechanism.

Increases in dopamine signaling in response to psychomotor drugs like cocaine have been documented for decades (see Sulzer 2011 for review). The results presented here indicate that DIR can occur at much lower concentrations of dopamine in the presence of 100 nM cocaine than with dopamine alone. As the EC50 of cocaine for monoamine transporters is between 10^{-7} M and 10^{-6} M (Hill et al. 2009), low concentrations of cocaine may increase the likelihood that dopamine in the VTA can cause activation of PKC, and subsequently decrease responsiveness to dopamine autoinhibition. This would result in less autoregulation, and excitatory inputs to the VTA would produce more increases in firing and more dopamine release in terminal fields receiving dopamine innervation. The linkage in the literature between increased dopamine and reward/reinforcement indicate that DIR could augment the reinforcing efficacy of drugs like cocaine by reducing autoinhibition produced by dopamine release in the VTA.The present results support the possible mechanism underlying the reversal of dopamine inhibition that is shown in Figure 10. Co-activation of D2 and D5 receptors causes activation of PLC (probably through a Gq-mediated mechanism (Grisshammer and Hermans 2001)), which in turn results in increases in DAG and IP3. IP3 acts to release calcium from intracellular stores and this also triggers an increase in SOCE. Both the IP3 receptor and the ryanodine receptor are important for controlling intracellular calcium levels in pDA VTA neurons (Morikawa et al. 2000). The increase in DAG, coupled with an increase in intracellular calcium, results in activation of PKC, likely the γ isoform, which in turn phosphorylates D2 receptors, decreasing D2-mediated inhibition (Figure 10). The involvement of Gq, the identification of the precise PKC isoform, and the nature of the phosphorylation event mediated by PKC remain to be fully characterized.

Desensitization of D2 dopamine receptors on dopamine neurons of the VTA is likely to have important ramifications on the excitability of these neurons, and would have consequences on information processing related to reward and reinforcement (Schultz 2010). Drugs like cocaine increase extracellular dopamine concentrations and can cause activation of D2 receptors on pDA

VTA neurons (Brodie and Dunwiddie 1990). One effect of this may be to desensitize D2 receptors, resulting in less autoinhibition. In addition, cocaine can cause a transient increase in AMPA-induced currents through redistribution or phosphorylation of existing AMPA receptors (Ungless et al. 2001; Saal et al. 2003); phosphorylation of AMPA receptors can be mediated by PKC (McDonald et al. 2001). In addition, phosphorylation of NMDA receptors related to LTP in the VTA can be mediated by PKC (Luu and Malenka 2008). It is possible that cocaine-induced increases in extracellular dopamine activate PKC through D5 and D2 receptor stimulation, and this activated PKC may phosphorylate AMPA and/or NMDA receptors, resulting in LTP. As all drugs of abuse can increase extracellular dopamine (Wise 1996; Di Chiara and Imperato 1988), activation of D5 and D2 receptors and induction of a form of LTP may be a common mechanism for long-term effects of abused substances on DA VTA neurons.

Disclosure/Conflicts of Interest:

None of the authors has any known conflicts of interest

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Titles and Legends for Figures:

Figure 1 Effect of cocaine on long-duration dopamine application. A-D, Mean ratemeter graphs of the effects of long duration application of dopamine and cocaine in single neurons. Vertical bars indicate the firing rate over 5 sec intervals. Horizontal bars indicate the duration of drug application (concentrations indicated above bar). (A) Dopamine alone (DA, final test concentration 10 μM) produced a decrease in firing rate of 83.0% which subsided over time so that the inhibition at 40 min (compared to control firing rate) was 41.6%. (B) In the presence of 100 nM cocaine, dopamine (1 μM) produced a 65.9% inhibition at 5 min, and this inhibition also subsided over time so that the inhibition at 40 min was 29.5%. (C) The mean concentration of DA used in the group of experiments similar to that shown in B was 0.88 μM; this concentration of DA given for 40 min produced a maximum decrease in firing rate of 16.8%, with no appreciable reversal. (D) A 40 min application of 100 nM cocaine alone produced a decrease in firing of 31.2% with no appreciable reversal. E. Relative change in firing rate (mean ± S.E.M.) is plotted as a function of time. In experiments similar to those shown in parts A-D above, the effect of dopamine at each time point was normalized by subtracting the change in firing rate (%) at the 5 min time point. A concentration of dopamine that produced more than 60% inhibition at 5 min time point was applied for 40 min (■). Dopamine ([DA] = 6.1 ± 1.4 μM (n = 7)) inhibition of firing rate at 5 min was followed by a significant decrease in dopamine-induced inhibition over time (One-way repeated measures ANOVA, $F_{(7,42)}$ = 34.42, $P < 0.01$). In the presence of cocaine (100 nM, o), a much lower concentration of dopamine ([DA] = 0.88 ± 0.7 μM, n = 8) also showed significant reversal of dopamine inhibition over time (One-way repeated measures ANOVA, $F_{(7,49)}$ = 19.61, $P < 0.01$). In the absence of cocaine, a low concentration of dopamine (0.88 μM, n = 11, ▼) produced a small but significant decrease in firing rate, in that the last three time points were significantly different from the 5 min time point (One-way repeated measures ANOVA, $F_{(7,70)}$ = 4.61, $P < 0.01$) In the absence of applied dopamine, 100

nM cocaine, (n = 8, □) produced a small but significant decrease in firing rate, in that the last two time points were significantly different from the 5 min time point (One-way repeated measures ANOVA, $F_{(11,77)} = 4.61$, $P < 0.01$)

Figure 2 Effects of activators of protein kinase A and protein kinase C on long-duration quinpirole application. A-D, Mean ratemeter graphs of the effects of protein kinase activators on long-duration quinpirole administration in single neurons. Vertical bars indicate the firing rate over 5 sec intervals. Horizontal bars indicate the duration of drug application (concentrations indicated above bar). Quinpirole (Q) alone (A) produced an inhibition of firing rate for the duration of quinpirole application, and the firing resumed after washout of the quinpirole. In the presence of either forskolin (B) or 8-Bromo-cAMP (C), there was no change in quinpirole-induced inhibition. In the presence of the phorbol ester PMA (D), a decrease in quinpirole inhibition was observed over the time course of quinpirole application. E, Relative change in firing rate (mean ± S.E.M.) in response to long-duration quinpirole application in the absence or presence of protein kinase activators is plotted as a function of time. Effect of quinpirole at each time point was normalized by subtracting the change in firing rate (%) at the 5 min time point. A concentration of quinpirole that after 5 min produced 50% inhibition or greater was applied for 40 min. Quinpirole alone (■, [quinpirole] = 40 ± 5.1 nM, n = 7) produced inhibition of the firing rate, which showed a significant decrease over the 40 min duration of quinpirole application (one-way repeated measures ANOVA, $F = 16.68$, $P < 0.01$). A significant increase in quinpirole inhibition was observed over time in the presence of 2.5 μM forskolin (▼, [quinpirole] = 72.43 ± 15.8 nM, n = 7) (one-way repeated measures ANOVA, $F = 2.58$, $P < 0.05$) and in the presence of 100 μM 8-Bromo-cAMP (∇, [quinpirole] = 60 ± 9.6 nM, n = 6) (one-way repeated measures ANOVA, $F = 7.04$, $P < 0.01$). In the presence of 100 nM PMA, there was a significant *reduction* in quinpirole inhibition over time (○, [quinpirole] = 35 ± 10 nM, n = 5),

with the last three time points significantly different from the 10 min time point (one-way repeated measures ANOVA, $F = 7.18$, $P < 0.001$; Tukey post-hoc test, $P < 0.05$).

