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D_2-like dopamine receptors depolarize dorsal raphe serotonin neurons through the activation of nonselective cationic conductance

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Running title: Mechanism of dopamine excitation of serotonin neurons

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Abbreviations: DR, Dorsal Raphe; 5-HT, serotonin; DA, Dopamine; TRPC, Transient Receptor Potential Canonical; TTX, Tetrodotoxin; 2-APB, 2-aminoethoxydiphenyl borate; BAPTA, 1,2-Bis(2-Aminophenoxy)Ethane-N, N, N', N'-Tetraacetic Acid

Abstract

The dorsal raphe (DR) receives a prominent dopamine (DA) input that has been suggested to play a key role in the regulation of central serotoninergic transmission. DA is known to directly depolarize DR serotonin neurons, but the underlying mechanisms are not well understood. Here we show that activation of D_2-like dopamine receptors on DR 5-HT neurons elicits a membrane depolarization and an inward current associated with an increase in membrane conductance. The DA-induced inward current (I_{DA}) exhibits a linear I-V relationship and reverses polarity at around -15 mV, suggesting the involvement of a mixed cationic conductance. Consistent with this notion, lowering the extracellular concentration of sodium reduces the amplitude of I_{DA} and induces a negative shift of its reversal potential to about -45 mV. This current is abolished by inhibiting G-protein function with $GDP_{\beta}S$. Examination of the downstream signaling mechanisms reveals that activation of the nonselective cation current requires the stimulation of phospholipase C but not an increase in intracellular calcium. Thus, pharmacological inhibition of phospholipase C reduces the amplitude of I_{DA}. In contrast, buffering intracellular calcium has no effect on the amplitude of I_{DA}. Bath application of transient receptor potential (TRP) channels blockers, 2-aminoethoxydiphenyl borate (2-APB) and SKF 96365 strongly inhibits I_{DA} amplitude suggesting the involvement of TRP-like conductance. These results reveal previously unsuspected mechanism by which D_2-like DA receptors induce membrane depolarization and enhance the excitability of DR 5-HT neurons.

Introduction

The dorsal raphe (DR) nucleus, a major source of serotonin (5-HT) in the mammalian brain plays an important role in the regulation of many physiological processes such as sleep/arousal, food intake and mood (Jacobs and Azmitia, 1992). In addition, a dysfunction of the 5-HT system is thought to underlie a variety of psychiatric disorders, including anxiety and depression (Arango et al., 2002). Drugs that increase serotonin tone in the brain are the major substances used clinically for the treatment of anxiety and depression disorders (Nemeroff, 2003)

5-HT neurons of the DR receive a dense dopaminergic innervation from midbrain dopamine (DA) neurons (Peyron et al., 1995; Kitahama et al., 2000), and express D_2-like (D2, D3) DA receptors (Mansour et al., 1990; Suzuki et al., 1998). These anatomical results suggest that DA input to the DR may play a critical role in the regulation of the function of DR 5-HT neurons. Consistent with this notion, in vivo neurochemical studies have reported that administration of DA receptor agonists increases the synthesis and release of 5-HT in the DR (Ferré et al., 1994; Matsumoto et al., 1996). Direct evidence for a role of DA in the regulation of 5-HT neurons function comes from recent *in vitro* electrophysiological study showing that activation of DA receptors induces a membrane depolarization and thereby, increases the excitability of DR 5-HT neurons (Haj-Dahmane, 2001). This excitatory effect is mediated via the activation of D_2-like DA receptors presumably located on DR 5-HT neurons.

D_2-like DA receptors, comprising D_2, D_3 and D_4 receptors, regulate neuronal excitability by modulating diverse ionic conductances and synaptic transmission (Greengard, 2001). These receptors are mainly coupled to G-protein of G_o/G_i family to inhibit adenelyly cyclase (Missale et al., 1998). Generally, activation of these receptors decreases neuronal excitability by inducing a membrane hyperpolarization (Lacey et al., 1987). The ionic mechanism underlying this inhibitory response is best characterized in midbrain DA neurons, where it is widely accepted that the D_2-like DA receptor-induced membrane hyperpolarization is mediated by an activation of an inward rectifier potassium current (Lacey et al., 1987). In contrast to midbrain DA neurons, activation of D_2-like DA receptors in the DR strongly depolarizes and enhances the excitability of 5-HT neurons (Haj-Dahmane, 2001). Similar membrane depolarization induced by these receptors has also been reported in several other brain regions, such as the nucleus accumbens (Uchimura et al., 1986), the thalamus (Munsch et al., 2005) and hypothalamus (Yang et al., 1991). However, the ionic and signaling mechanisms underlying this excitatory response remain unknown. In the present study, we show that activation of D_2-like DA receptors in the DR depolarizes 5-HT neurons via activation of nonselective cation current. The D_2-like DA receptors-induced cationic conductance is signaled through phospholipase C pathway and exhibits pharmacological and electrical properties similar to TRPC channels. As such, these results outline a novel mechanism by which D_2-like DA receptors increase the intrinsic excitability of DR 5-HT neurons.

Methods

Brain slices preparation: Coronal brainstem slices containing the DR were prepared from 4 to 6 weeks old male Sprague-Dawley rats using methods detailed previously (Haj-Dahmane, 2001). All experiments were conducted in compliance with the National Institutes of Health and American Association for Accreditation of Laboratory Animal Care guidelines, and were approved by the University at Buffalo Institutional Animal Care and Use Committee. Briefly, animals were anaesthetized with halothane and killed by decapitation. The brain was quickly removed and cooled in ice-cold standard artificial cerebrospinal fluid (aCSF) solution of the following composition (in mM): NaCl, 119; KCl, 2.5; $CaCl_2$, 2.5; $MgSO_4$, 1.3; NaH_2PO_4, 1; $NaHCO_3$, 26.2; glucose, 11 and saturated with 95% O_2 / 5% CO_2. A block of brain tissue containing the DR was dissected, and coronal slices (300-400 μm) were cut with a vibratome (Lancer series 1000, Ted Pella Inc., Irvine, CA). Slices were stored for at least one hour at room temperature in a holding chamber filled with the aCSF solution and continuously bubbled with 95% O_2 / 5% CO_2. Slices containing the DR were then transferred one at a time to a recording chamber, in which the slice was held submerged between two nylon nets and continuously perfused with the standard aCSF solution (1 to 2 mL/min) saturated with 95%O_2 / 5% CO_2 at 30 ± 1ºC.

Whole-cell recordings: Whole-cell recordings were performed from DR neurons using the “blind” patch clamp technique. Low resistance path electrodes (3-5 MΩ) were made

from borosilicate glass tubing (Warner Instruments Inc, Hamden, CT) using a P97 horizontal puller (Sutter Instruments, Novato, CA). Recording pipettes were filled with an internal solution containing (in mM): potassium gluconate, 120; KCl, 10; Na_2-phosphocreatine, 10; HEPES, 10; $MgCl_2$, 1; EGTA, 1; Na_2-ATP; 2 and Na-GTP, 0.25 (pH 7.3, adjusted with KOH and osmolarity, 280 mOsmol/L). In some experiments aimed at examining the ionic mechanism of the DA-induced current, recordings were performed using a cesium gluconate based intracellular solution of the following composition (in mM): D-gluconic acid, 120; CsCl, 10; Na_2- phosphocreatine, 20; HEPES, 10; $MgCl_2$, 1; EGTA, 1; Na_2-ATP; 2 and Na-GTP, 0.25 (pH 7.3, adjusted with CsOH ; osmolarity, 280 mOsmol/L). Intracellular electric signals were recorded using an Axoclamp 2B amplifier (Axon Instrument, Foster City, CA, USA) in the bridge mode or in the continuous, single-electrode voltage clamp mode. Membrane voltages and currents were filtered at 3 -10 kHz and recorded online using a paper chart recorder (model TA 240, Gould Instrument, Valley View, OH). Fast electric events such as voltage ramps were digitized at 10 KHz using the Digidata 1200 interface board (Axon Instruments, Foster City, CA) and stored on a hard disk using Pclamp 9.0 software (Axon Instruments, Foster City, CA). DR 5-HT neurons were identified by their electrophysiological properties as described previously (Haj-Dahmane, 2001). Access resistance (10 to 20 MΩ) was monitored online during the course of the experiments with hyperpolarizing steps (- 50 to -100 pA, 200-500 ms). Experiments were discarded when series resistance changed by more than 10%.