Figure 3 Effects of protein kinase C inhibitors on long-duration dopamine application. Relative change in firing rate (mean ± S.E.M.) is plotted as a function of time. Effect of dopamine at each time point was normalized by subtracting the change in firing rate (%) at the 5 min time point. A concentration of DA that after 5 min produced more than 60% inhibition was applied for 40 min alone (■, [DA] = 6.1 ± 1.4 μM; n = 7), or in the presence of 10-25 μM chelerythrine (∇, [DA] = 4.75 ± 1.26 μM; n = 8), 1 μM BIS-I (▼, [DA] = 8.57 ± 0.74 μM; n = 7), 1 μM Gö6976 (●, [DA] = 4.9 ± 1.62 μM; n = 5), or 1 μM PKCβ inhibitor (○, [DA] = 4.33 ± 1.3 μM; n = 6). The effect of dopamine alone is shown for comparison (open symbol and dashed line, data from Fig. 1 for reference only). Dopamine produced inhibition in firing rate without a significant reversal when BIS-I (one-way repeated measures ANOVA, $F=0.64$, $P > 0.05$) or chelerythrine (one-way repeated measures ANOVA, $F=1.35$, $P > 0.05$) was present. In the presence of Gö6976, dopamine produced a significant increase of inhibition in firing rate over the 40 min time course (one-way repeated measures ANOVA, $F=11.23$, $P < 0.01$). In contrast, in the presence of the PKCβ inhibitor, a significant reduction in dopamine-induced inhibition over the 40 min time course was observed (one-way repeated measures ANOVA, $F=8.01$, $P < 0.01$)

Figure 4 Effect of phospholipase C inhibitor on long-duration dopamine application. Relative change in firing rate (mean ± S.E.M.) is plotted as a function of time. Effect of dopamine at each time point was normalized by subtracting with the change in firing rate (%) at the 5 min time point. The response to a concentration of dopamine that produced reversal of dopamine-induced inhibition (from Fig. 1) is shown for reference (open symbols and dashed line). A concentration of dopamine

that produced more than 60% inhibition at 5 min time point was applied for 40 min. In the presence of PLC inhibitor U73122 (2 μM), there was no significant change in the inhibition produced by dopamine ([DA] = 7.8 ± 0.7 μM, n = 8) over the time course (one-way repeated measures ANOVA, $F = 1.55$, $P > 0.05$).

Figure 5 Effects of inhibitors of ryanodine and IP3- receptors on long-duration dopamine application. Relative change in firing rate (mean ± S.E.M.) is plotted as a function of time. Effect of dopamine at each time point was normalized by subtracting the change in firing rate (%) at the 5 min time point. The response to a concentration of dopamine that produced reversal of dopamine-induced inhibition (from Fig. 1) is shown for reference (open symbols and dashed line). A concentration of dopamine that produced more than 60% inhibition at 5 min time point was applied for 40 min. A, In the presence of IP3 receptor inhibitor 2-APB (10 μM), there was no significant change in the inhibition produced by dopamine ([DA] = 6.0 ± 1.0 μM, n = 5) over the 40 min time course (one-way repeated measures ANOVA, $F = 0.26$, $P > 0.05$). B, In the presence of the ryanodine receptor inhibitor ryanodine (10 μM), a significant increase in dopamine induced inhibition ([DA] = 7.5 ± 1.12 μM, n = 6) was observed (one-way repeated measures ANOVA, $F = 4.43$, $P < 0.01$).

Figure 6 Effects of depletion of calcium from intracellular stores on long-duration dopamine application. Relative change in firing rate (mean ± S.E.M.) is plotted as a function of time. Effect of dopamine at each time point was normalized by subtracting the change in firing rate (%) at the 5 min time point. The response to a concentration of dopamine that produced reversal of dopamine-induced inhibition (from Fig. 1) is shown for reference (open symbols and dashed line). A, Effect of caffeine on long-duration dopamine application. A concentration of dopamine that produced more than 60% inhibition at 5 min time point was applied for 40 min in aCSF containing 5 mM caffeine. A significant increase in dopamine-induced inhibition ([DA] = 4.2 ± 0.8 μM, n = 5) was observed over

the 40 min time course (one-way repeated measures ANOVA, $F = 9.4$, $P < 0.01$). B, Effect of thapsigargin on long-duration dopamine application. Thapsigargin (30 μM) was added to the microelectrode filling solution and these electrodes were used to measure the change in firing rate. After 20 min of recording, a concentration of dopamine that produced more than 60% inhibition at the 5 min time point was applied for 40 min. There was no significant change in the inhibition induced by dopamine ([DA] = 6.0 ± 1.3 μM, n = 5) over the 40 min time course (one-way repeated measures ANOVA, $F = 1.76$, $P > 0.05$).

Figure 7 Effects of inhibitors of voltage-gated calcium channels and store-operated calcium entry (SOCE) on long-duration dopamine application. Relative change in firing rate (mean ± S.E.M.) is plotted as a function of time. Effect of dopamine at each time point was normalized by subtracting the change in firing rate (%) at the 5 min time point. The response to a concentration of dopamine that produced reversal of dopamine-induced inhibition (from Fig. 1) is shown for reference (open symbols and dashed line). The tested drugs were added to the microelectrode filling solution and these electrodes were used to measure the change in firing rate. After 20 min of recording, a concentration of dopamine that produced more than 60% inhibition at the 5 min time point was applied for 40 min. In the presence of the voltage-gate calcium channel blocker nifedipine (▼, 10 μM), a significant reduction in dopamine inhibition over the 40 min time course was observed ([DA] = 5.3 ± 1.1 μM, n = 10), with the last 4 time points significantly different from the 10 min time point (one-way repeated measures ANOVA, $F = 17.71$, $P < 0.01$; Tukey post-hoc test, $P < 0.05$). In the presence of the SOCE inhibitor SKF96365 (■, 100 μM), there was no significant change in the inhibition induced by dopamine ([DA] = 8.4 ± 1.2 μM, n = 8) over the 40 min time course (one-way repeated measures ANOVA, $F = 1.37$, $P > 0.05$).

Figure 8 Effect of low extracellular calcium buffer on long-duration dopamine application. Relative change in firing rate (mean ± S.E.M.) is plotted as a function of time. Effect of dopamine at each time point was normalized by subtracting the change in firing rate (%) at the 5 min time point. The response to a concentration of dopamine that produced reversal of dopamine-induced inhibition (from Fig. 1) is shown for reference (open symbols and dashed line). A concentration of DA that after 5 min produced more than 60% inhibition was applied for 40 min in low calcium buffer ([Ca2+] = 0.24 mM). No significant reduction in dopamine-induced inhibition over the 40 min time course was observed when dopamine (6.25 ± 1.3 μM; n = 10) was applied in low calcium buffer (one-way repeated measures ANOVA, $F = 0.64$, $P > 0.05$).

Figure 9 Age-dependence of DA inhibition reversal. The pDA VTA neurons in brain slices of rat pups of 12 to 18 days postnatal age were examined. Relative change in firing rate (mean ± S.E.M.) is plotted as a function of time. Effect of dopamine at each time point was normalized by subtracting the change in firing rate (%) at the 5 min time point. A concentration of dopamine that produced more than 60% inhibition at 5 min time point was applied for 40 min. Dopamine produced a steady (age 13) or increasing (ages 12 and 14) inhibition of firing over the 40 min time course of application in pDA VTA neurons from rat pups ages 12 (□, one-way repeated measures ANOVA, $F=4.05$, $P < 0.01$), 13 (▼,one-way repeated measures ANOVA, $F=1.23$, $P > 0.05$) and 14 days (○, one-way repeated measures ANOVA, $F=5.74$, $P > 0.05$). At age 15 days postnatal, there was a small but significant reversal in dopamine-induced inhibition over the 40 min time course of administration (■,one-way repeated measures ANOVA, $F=3.89$, $P < 0.01$). A significant reversal of DA inhibition was observed in pDA VTA neurons from rat pups ages 16 (∇, one-way repeated measures ANOVA, $F=7.62$, $P < 0.01$) and 18 (●,one-way repeated measures ANOVA, $F=12.05$, $P < 0.01$) days postnatal.

Figure 10 Possible mechanism underlying the reversal of dopamine inhibition of pDA VTA neurons. Concurrent stimulation of D2 and D5 receptors causes an activation of PLC (probably through Gq-mediated mechanism), resulting in increases in DAG and IP3 formation. Calcium release from the intracellular stores is induced by IP3 receptor activation, which triggers an influx of calcium through the SOCE. The activated DAG and the increase in intracellular calcium stimulate cPKC, likely the γ isoform, which may further phosphorylate D2 receptors, decreasing D2-mediated inhibition.