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Voltage clamp recordings were performed using the continuous voltage-clamp mode. The access resistance was compensate by up to 80% using the bridge circuit of the amplifier and the settling time of the membrane current in response to 10 mV hyperpolarization was optimized to <5 ms. The steady state current-voltage (I-V) plots were obtained using a slow voltage ramps (6 mV/s) from -120 mV to 10 mV before and during DA application. These experiments were conducted in the presence of TTX (1 μM) and $CdCl_2$ (200 μM) to block voltage-dependent sodium and calcium current, respectively. The net current-voltage plot of the DA-induced inward current was obtained by digital subtraction of the control I-V curve from that obtained in the presence of DA. The values of membrane potential were corrected for the liquid junction potential that was present between the pipette and the bath solutions (approximately + 6 mV and + 12 mV for the potassium and cesium gluconate based intracellular solutions, respectively).

To examine the effect of low extracellular sodium ($[Na^+]_o$ = 27 mM) on the amplitude and I-V curve of DA-induced inward current, aCSF contained low sodium was prepared by substituting NaCl with equimolar concentration of choline-chloride. Because high concentration of choline can activate nicotinic and muscarinic receptors, theses experiments were conducted in the presence of atropine (3 μM) to block any potential muscarinic receptor-induced responses.

In some experiments, the calcium buffering capacity of the internal solution was increased by replacing 25 mM potassium gluconate with 25 mM 1,2-Bis (2-

Aminophenoxy) Ethane-N,N,N',N'-Tetraacetic Acid (BAPTA). In experiments examining the role of postsynaptic G-proteins in the signaling of DA induced membrane depolarization/inward current, GTP was replaced with either $GDP_{\beta}S$ (200 μM) or $GTP_{\gamma}S$ (200 μM).

Drugs and drug application: DA and others drugs were administered by bath application in the aCSF solution at known concentrations. Sodium metabusulphite (100 μM), which had no effect on the excitability of DR 5-HT neurons, was routinely added to the aCSF solution to prevent DA oxidization. In order to examine the involvement of phospholipase C (PLC) in the signaling of DA-induced inward current, slices were pretreated with a PLC inhibitor, 1-(6-((17b-3-methoxyestra-1,3,5 (10)-trien-17-yl)amino)hexyl)1-H-pyrole-2,5-dione (U 73122, 10 μM) or its inactive analog, 1-(6-((17b-3-methoxyestra-1,3,5 (10)-trien-17-yl)amino)hexyl) 2,5 pyrrolidine-dione (U 73343, 10 μM) for at least one hour before recording. U 73122 and U 73343 were first reconstituted in chloroform into single-use aliquots. The aliquots were evaporated to dryness, dissolved in DMSO, and then added to the aCSF solution at the desired concentration. The final dilution of DMSO was 0.001% which had no significant effect on the excitability of DR 5-HT neurons. Most chemicals and drugs, including forskolin, 8-BrcAMP and 2-aminoethoxydiphenyl borate (2-APB) were purchased from Sigma-Aldrich (St Louis, MO, USA). Quinpirole and sulpiride were obtained from Research Biochemical Incorporated (Natick, MA, USA). Tetrodotoxin (TTX) was purchased from Alomome Labs (Jerusalem, Israel). U 73122, U 73343, and SKF-93635 (1-[2-(4-methoxyphenyl)-2-[3-(4methoxyphenyl)propoxy]ethyl-1H-imidazole hyfrochloride) were

obtained from Biomol (Plymouth Meeting, PA, USA). Ro-32-0432 and chelerythrine chloride were obtained from Calbiochem (San Diego, CA, USA). Results in the text and figures are presented as mean ± SEM. Statistical analysis were performed using the Student's paired t-test for within group comparison and the Student's unpaired t-test for comparison between groups.

Results

DA depolarizes DR 5-HT neurons via the activation of D_2-like DA receptors

The effects of DA were examined on 150 DR neurons that exhibited electrophysiological features of 5-HT neurons, which included slow spiking activity in response to suprathreshold depolarizing pulses, a large afterhyperpolarizing potential (AHP) and a membrane hyperpolarization induced by stimulation of somatodendritic 5-HT_{1A} receptors (Aghajanian and Vandermaelen, 1982; Haj-Dahmane, 2001). Consistent with earlier findings (Haj-Dahmane, 2001), bath application of DA (3 to 100 μM) elicited a reversible slow membrane depolarization. Generally, the membrane depolarization induced by DA (30 μM) was sufficient to activate voltage-dependent sodium current and initiate a train of action potentials (Fig $1A_1$, n = 30). The ability of DA to depolarize DR 5-HT neurons persisted in the presence of TTX (1 μM) and cadmium chloride (200 μM), a manipulation that profoundly reduces neurotransmitter release. Under these conditions DA (30 μM) depolarized 5-HT neurons by 10.5 ± 3.8 mV (Fig. $1A_2$, n = 6), indicating that the DA-induced membrane depolarization is mediated by activation of postsynaptic DA receptors located on 5-HT neurons.

To determine the receptor subtype underlying the DA-induced membrane depolarization, we examined the effects of selective D_1 and D_2-like DA receptor agonists on the resting membrane potential of DR 5-HT neurons. Bath application D_1-like DA receptor agonist SKF 38393 (3 to 100 μM) failed to induce a membrane depolarization (Fig 1D, n = 8). In contrast, administration of (-) quinpirole (30 μM), a non selective D_2-like DA receptor agonist, which exhibits dissociation constants of 4.8 and 24 nM for D_2 and D_3 receptors (Seeman and Van Tol, 1994), respectively, elicited a slow reversible membrane depolarization (Fig. $1B_1$, n = 8). Similar to the effect of DA, the ability of (-) quinpirole to depolarize DR 5-HT neurons persisted in the presence of TTX and cadmium chloride (Fig. $1B_2$, control = 9.5 $\pm$ 4.6 mV; in TTX + $CdCl_2$ = 8 $\pm$ 2.5 mV, n = 6, $p > 0.05$), suggesting that the membrane depolarization is mediated by the stimulation of postsynaptic D_2-like DA receptors.

To further confirm the involvement of D_2-like DA receptors, we tested the effect of sulpiride, a non selective D_2-like DA receptor antagonist which binds to both D_2 and D_3 receptors with equal affinity (Seeman and Van Tol, 1994), on the ability of DA and (-) quinpirole to induce a slow membrane depolarization. As illustrated in figure 1C, pretreatment of slices with sulpiride (3 μM) totally abolished the membrane depolarization induced by DA (30 μM). The average amplitude of the membrane depolarization induced by DA (30 μM) obtained in control condition and in the presence of sulpiride was 10.5 $\pm$ 3.8 mV and 0.25 $\pm$ 0.34 mV (Fig 1D, n = 8, $p < 0.001$). Similarly, in the presence of sulpiride, the selective D_2-like agonist, quinpirole (30 μM) failed to depolarize DR 5-HT neurons (Fig 1D, control = 9.5 $\pm$ 4.6 mV, in sulpiride = 0.35 $\pm$ 0.16,

n = 7, $p < 0.01$). Taken together, these results strongly indicate that DA depolarizes DR 5-HT neurons via activation of post-synaptic D_2-like DA receptors.