TABLE 1

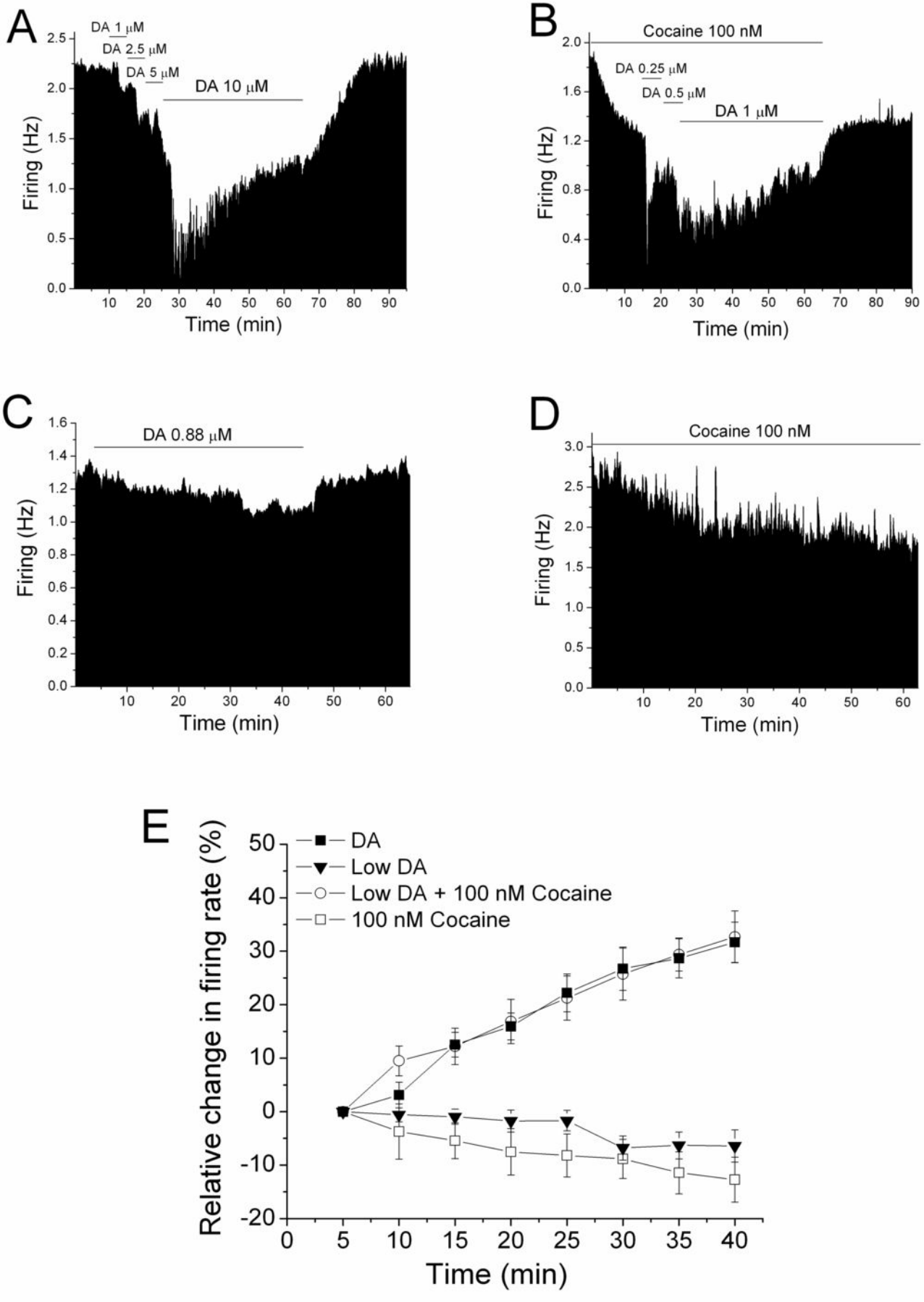
Chemical name	Chemical conc. (μM) (in delivery tubing)	No. of cells	Mean firing rate at baseline (Hz)	Mean firing rate at 20 min (Hz)	Mean firing rate at 55 min (Hz)	P value
Forskolin	2.5	5	2.18 ± 0.6	2.32 ± 1.5	2.19 ± 0.6	> 0.05
8-Bromo-cAMP	100	6	1.92 ± 0.3	1.96 ± 0.8	2.00 ± 0.3	> 0.05
PMA	0.1	5	2.08 ± 0.4	1.87 ± 0.6	1.77 ± 0.2	> 0.05
PKC-β inhibitor	1	5	1.75 ± 0.2	1.73 ± 0.4	1.79 ± 0.2	> 0.05
Bis-I	1	5	1.95 ± 0.1	1.95 ± 0.2	1.80 ± 0.2	< 0.05
Gö6976	1	7	1.25 ± 0.4	1.22 ± 0.3	1.16 ± 0.1	> 0.05
Chelerythrine	10	4	2.66 ± 0.3	2.56 ± 0.7	2.75 ± 0.4	> 0.05
U73122	2	8	2.27 ± 0.3	2.27 ± 0.9	2.08 ± 0.3	> 0.05
Ryanodine	10	5	0.97 ± 0.1	0.86 ± 0.2	0.90 ± 0.03	> 0.05
2-APB	10	7	2.03 ± 0.3	1.87 ± 0.9	1.77 ± 0.4	> 0.05

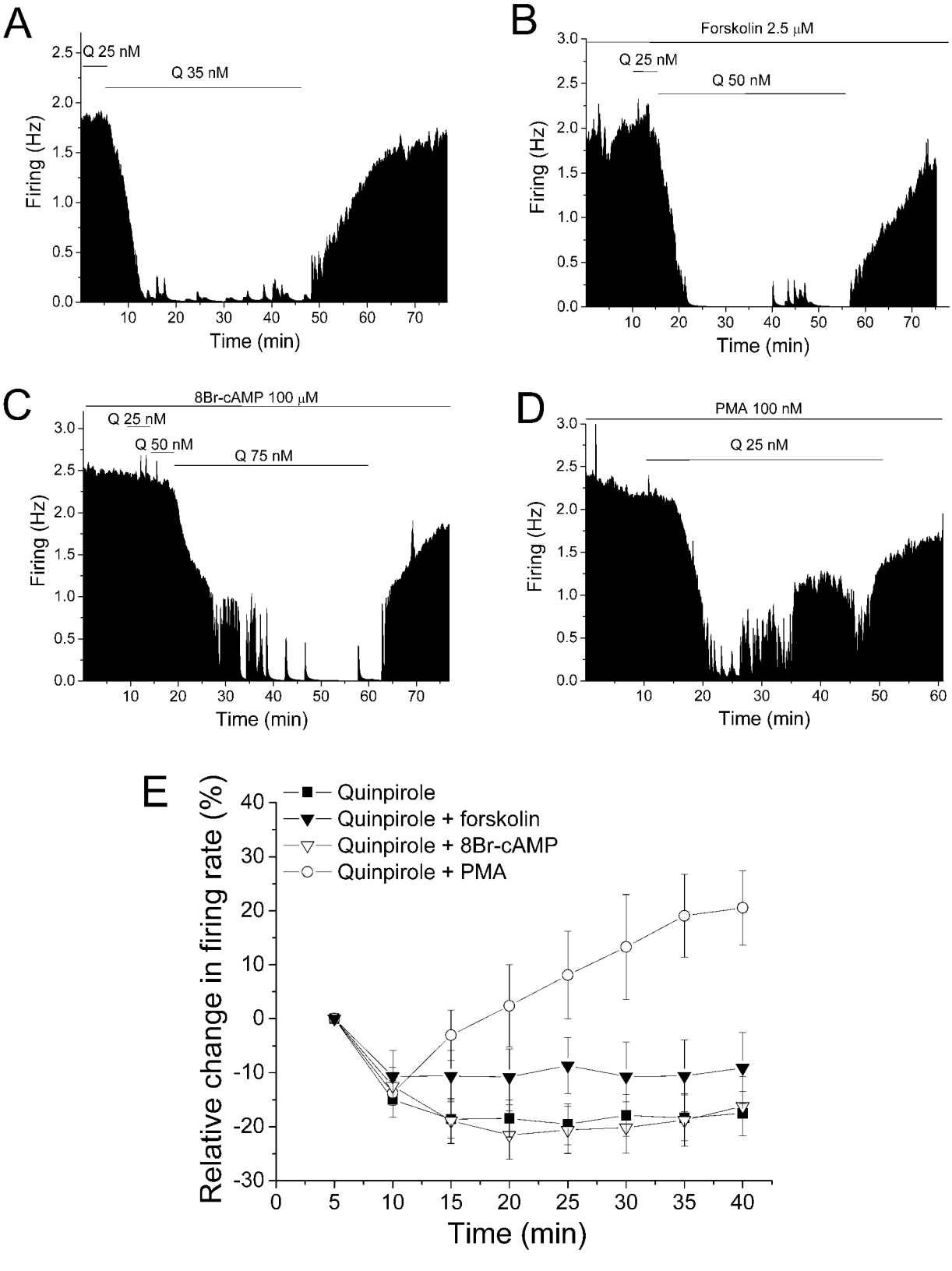
Chemical name	Chemical conc. (μM) (in pipette)	No. of cells	Mean firing rate at baseline (Hz)	Mean firing rate at 25 min (Hz)	Mean firing rate at 60 min (Hz)	P value
Nifedipine	10	6	1.15 ± 0.2	1.06 ± 0.3	1.05 ± 0.2	> 0.05
SKF96365	100	5	2.88 ± 0.3	2.79 ± 0.8	2.68 ± 0.3	> 0.05
Thapsigargin	30	9	1.04 ± 0.1	1.0 ± 0.38	1.07 ± 0.1	< 0.05