DA activates a nonselective cationic conductance

Previous studies in other neuronal preparations have suggested that activation of D_2-like DA receptors can induce a membrane depolarization through a reduction in potassium conductance (Uchimura et al., 1986; Higashi et al., 1989). To test whether a similar ionic mechanism also mediated the D_2-like receptor induced membrane depolarization in DR 5-HT neurons, we first assessed the impact of DA on the holding current and membrane conductance of DR 5-HT neurons in voltage clamp mode. As illustrate in figure $2A_1$, in DR 5-HT neurons voltage clamped at -70 mV, bath application of DA (30 μM) elicited a reversible inward shift of the holding current. The average amplitude of the DA-induced inward current (I_{DA}) was 65 ± 6.5 pA (n = 15). Further examination of the changes in membrane conductance using a hyperpolarizing voltage step (10 mV, 500 ms) applied before and during DA application, revealed that the I_{DA} was consistently associated with a significant increase in membrane conductance (Fig. $2A_2$-A_3; G_m control = 1.89 ± 0.32 nS, G_m DA = 2.75 ± 0.56 nS, n = 6, $p < 0.05$). We next examined the ability of DA to induce an inward current using cesium gluconate based intracellular solution and in the presence of extracellular cesium (2 mM), a manipulation that blocks most potassium channels. We found that bath application of DA (30 μM) still induced a robust inward current in all DR 5-HT neurons tested (Fig. 2 B, n = 7). The average amplitude of I_{DA} recorded under these conditions was essentially comparable to that obtained using potassium gluconate based intracellular solution (Fig. 2C, I_{DA}

control = 65 ± 6.9 pA, I_{DA} Cesium = 68 ± 7.5 pA, $p > 0.05$, $n = 7$). These results suggest that I_{DA} in DR 5-HT neuron is unlikely to be mediated by a blockade of potassium current.

To characterize the ionic mechanism of I_{DA} we assessed its current-voltage (I-V) relationship using a slow voltage ramps (6 mV/s) from -120 to 10 mV applied before DA (30 μM) application and at the peak of the inward current. As illustrated in figure 3B, the I-V relationship of I_{DA} recorded with a cesium gluconate based intracellular solution and in the presence of TTX (1μM) was linear and exhibited a positive slope conductance at potential between -120 mV and 0 mV. The average reversal potential of I_{DA} was -15 ± 8 mV (Fig 3B, $n = 8$), suggesting the involvement of mixed cationic conductance. To further confirm that I_{DA} is mainly mediated by a nonselective cation current, we next examined the impact of lowering the extracellular concentration of sodium on the amplitude and the reversal potential of I_{DA}. As illustrated in figure 3C, replacing sodium chloride in the extracellular solution with choline chloride profoundly reduced the amplitude of I_{DA}. The average amplitudes of I_{DA} recorded in control condition and in low extracellular sodium were 65 ± 10 pA and 15 ± 8 pA, respectively (Fig. 3E, $p < 0.05$, $n = 8$). Lowering the extracellular concentration of sodium also resulted in a negative shift of the reversal potential of the I_{DA} (Fig. 3D, E_{rev} control = -15 ± 8 mV; E_{rev} in low sodium = -45 ± 10 mV, $n = 8$, $p < 0.05$). Because sodium substitution per se had no effect on the cell input resistance, the reduction in I_{DA} amplitude and the shift of its reversal potential was unlikely to be attributed to a loss of voltage control of the dendritic arbor. Taken

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together, these results indicate that I_{DA} is mainly mediated by an activation of nonselective cationic conductance permeable to sodium ions.

I_{DA} is signaled via G-protein and phospholipase C pathway

To determine whether the signaling of I_{DA} required G-protein activation, we first tested the impact of intracellular application of $GDP_{\beta}S$, a membrane impermeable inhibitor of G-proteins, on the ability of DA to induce I_{DA}. To insure that this manipulation was effective in inhibiting the function of G-proteins, we also monitored the 5-HT-induced outward potassium current, a response known to be mediated by activation of G-proteins (Innis et al., 1988). As illustrated in figure 4A, in 5-HT neurons recorded with a pipette solution containing GTP (250 μM) bath application of 5-HT (30 μM) induced an outward current, whereas, administration of DA (30 μM) elicited a prominent inward current. Replacing GTP with $GDP_{\beta}S$ (250 μM) in the pipette solution completely blocked the 5-HT induced potassium current (Fig. 4B, n = 6), indicating an effective blockade of G-proteins. Under these conditions, bath application of DA (30 μM) failed to induce an inward current (Fig. 4B, n = 6). Overall, the amplitude of I_{DA} recorded with GTP and $GDP_{\beta}S$ was 63 ± 8 pA and 6 ± 5 pA, respectively (Fig. 4D, n = 6, $p < 0.01$). We next examined the effect of $GTP_{\gamma}S$, an irreversible activator of G-protein, on the DA-induced inward current. As expected for a G-protein mediated response, in 5-HT neurons loaded with $GTP_{\gamma}S$, bath application of DA (30 μM) induced a persistent inward current (Fig. 4C, n = 4). Combined, these results demonstrate that activation of G-proteins is required for the signaling of I_{DA} in DR 5-HT neurons.

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Results from biochemical studies have demonstrated that D_2-like DA receptors are negatively coupled to adenylyl cyclase through a G_o/G_i type G-protein (Missale et al., 1998). As such, it is conceivable that this pathway may be involved in the signaling of I_{DA}. If inhibition of adenylyl cyclase is required for the signaling of I_{DA}, activation of this enzyme should at least reduce the amplitude I_{DA}. To test this possibility, we examined the effect of forskolin (10 μM), a lipophilic adenylyl cyclase activator on the amplitude of I_{DA}. We found that in slices treated with forskolin, bath application of DA (30 μM) still induced a robust inward current with a comparable amplitude to that obtained in control slices (I_{DA} control = 62 ± 4.5 pA; I_{DA} forskolin = 68 ± 6.5 pA, n = 5, $p > 0.05$, data not shown). Similarly, treatment of slices with the membrane permeable cAMP analog, 8-BrcAMP, which presumably can increase intracellular cAMP level failed to significantly affect the amplitude of I_{DA} (I_{DA} control = 62 ± 4.5 pA; I_{DA} in 8-BrcAMP = 65 ± 6.8 $p > 0.05$; n = 5). Together, these results suggest that inhibition of adenylyl cyclase is unlikely to contribute to the signaling of I_{DA} in DR 5-HT neurons.

Several reports have provided evidence that D_2-like DA receptors can also activate phospolipase C (PLC) and increase the hydrolysis of phosphatidylinositol-4, 5 biphosphate (PIP2) (Vallar et al., 1990; MacKenzie et al., 1994). We thus investigated whether pharmacological inhibition of PLC/PIP_2 pathway could affect I_{DA}. As shown in Figure $5A_2$, pretreatment of slices with U 73122 (10 μM), an inhibitor of PLC profoundly reduced the amplitude of I_{DA} (I_{DA} control = 61.66 ± 4.2 pA; I_{DA} in U 73122 = 10.1 ± 4.1, $p < 0.05$; n = 8, Fig. 5B). In contrast, treatment of slices with U 73433 (10 μM), an inactive analog of U 73122, had no significant effect on I_{DA} amplitude (I_{DA} control = 61.66 ± 4.2

pA; I_{DA} in U 73433 = 59 ± 9.1 pA, n = 4, P > 0.05, Fig $4A_3$, B). These results suggest that activation of PLC is required for the signaling of I_{DA}.

Activation of PLC generates two second messengers, IP_3 and diacylglycerol (DAG). IP_3 increases intracellular calcium via calcium release from the internal stores and DAG activates protein kinase C (PKC). To examine the role of these second messenger systems in the signaling of I_{DA}, we tested whether pharmacological inhibition of PKC abolishes the effect of DA. We found that in slices treated with the PKC inhibitor, Ro-32-0432 (10 μM), administration of DA (30 μM) still induced a robust inward current with similar amplitude to that obtained in control slices (Fig. $4A_4$-B, control = 61.66 ± 4.21 pA; in Ro-32-0432 = 58 ± 13.6 pA, p > 0.05, n = 5). Similar results were also obtained with another PKC inhibitor chelerythrine chloride (10 μM, n = 4, data not shown). To further test the role of PKC in the signaling of DA-induced current, we examined whether PKC activator can mimic the DA-induced inward current. In all DR 5-HT neurons tested, application of PKC activator, phorbol 12-myristate 13-acetate (300 nM-1 μM) did not induce an inward current (n = 4, data not shown), suggesting that activation of PKC is not involved in the gating of the DA-induced cationic conductance.

We next examined whether a rise in intracellular calcium was required for the activation of I_{DA}. To this end, DR 5-HT neurons were recorded with a potassium gluconate based intracellular solution containing a high concentration of BAPTA (25 mM), a fast calcium chelator. The ability of BAPTA to buffer the transient increase in intracellular calcium was monitored by the amplitude of the current underlying the

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afterhypepolarization potential (I_{AHP}), a response known to be mediated by calcium-activated potassium current (Pan et al., 1994). We found that dialysis of 5-HT neurons with BAPTA (25 mM) for at least 10 min strongly reduced the amplitude of the I_{AHP} (I_{AHP} at 1 min = 155 ± 15 pA; I_{AHP} at 10 min = 25 ± 12 pA; n = 6), but failed to affect the ability of DA (30 μM) to induce an inward current. Overall, the amplitude of I_{DA} recorded with an internal solution containing BAPTA (25 mM) was similar to that obtained using a pipette solution containing a low concentration of calcium chelator EGTA (1 mM) (Fig. 5D; I_{DA} control = 58.33 ± 9.21 pA; I_{DA} in BAPTA = 56.66 ± 11.8 pA, $p > 0.05$, n = 6). These findings indicate that a rise in cytosolic calcium is not required for the activation of I_{DA} in DR 5-HT neurons.

TRP channel blockers profoundly reduce I_{DA}

The transient receptor potential canonical (TRPC) is a family of proteins that forms a diverse group of nonselective cation currents (Herteneck et al., 2000; Clapham et al., 2001). The TRPC family consists of seven members (TRPC1-TRPC7), which are widely distributed within the mammalian brain (Strübing et al., 2001; Riccio et al., 2002). One prominent feature of TRPC channels is that they are activated in response to receptor-mediated stimulation of different PLC isoforms (Clapham et al., 2001). Our findings that the I_{DA} requires PLC activation and exhibit an I-V relationship comparable to some TRPC currents raised the possibility that the cation channels activated by D_2-like DA receptors may belong to TRPC channel family. To test this possibility, we examined the effect of 2-Aminoethoxydi-phenylborate (2-APB) and SKF -93635, which are known to block TRPC channels (Li et al., 1999; Clapham et al., 2001) on the

amplitude of I_{DA}. As illustrated in figure 6A, treatment of brain slices with either 2-APB or SKF 96365 (100 μM) strongly reduced the amplitude of I_{DA}, albeit the blockade of I_{DA} by 2-APB was less pronounced than that obtained with SKF-93635 (I_{DA} control = 54.5 ± 6.9 pA; I_{DA} in 2-APB = 15.6 ± 4.7 pA, I_{DA} in SKF 96365 = 5.45 ± 5.2 pA, $n < 0.05$, $n = 8$, Fig. 6B). Because 2-APB is also a blocker of IP3 receptors, it is conceivable that the reduction of I_{DA} amplitude simply reflects a blockade of calcium release from IP3 sensitive store. However, this possibility is highly unlikely since an increase in intracellular calcium is not required for the activation of I_{DA}. Taken together, these pharmacological results suggest that I_{DA} may be mediated by TRPC channels.

Discussion

The present study demonstrates that in the DR, administration of DA to 5-HT neurons causes a slow membrane depolarization. This response is mediated by D_2-like DA receptors, as it was mimicked by quinpirole, a selective D_2-like DA receptors and blocked by sulpiride, a D_2-like DA receptor antagonist. A novel finding of the present study is that D_2-like DA receptors depolarize DR 5-HT neurons via the activation of a nonselective cation current, which displays a linear steady-state I-V relationship at membrane potential ranging from -130 to -10 mV. We have also shown that the DA-induced nonselective cation current is signaled via a G-protein-dependent process and requires the activation of PLC. However, neither activation of PKC nor the increase in intracellular calcium seems to be required for the signaling of I_{DA}. Finally, pharmacological analysis revealed that TRPC channel blockers strongly reduced the amplitude of I_{DA}, suggesting that TRPC channels may mediate the DA-induced

nonselective cation current. As such, these results reveal previously unsuspected ionic mechanism by which D_2-like DA receptors increase the excitability of DR 5-HT neurons.

Ionic mechanism of the D_2-like DA receptor-induced membrane depolarization

Stimulation of D_2-like DA receptors has been shown to induce a slow membrane depolarization in several other brain regions (Uchimura et al., 1986; Yang et al., 1991; Munsch et al., 2005). Results from a previous study in accumbens neurons have suggested that this excitatory response is mediated by a decrease in potassium conductance (Uchimura et al., 1986). This conclusion, however, was based on the finding that the membrane depolarization was associated with a small or no increase in membrane resistance. Unlike these neurons, we find that in DR 5-HT neurons, the D_2-like DA receptor induced membrane depolarization is predominantly mediated by an increase in nonselective cationic conductance.

Several lines of evidence support this conclusion. First, the D_2-like DA receptor induced membrane depolarization and inward current were consistently associated with a significant increase in membrane conductance. In addition, blockade of potassium currents with cesium had no effect on the amplitude of I_{DA}. These observations exclude the possibility that I_{DA} is mediated by a reduction in potassium conductance. Second, the amplitude of I_{DA} always increased linearly with membrane hyperpolarization and displayed a reversal potential at -15 mV. The reversal potential determined within the technical limitation inherent to clamping neurons in brain slices, exhibited an intermediate value between the equilibrium potentials for sodium (E_{Na} = +56 mV) and

potassium ions (E_k= -104 mV), suggesting the involvement of a mixed cationic conductance. Consistent with notion, lowering the extracellular concentration of sodium reduced the amplitude of I_{DA} and induced a negative shift of it reversal potential, thus indicating that sodium ion is a main charge carrier.

Cation nonselective currents have been shown to mediate slow membrane depolarization in a several other brain regions (Shen and North, 1992; 1993, Haj-Dahmane and Andrade, 1996). In some neuronal preparations, these cationic current are voltage-dependent (Haj-Dahmane and Andrade, 1996; Faber et al., 2006), while in others they appear to be voltage independent (Shen and North, 1992; Crepel et al., 1994). In the present study, we find that I_{DA} in DR 5-HT neurons is not voltage-dependent, as its amplitude increases with membrane hyperpolarization and exhibits a linear I-V relationship. A similar voltage-independent nonselective cation current has recently been shown to mediate the membrane depolarization of DR 5-HT neurons induced by orexin receptors (Liu et al., 2002). Thus, our results further support the notion that nonselective cation currents play a critical role in the regulation of the excitability of DR 5-HT neurons.

Most cation nonselective currents known to mediate slow membrane depolarization, with the exception of cyclic nucleotide gated cation channels and hyperpolraization activated cation current (I_H), are though to be activated in response to the stimulation of G_q/G_{11} coupled receptors, such as, M_1 muscarinic receptor (Haj-Dahmane and Andrade, 1996), group I metabotropic glutamate receptors (Kim et al., 2003), and orexin

receptors (Liu et al., 2002). In the present study, we find that the DA-induced cationic current (I_{DA}) in DR 5-HT neurons is signaled by D_2-like DA receptors, which are mainly coupled to G_o/G_i type G-proteins (Missale et al., 1998), indicating that cation nonselective conductances can also be activated by G_o/G_i coupled receptors. Such ionic mechanism may also mediate the membrane depolarization induced by D_2-like DA receptors in other brain areas (Yang et al., 1991; Munsch et al., 2005).

Previous studies have shown that an increase in intracellular calcium is required for the activation of some nonselective cationic conductances (Swandulla and Lux, 1985; Crepel et al., 1994). These calcium-activated cationic currents, commonly called CAN currents (For review see, Partridge et al., 1994), have been shown to mediate the membrane depolarization induced by group I metabotropic receptor in CA1 pyramidal neurons (Crepel et al., 1994). In the present study, however, we find that activation of I_{DA} does not require an increase in intracellular calcium, because loading 5-HT neurons with high concentration of calcium chelator BAPTA (25 mM) had no effect on the amplitude the DA-induced cationic current. This finding indicates that I_{DA} is unlikely to be mediated by calcium activated nonselective cation current (CAN).

Signaling mechanisms of I_{DA}

Previous studies have shown that D_2-like DA receptors are primarily coupled to G_o/G_i type G-proteins (Missale et al., 1998). As expected for a G-protein mediated response, we find that intracellular application of $GDP_\beta S$, which blocks G-protein function, totally abolishes I_{DA}, whereas, in the presence of $GTP_\gamma S$, which irreversibly activates G-protein,

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stimulation of D_2-like DA receptors induces a persistent I_{DA}. These results demonstrate that I_{DA} is signaled via G-protein dependent mechanisms.