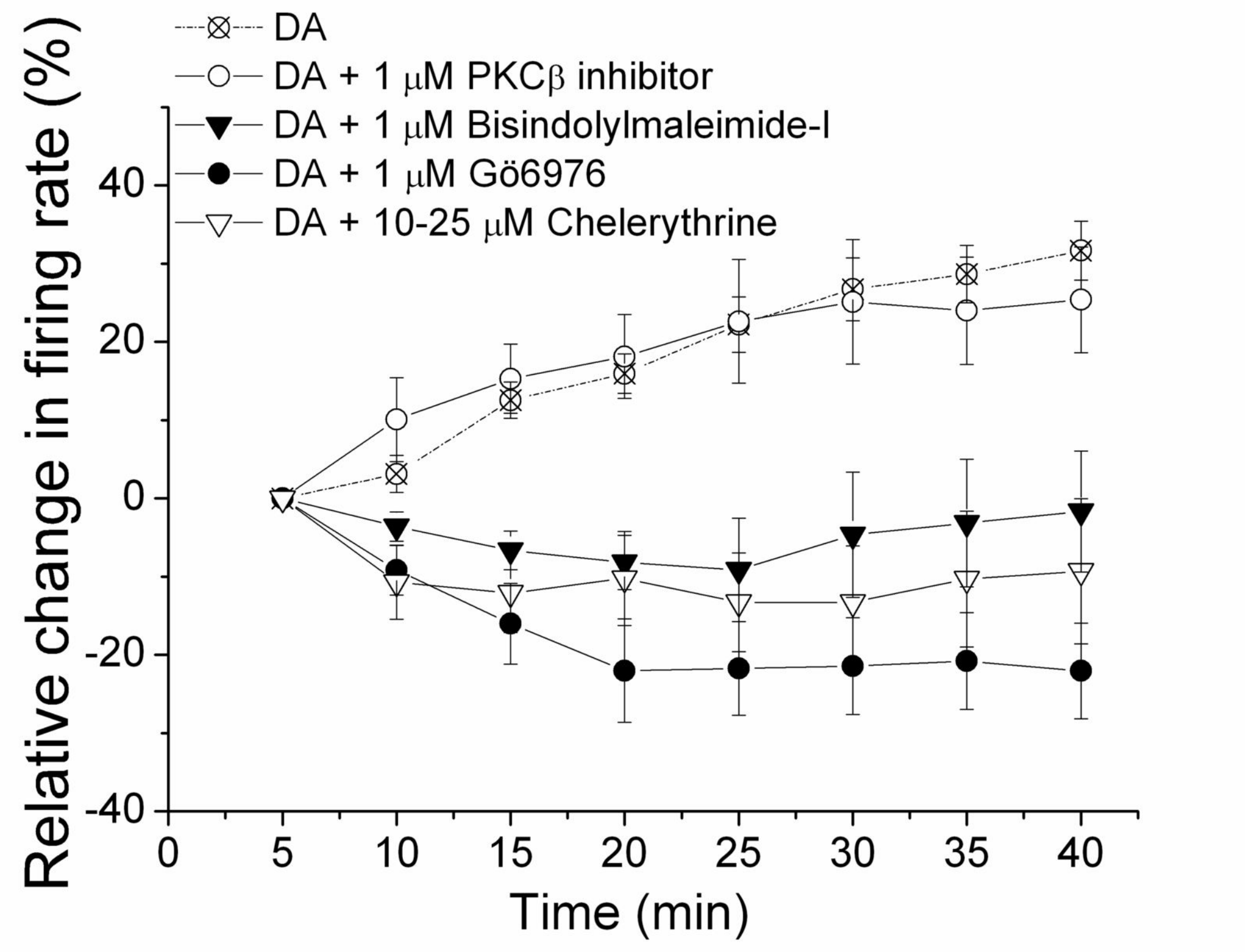
Chemical name	Chemical conc. (in delivery tubing)	No. of cells	Mean firing rate at baseline (Hz)	Mean firing rate at 55 min (Hz)	F value	P value	Significant at 0.05
Forskolin	2.5 μM	5	2.18258	2.18556	0.00806	1.00000	No
8-Bromo-cAMP	100 μM	6	1.9198	2.00107	0.02747	1.00000	No
PMA	100 nM	5	2.07778	1.77492	0.08239	0.99995	No
PKC-β inhibitor	1 μM	5	1.75466	1.79626	0.02931	1.00000	No
BIS-I	1 μM	5	1.94664	1.80552	0.23183	0.99401	No
Go6976	1 μM	7	1.25116	1.1591	0.07652	0.99997	No
Chelerythrine	10 μM	4	2.65775	2.75218	0.02552	1.00000	No
U73122	2 μM	8	2.265	2.07574	0.0715	0.99998	No
Ryanodine	10 μM	5	0.97052	0.90352	0.56096	0.85029	No
2-APB	10 μM	7	2.03394	1.76831	0.0699	0.99998	No

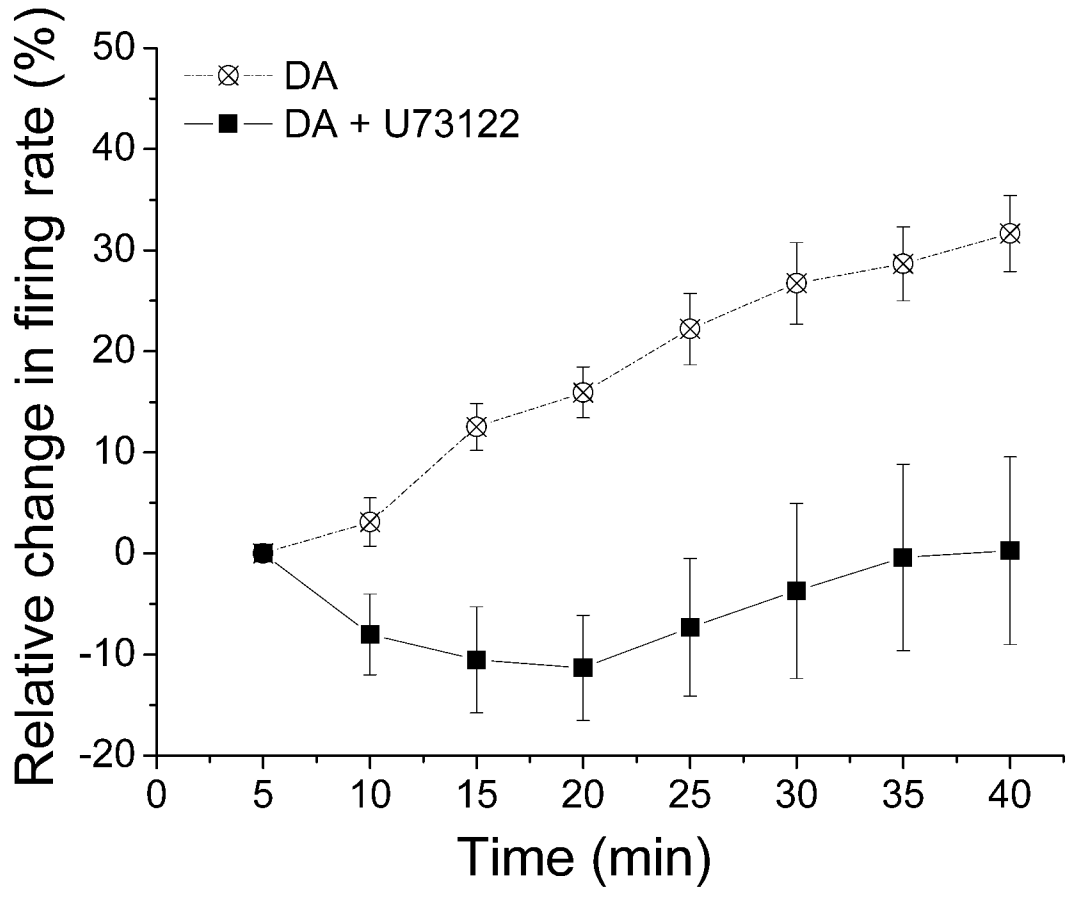
Chemical name	Chemical conc. (in pipette)	No. of cells	Mean firing rate at baseline (Hz)	Mean firing rate at 60 min (Hz)	F value	P value	Significant at 0.05
Nifedipine	10 μM	6	1.15312	1.05135	0.0589	1.00000	No
SKF96365	100 μM	5	2.88138	2.67968	0.04481	1.00000	No
Thapsigargin	30 μM	9	1.04338	1.06904	0.04614	1.00000	No