We have shown that activation of adenyly cyclase with forskolin has no significant effect on the amplitude of I_{DA}. In addition, the ability of D_2-like DA receptors to activate I_{DA} is also preserved in the presence of 8-BrcAMP, a membrane permeable analog of cAMP. Hence, although stimulation of D_2-like DA receptor is known to inhibit adenylyl cyclase (Missale et al., 1998), our data suggest that this pathway is unlikely to be involved in the signaling of I_{DA}. Instead, we find that pharmacological inhibition of PLC with U 73122 almost abolished I_{DA}, indicating that activation of PLC is required for the signaling of I_{DA}. Interestingly, activation of PLC pathway has also been shown to mediate the D_2-like receptors induced inhibition of calcium current in striatal neurons (Hernandez-Lopez et al., 2000). Thus, our results are consistent with the notion that D_2 like DA receptors can activate PLC pathway (Vallar et al., 1990; MacKenzie et al., 1994) and through this pathway they modulate a variety of membrane conductances including the nonselective cation current described in the present study.

Although I_{DA} is activated via stimulation of PLC, we find that PKC activators and inhibitors fail to mimic and inhibit DA-induced current, respectively, suggesting that I_{DA} is unlikely to be gated by PKC –dependent protein phosphorylation. Similarly, because elevation of intracellular calcium is not required for the activation of I_{DA}, it is unlikely that IP_3-induced calcium release from internal stores contribute to the activation of I_{DA}. As

such, it is possible that I_{DA} is directly activated by DAG, or by yet unknown second messengers system downstream from PLC.

Possible involvement of TRP channels

Although cation nonselective currents have been widely described in the mammalian brain, the molecular identities of these channels remain unknown. Recently, convergent lines of evidence indicate that TRPC proteins are primary candidate. Thus, all seven member of the TRPC family are widely expressed in the mammalian brain (Strübing et al., 2001; Riccio et al., 2002). Moreover, depending on the subunit composition, in vitro expression of TRPC proteins leads to a diverse membrane conductances that resemble many feature of native nonselective cationic currents recorded in CNS neurons. For instance, co-expression of TRPC1 with TRPC4 or TRPC5 forms heteromultimers that generate voltage dependent nonselective cation current (Strubing et al., 2001) with an I-V curve strikingly similar to the native cationic current activated by muscarinic receptors in cortical neurons (Haj-Dahmane and Andrade, 1996). In contrast, expression of homommeric TRPC proteins forms cation nonselective currents which display linear I-V relationships at negative membrane potentials (Hofmann et al., 2002; Plant and Schaefer, 2003). In the present study, we find that I_{DA} also exhibits a linear I-V curve. In addition, like TRPC channels, I_{DA} is also signaled via PLC dependent pathway, and is profoundly reduced by 2-APB and SKF 96365. These pharmacological results combined with the functional similarities between I_{DA} and TRPC channels, suggest that I_{DA} is most likely mediated by TRPC channels. This notion is also supported by a recent study showing that DR 5-HT neurons express at least four members of TRPC family

(TRPC1, TRPC3, TRPC5, and TRPC6) (Sergeeva et al., 2003). However, future studies using molecular approaches are required to confirm that I_{DA} is indeed meditated by TRPC channels and determine which TRPC protein(s) is the target of DA modulation in the DR 5-HT neurons.

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Footnotes

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Legends for Figures

Figure 1: D_2-like DA receptor activation induces a direct membrane depolarization of DR 5-HT neurons. A_1-A_2, Current-clamp recordings from DR 5-HT neurons depicting the membrane depolarization induced by DA (30 μM) in control condition (A_1) and in the presence of TTX (1μM) and $CdCl_2$ (200 μM) (A_2). Note that the DA-induced depolarization persisted in the presence of agents that profoundly reduce synaptic transmission. B_1-B_2, Effect of the selective D_2-like DA receptor agonist, quinpirole, on the resting membrane potential of DR 5-HT neuron obtained in control (B_1) and in the presence of TTX (1μM) and $CdCl_2$ (200 μM) (B_2). In both conditions, bath application of quinpirole (30 μM) elicited strong membrane depolarization. C, Sulpiride, a selective D_2-like DA receptor antagonist, blocked the depolarization induced by DA (30 μM) and quinpirole (30μM). Calibration bars: 10 mV, 1 min. D, Summary histograms representing the average membrane depolarization induced by D_1-like agonist, SKF 38393 (100 μM), DA (30 μM) in control condition and in the presence of sulpiride (3 μM), and by the D_2-like agonist, quinpirole (30 μM) in the absence and presence of sulpiride (** $p < 0.01$)

Figure 2: The DA-induced membrane depolarization/inward current is not mediated by a blockade of potassium current. A, The DA-induced inward current is associated with an increase in membrane conductance. A_1, A typical whole-cell voltage clamp recording showing the inward current induced by bath application of DA (30 μM) in a 5-HT neuron voltage clamped at -65 mV. Calibration bars, 50 pA, 2 min. A_2, Average current traces induced by hyperpolarizing voltage steps (10 mV, 600 ms) and recorded in control condition and during DA application. Calibration bars, 100 pA, 200 ms. A_3,Time course

of the DA effect on the membrane conductance of the 5-HT neuron illustrated in panel A_1. Each data point represents the average membrane conductance assessed by ten consecutive hyperpolarizing voltage steps. Note that bath application of DA (30 μM) induced a reversible increase in membrane conductance. B, Blockade of potassium currents with intracellular cesium had no effects on DA-induced inward current. Left and right traces depict the inward current induced by DA (30 μM) in 5-HT neurons voltage clamped at -65 mV and recorded with potassium gluconate and cesium gluconate based intracellular solution, respectively. Intracellular application of cesium had no significant effect on the amplitude of DA-induced inward current C, Summary graph of the average amplitude of DA-induced current recorded with potassium gluconate and cesium gluconated based pipette solutions.

Figure 3: DA activates a nonselective cation current in DR 5-HT neurons.
A, Current trace from 5-HT neuron voltage clamped at -65 mV showing the DA-induced inward current recorded with cesium based intracellular solution and in the presence of TTX (1μM). Scale bars, 50pA, 1 min. B, Current-voltage (I-V) curve of the net DA-induced current, which was obtained by subtracting the control I-V curve from that obtained in the presence of DA using slow voltage ramps from -120 to 10 mV applied at the time point indicated by numbers in figure A. Note that the DA-induced current exhibited a linear I-V curve at membrane potential ranging from -120 to -20 mV and reversed polarity at around -15 mV. C, The amplitude of the DA-induced inward current depends on the extracellular concentration of sodium. Current traces depicting the inward current induced by DA (30 μM) recorded from a 5-HT neuron voltage clamped at

-65 mV, in control condition (left trace) and in low extracellular sodium (right trace). Substitution of extracellular sodium markedly reduced the amplitude of the DA-induced current. D, I-V curves of the DA-induced current recorded from the same neuron illustrated in panel C, in control condition and in low extracellular sodium. Reducing extracellular sodium induced a negative shift of the reversal potential of DA-induced inward current to -45 mV. E, summary graph of the average amplitudes of the DA-induced current recorded in control condition and in low extracellular sodium (* $p < 0.05$, $n = 8$).

Figure 4: Inhibition of postsynaptic G-protein signaling abolishes the DA-induced inward current.

A, Current trace recorded from a 5-HT neuron using potassium based intracellular solution containing GTP (250 μM). Bath application of 5-HT (30 μM) induced an outward potassium current, whereas application of DA (30 μM) elicited an inward current. B, Replacing GTP with $GDP_{\beta}S$ (250 μM) abolished the effects of both 5-HT (30 μM) and DA (30 μM). C, Intracellular application of $GTP_{\gamma}S$ (250 μM) resulted in a persistent DA-induced inward current. D, Average amplitude of DA-induced current recorded with an internal solution containing either GTP or $GDP_{\beta}S$ (** $p < 0.01$, $n = 6$).

Figure 5: D_2-like DA receptors activate a nonselective cation current via PLC.

Sample traces of inward current induced by DA (30 μM) recorded in control condition (A_1), and in slices treated with PLC inhibitor, U-73122 (10 μM) (A_2) or its inactive analog, U-73343 (10 μM) (A_3). Note that U-73122 almost abolished the DA-induced

inward current, whereas, U-73343 failed to affect the amplitude of the DA-induced current. A_4, sample current trace depicting the effect of DA in slice treated with RO-32-0432 (3 μM), an inhibitor of protein kinase C (PKC). Note that in the presence of PKC inhibitor, bath application of DA (30 μM) still induced a robust inward current. B, Summary graph of the average amplitudes of the DA-induced inward current obtained in control slices, and in slices treated with either, U-73122, U-73343, or Ro-32-0432 (** $p < 0.01$, $n = 8$). C, A rise in intracellular calcium is not required for the activation of DA-induced inward current. Sample current traces depicting the DA-induced inward current recorded with an internal solution containing either EGTA (1 mM, upper panel) or BAPTA (25 mM, lower panel). Increasing the calcium-buffering capacity of the pipette solution had no significant effects on the amplitude of DA-induced inward current. D, Summary graph of the amplitude of DA-induced current recorded with an internal solution containing either EGTA (1 mM, $n = 7$) or BAPTA (25 mM, $n = 7$).

Figure 6: TRPC channels blockers profoundly reduce the amplitude of I_{DA}.
A, Sample traces of I_{DA} induced by bath application of DA (30 μM) in control slices (left trace), slices treated with either 2-APB (100 μM) (middle trace) or SKF-93635 (100 μM) (right trace). Note that both 2-APB and SKF-93635 strongly reduced the amplitude of I_{DA}. B, Summary graph depicting the average amplitudes of I_{DA} obtained in control condition and in slices treated with 2-APB or SKF 93635(* $p < 0.05$; ** $p < 0.01$)

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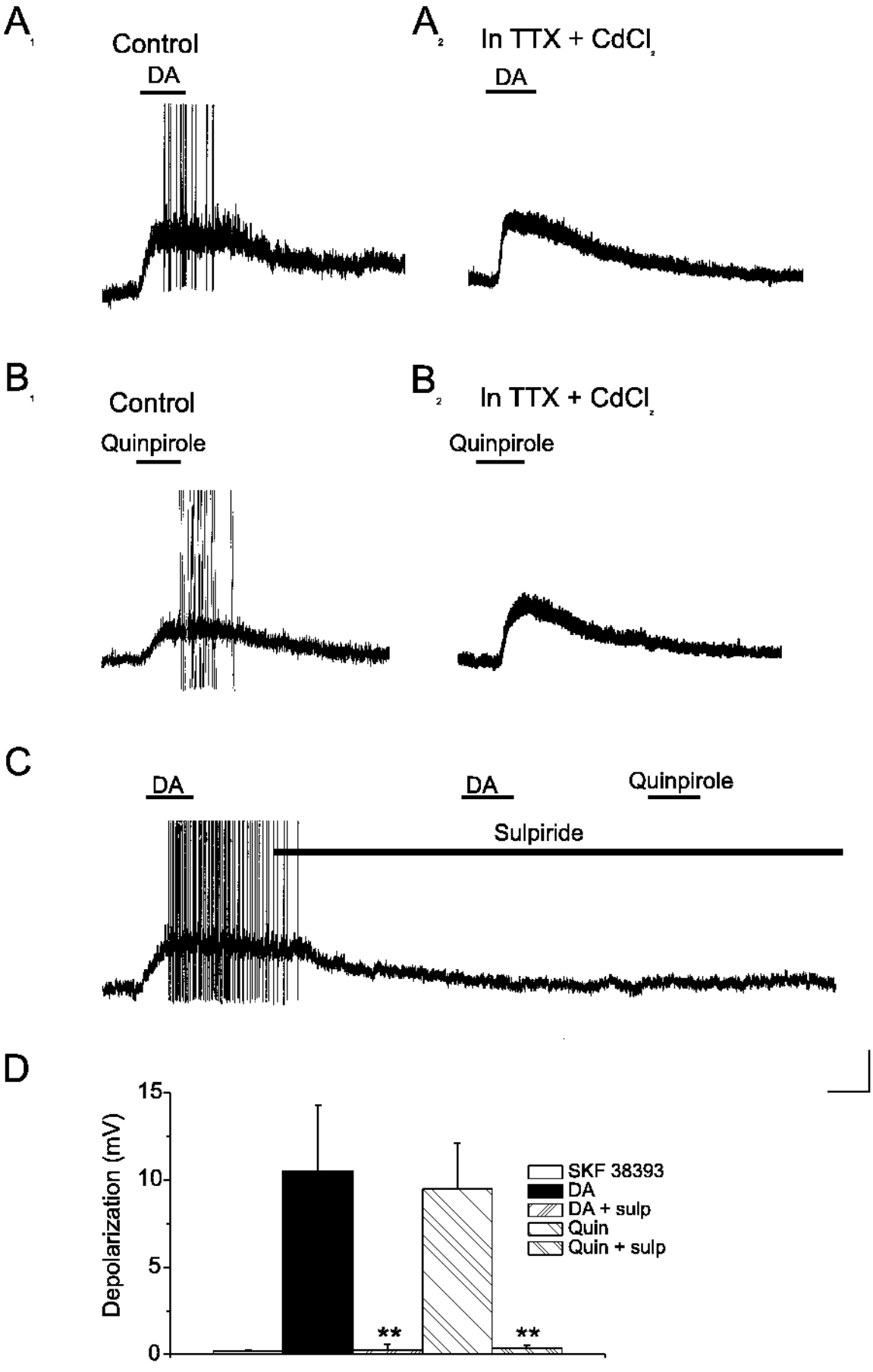

Fig. 1

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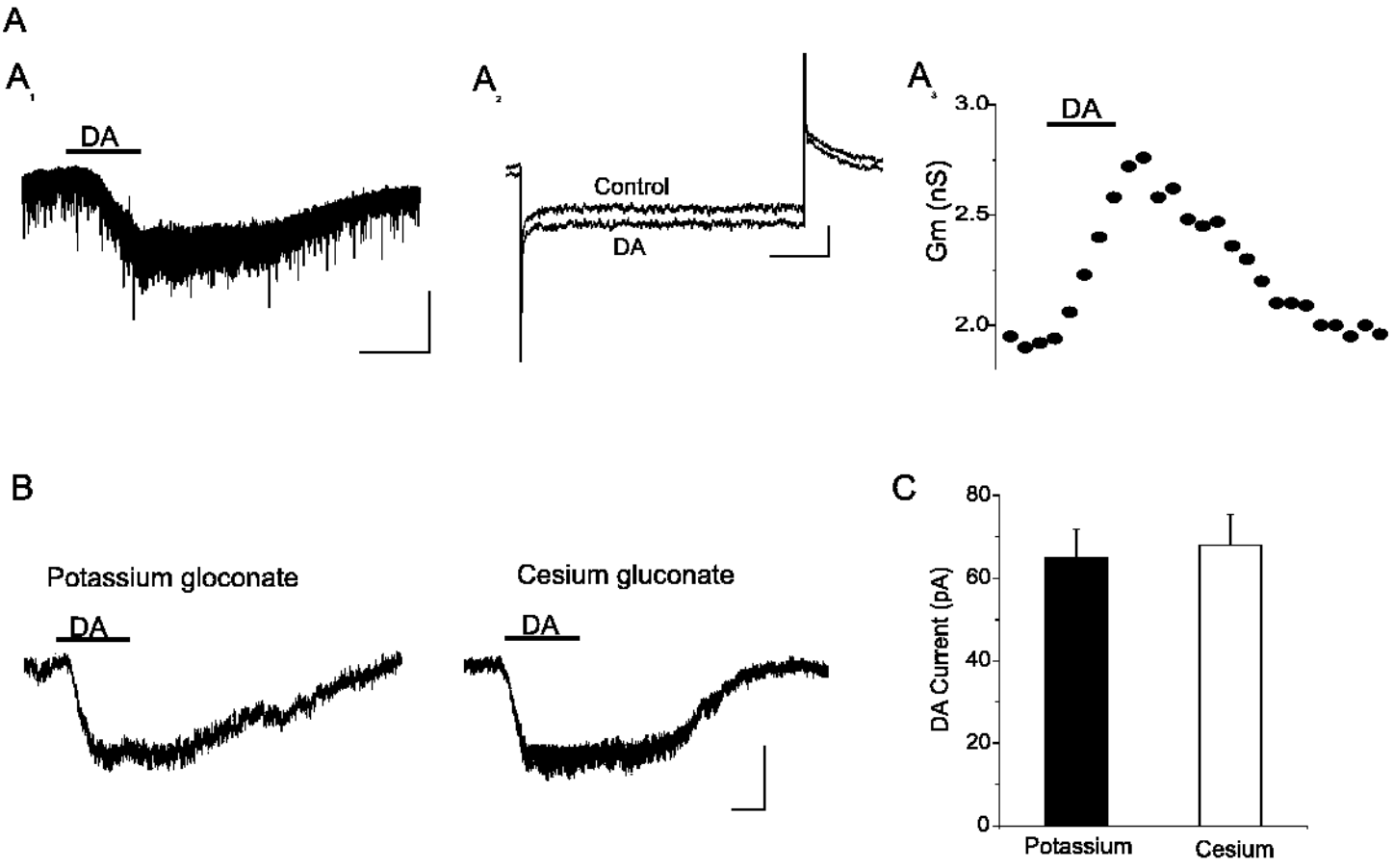