Experiment	Concentration	Mean Baseline (Hz)	Drugs at 15 min (Hz)	F value	P value	Significant at 0.05
Forskolin	2.5 uM	2.42877	2.54703	0.34509	0.56779	No
8Br-cAMP	100 uM	2.04068	2.2515	1.90277	0.19783	No
PMA	100 nM	1.53504	1.38402	0.20402	0.66349	No
PKC beta inhibitor	1 uM	2.14745	2.11883	0.00666	0.93655	No
BIS-I	1 uM	2.14231	2.15874	0.00569	0.94109	No
Go6976	1 uM	2.0642	1.98844	0.0133	0.91104	No
Chelerythrine	10-25 uM	1.88546	1.73092	0.53021	0.47852	No
U73122	2 uM	2.03871	2.15541	0.1105	0.7445	No
Ryanodine	10 uM	2.0662	2.0731	0.00082	0.97775	No
2-APB	10 uM	2.0311	1.86138	0.10045	0.75941	No
Experiment	**Concentration**	**Mean Baseline (Hz)**	**Drugs at 20 min (Hz)**	**F value**	**P value**	**Significant at 0.05**
Nifedipine	10 uM	1.89005	1.8687	0.00678	0.93531	No
SKF96365	100 uM	1.92548	1.96769	0.01873	0.8931	No
Thapsigargin	30 uM	1.27092	1.29412	0.03581	0.85462	No

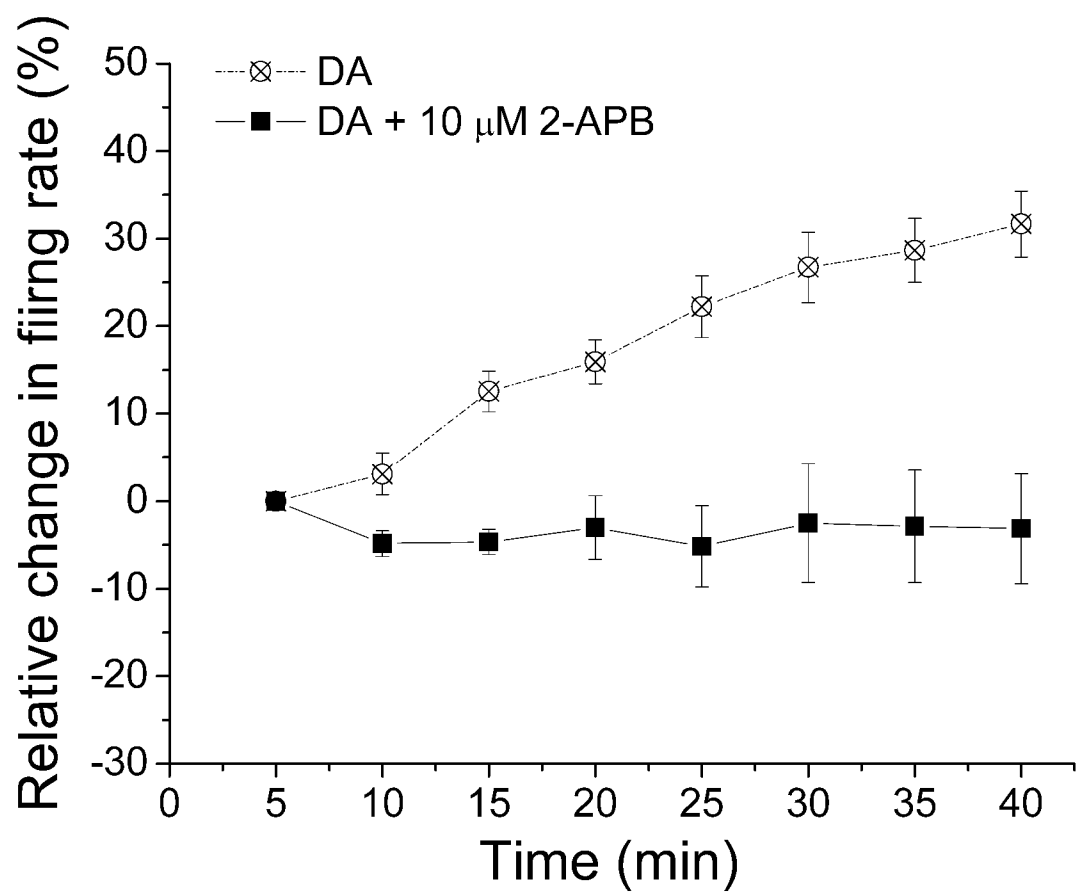

A
DA 1 μM
DA 2.5 μM
DA 5 μM
DA 10 μM
Firing (Hz)
Time (min)
B
Cocaine 100 nM
DA 0.25 μM
DA 0.5 μM
DA 1 μM
Firing (Hz)
Time (min)
C
DA 0.88 μM
Firing (Hz)
Time (min)
D
Cocaine 100 nM
Firing (Hz)
Time (min)
E
DA
Low DA
Low DA + 100 nM Cocaine
100 nM Cocaine
Relative change in firing rate (%)
Time (min)


A
Q 25 nM
Q 35 nM
Firing (Hz)
Time (min)
B
Forskolin 2.5 μM
Q 25 nM
Q 50 nM
Firing (Hz)
Time (min)
C
8Br-cAMP 100 μM
Q 25 nM
Q 50 nM
Q 75 nM
Firing (Hz)
Time (min)
D
PMA 100 nM
Q 25 nM
Firing (Hz)
Time (min)
E
Quinpirole
Quinpirole + forskolin
Quinpirole + 8Br-cAMP
Quinpirole + PMA
Relative change in firing rate (%)
Time (min)