Fig. 2

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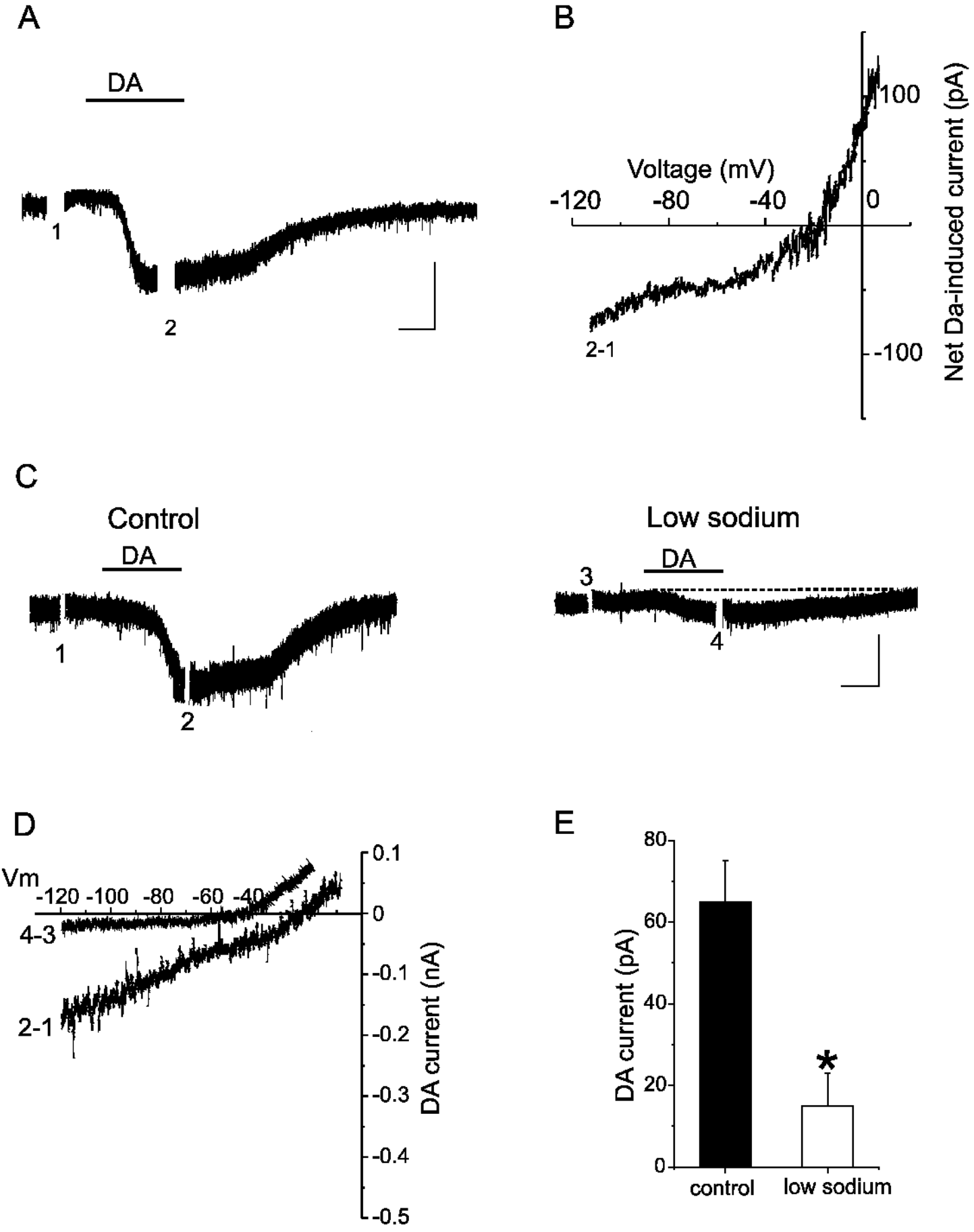

Fig. 3

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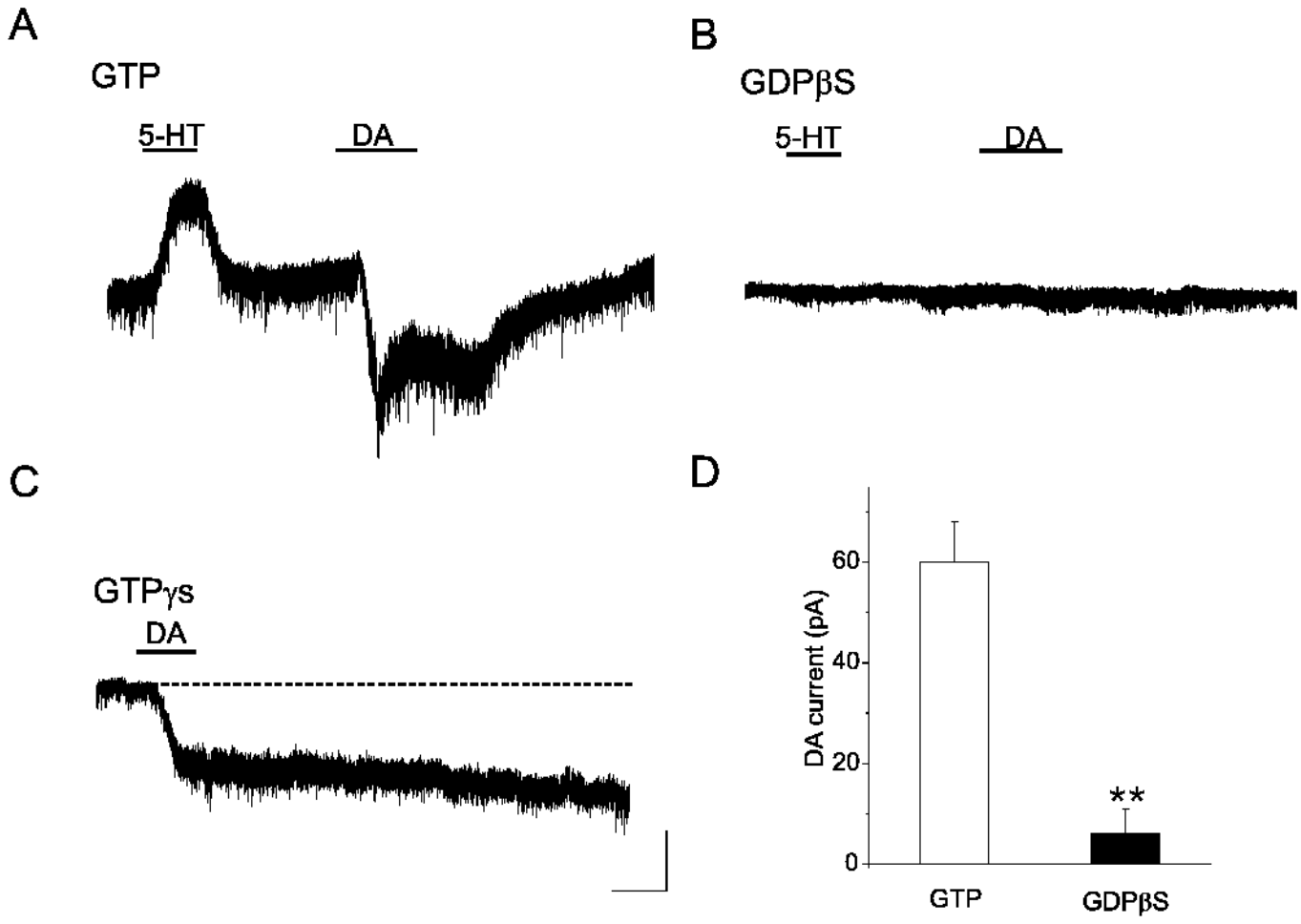

Fig. 4

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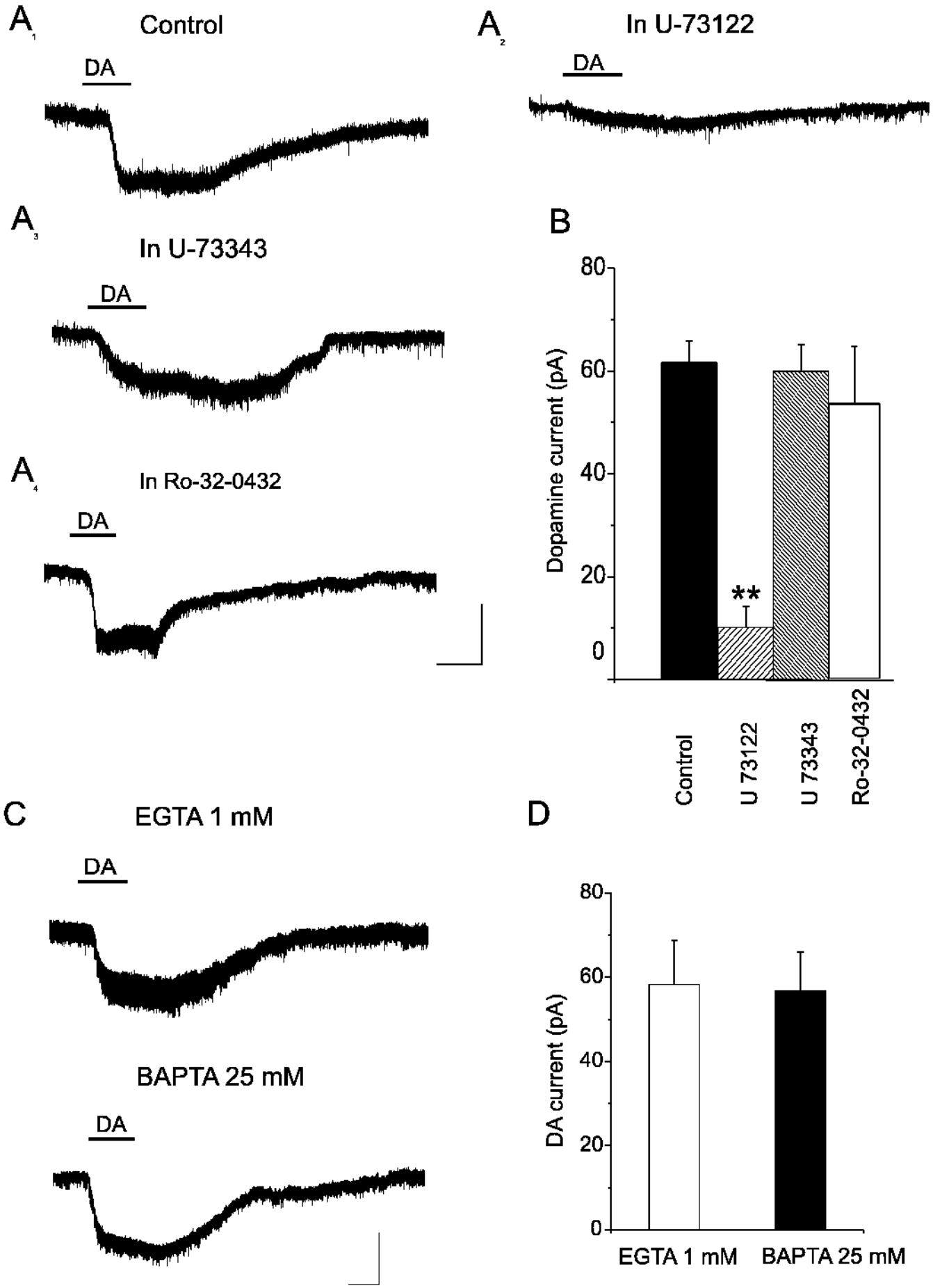

Fig. 5

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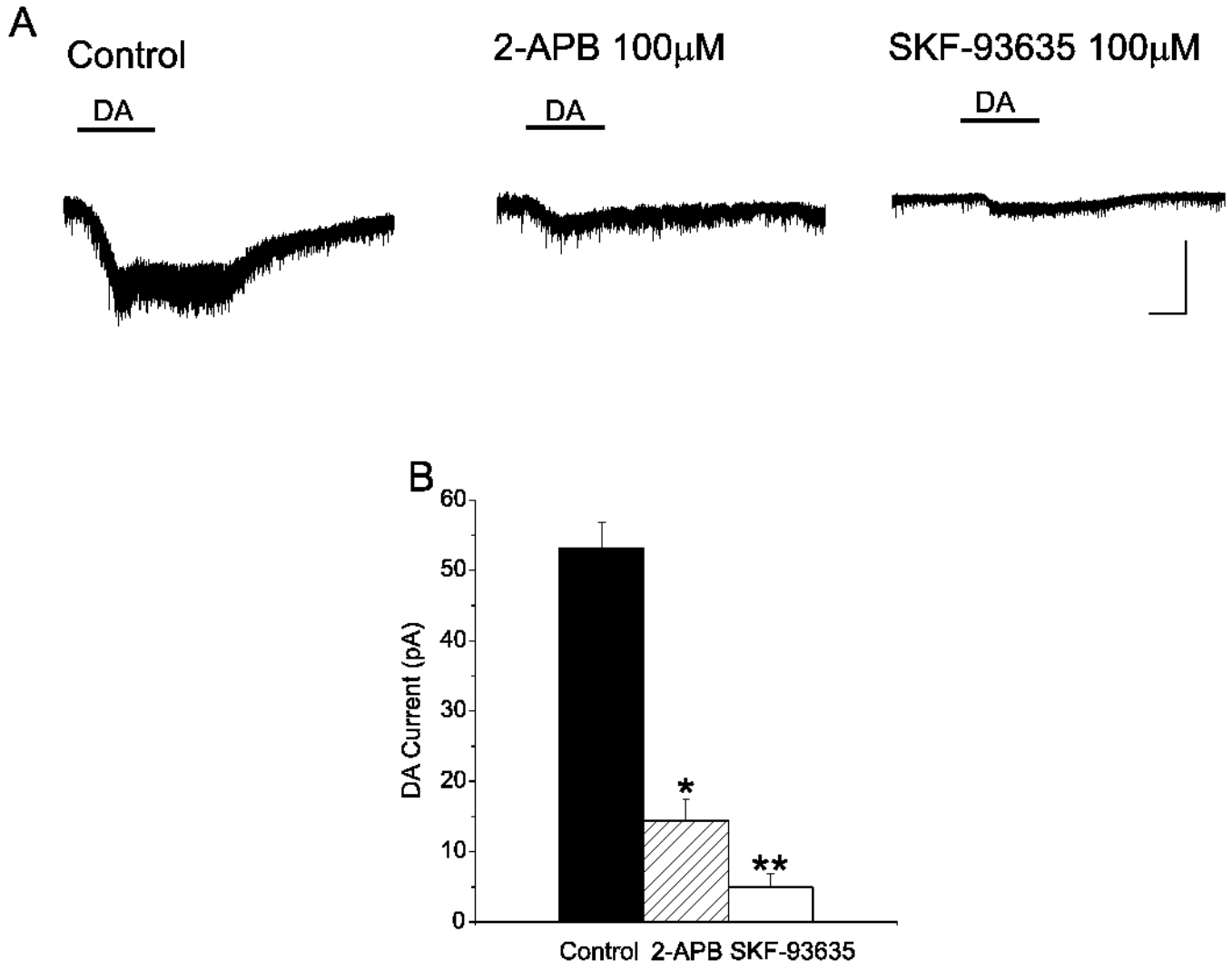

Fig. 6