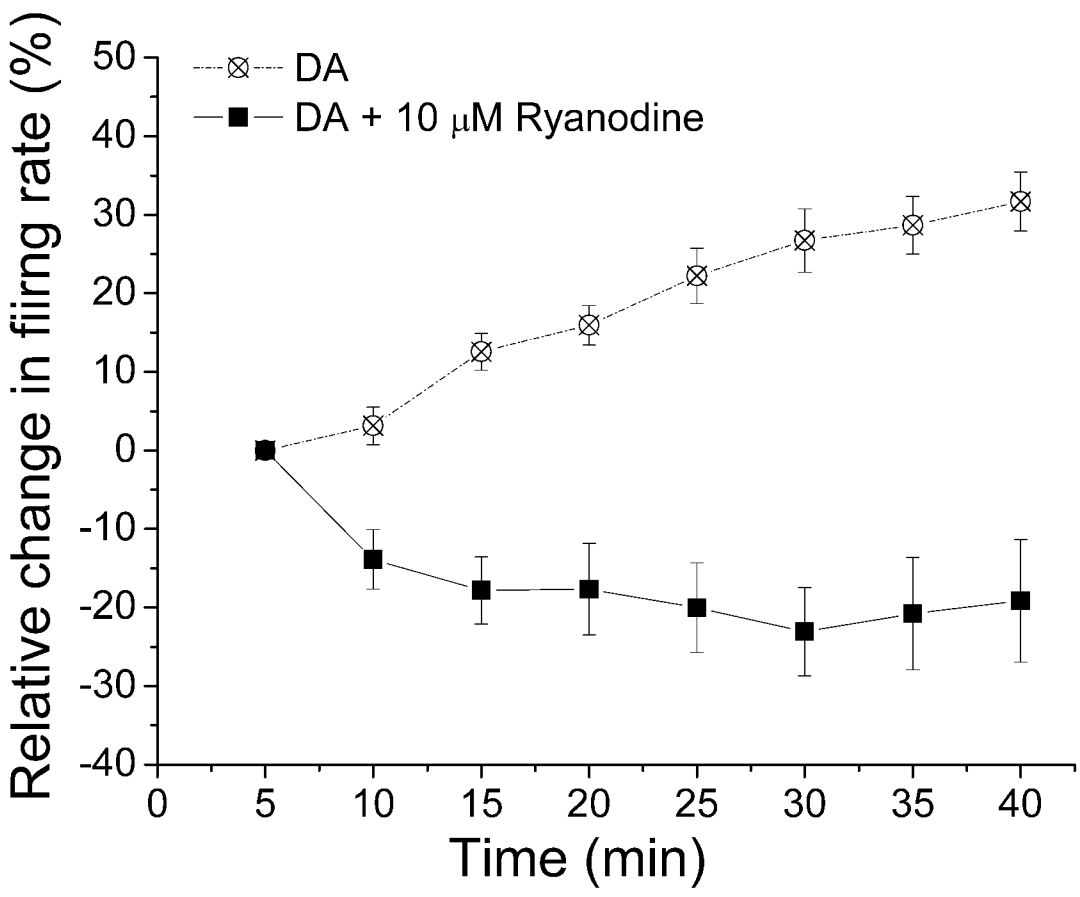

DA
DA + 1 μM PKCβ inhibitor
DA + 1 μM Bisindolylmaleimide-I
DA + 1 μM Gö6976
DA + 10-25 μM Chelerythrine
Relative change in firing rate (%)
40
20
0
-20
-40
0
5
10
15
20
25
30
35
40
Time (min)


DA
DA + U73122
Relative change in firing rate (%)
Time (min)
50
40
30
20
10
0
-10
-20
0
5
10
15
20
25
30
35
40

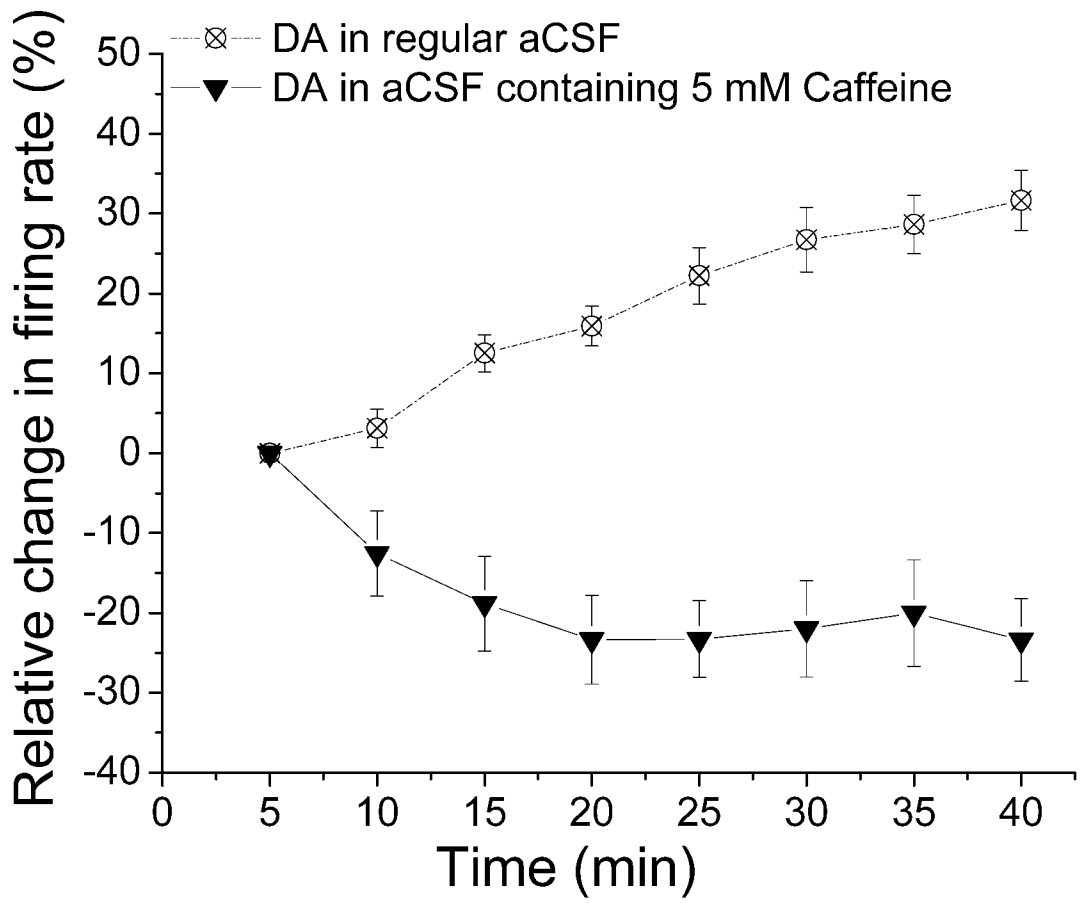
A


DA
DA + 10 μM 2-APB
Relative change in fiirng rate (%)
50
40
30
20
10
0
-10
-20
-30
0
5
10
15
20
25
30
35
40
Time (min)

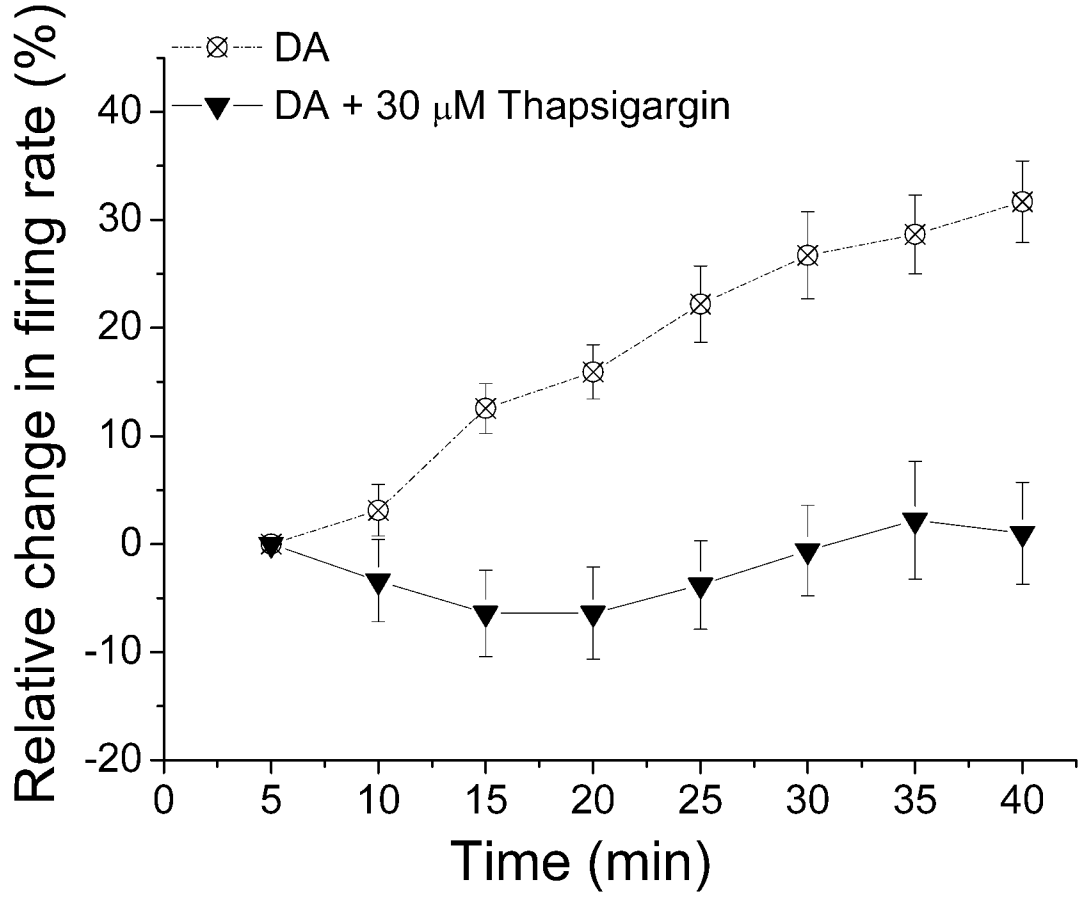
B

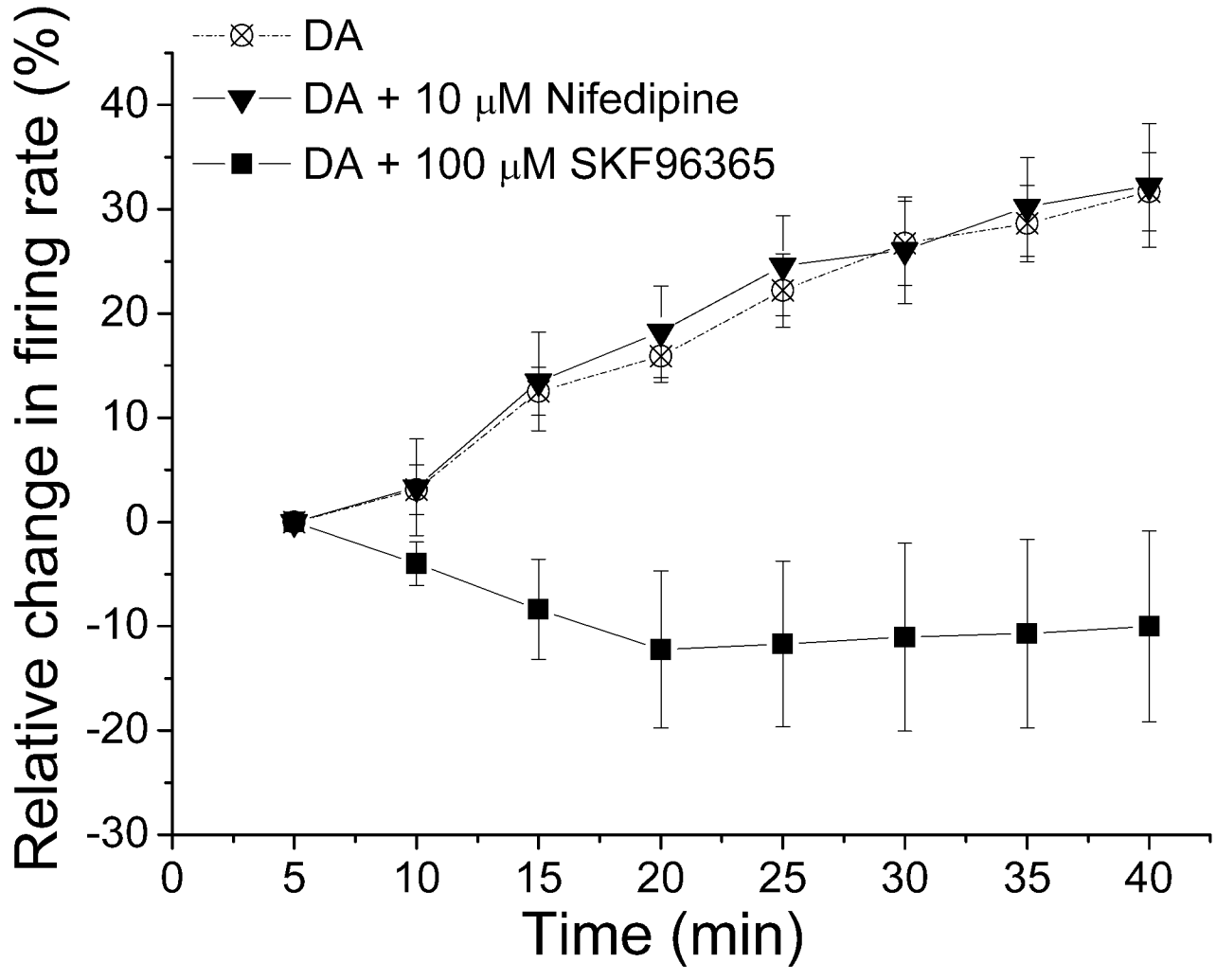

DA
DA + 10 μM Ryanodine
Relative change in fiirng rate (%)
50
40
30
20
10
0
-10
-20
-30
-40
0
5
10
15
20
25
30
35
40
Time (min)

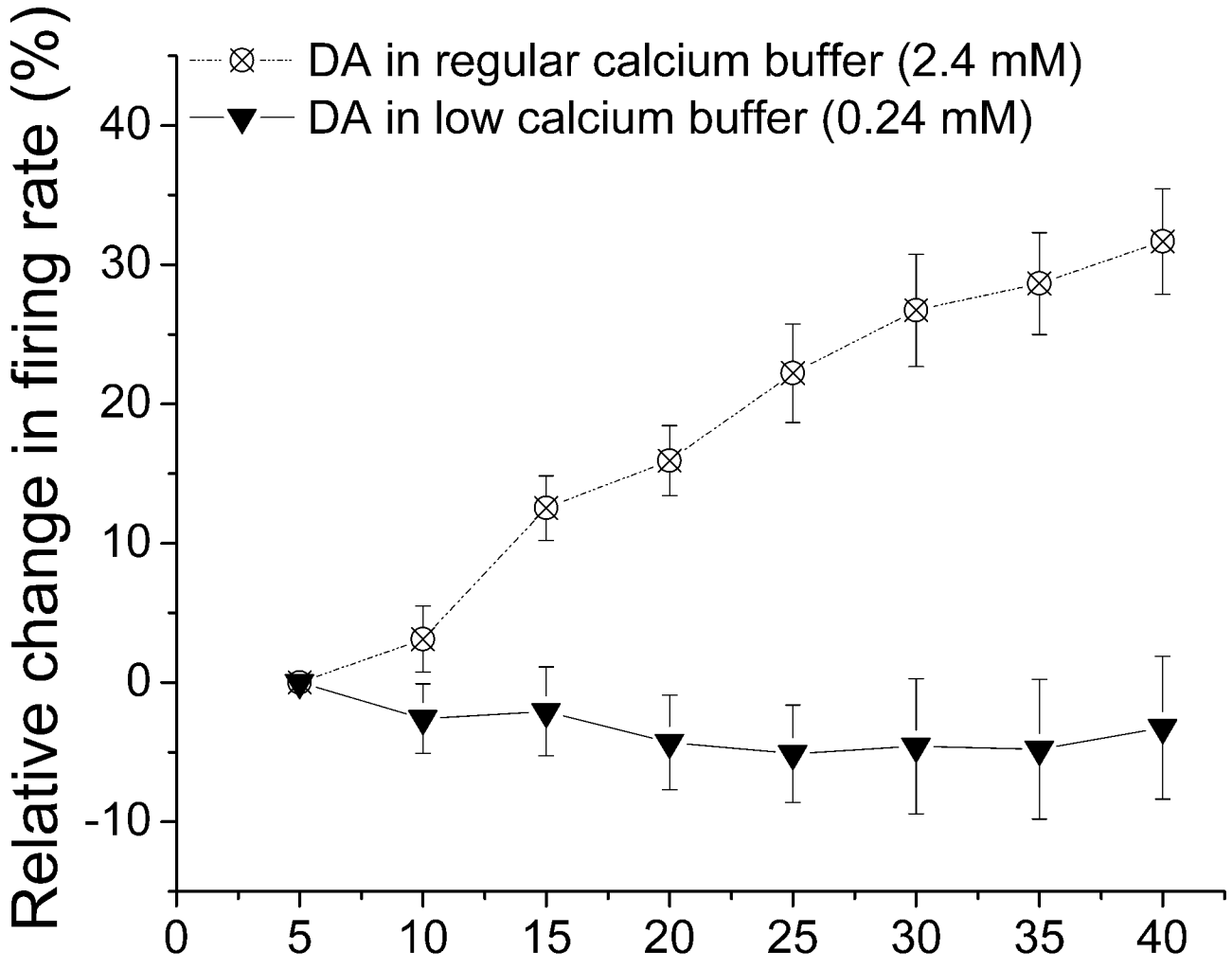
A

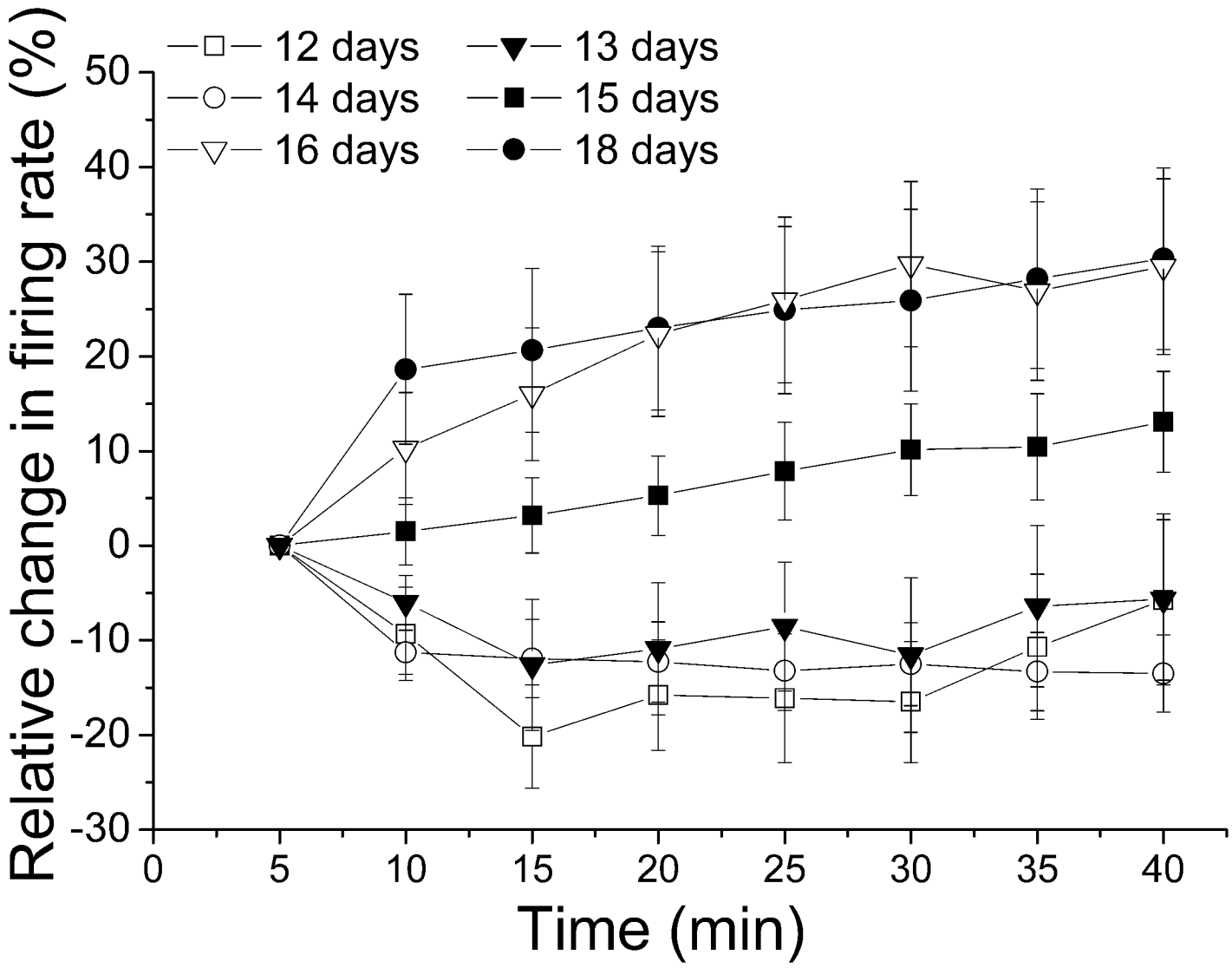

DA in regular aCSF
DA in aCSF containing 5 mM Caffeine
Relative change in firing rate (%)
50
40
30
20
10
0
-10
-20
-30
-40
0
5
10
15
20
25
30
35
40
Time (min)

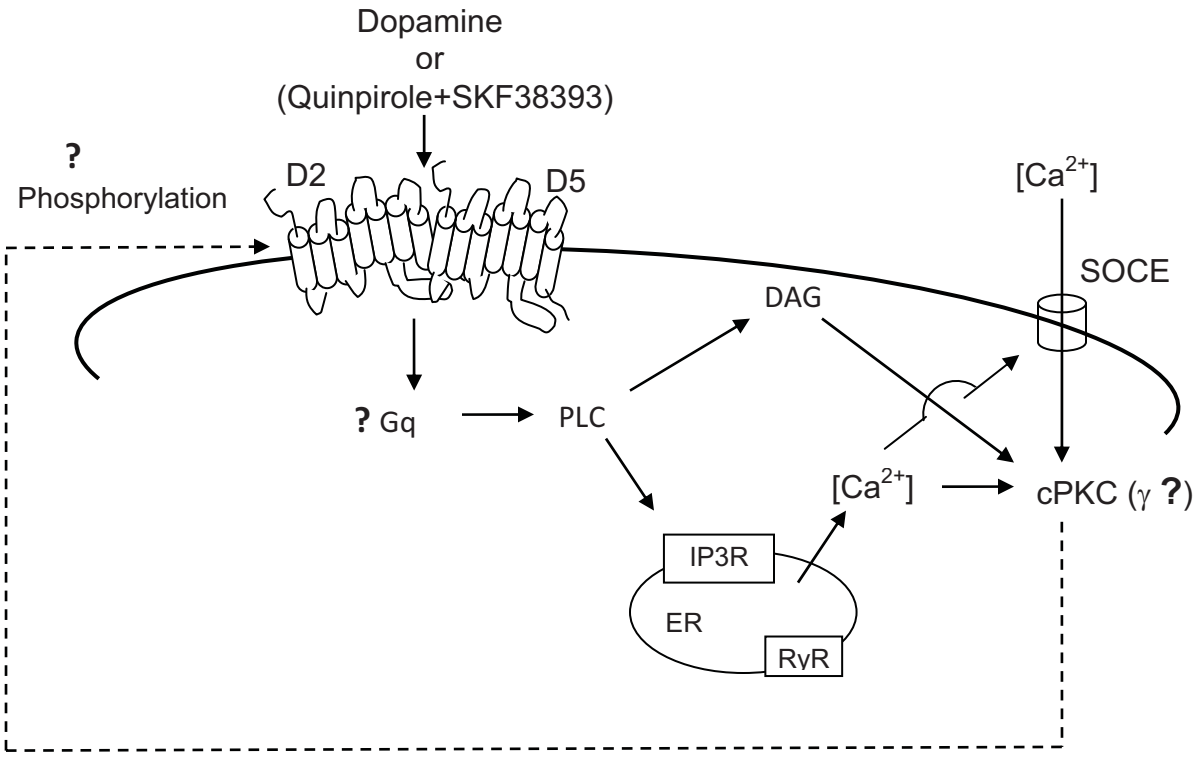
B


DA
DA + 30 μM Thapsigargin
Relative change in firing rate (%)
40
30
20
10
0
-10
-20
0
5
10
15
20
25
30
35
40
Time (min)


DA
DA + 10 μM Nifedipine
DA + 100 μM SKF96365
Relative change in firing rate (%)
40
30
20
10
0
-10
-20
-30
0
5
10
15
20
25
30
35
40
Time (min)


DA in regular calcium buffer (2.4 mM)
DA in low calcium buffer (0.24 mM)
Relative change in firing rate (%)
40
30
20
10
0
-10
0
5
10
15
20
25
30
35
40
Time (min)


12 days
13 days
14 days
15 days
16 days
18 days
Relative change in firing rate (%)
50
40
30
20
10
0
-10
-20
-30
0
5
10
15
20
25
30
35
40
Time (min)


Dopamine
or
(Quinpirole+SKF38393)
?
Phosphorylation
D2
D5
[Ca2+]
SOCE
DAG
? Gq
PLC
[Ca2+]
cPKC (γ ?)
IP3R
ER
RyR