Published in final edited form as:

Brain Res Mol Brain Res. 2004 November 24; 131(0): 65–72. doi:10.1016/j.molbrainres.2004.08.007.

L-type Ca^{2+} channel blockers promote Ca^{2+} accumulation when dopamine receptors are activated in striatal neurons

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Abstract

Dopamine (DA) receptor-mediated signal transduction and gene expression play a central role in many brain disorders from schizophrenia to Parkinson's disease to addiction. While trying to evaluate the role of L-type Ca^{2+} channels in dopamine D_1 receptor-mediated phosphorylation of the transcription factor cyclic AMP response element-binding protein (CREB), we found that activation of dopamine D_1 receptors alters the properties of L-type Ca^{2+} channel inhibitors and turns them into facilitators of Ca^{2+} influx. In D_1 receptor-stimulated neurons, L-type Ca^{2+} channel blockers *promote* cytosolic Ca^{2+} accumulation. This leads to the activation of a molecular signal transduction pathway and CREB phosphorylation. In the absence of dopamine receptor stimulation, L-type Ca^{2+} channel blockers inhibit CREB phosphorylation. The effect of dopamine on L-type Ca^{2+} channel blockers is dependent on protein kinase A (PKA), suggesting that protein phosphorylation plays a role in this phenomenon. Because of the adverse effect of activated dopamine receptors on L-type Ca^{2+} channel blocker action, the role of L-type Ca^{2+} channels in the dopamine D_1 receptor signal transduction pathway cannot be assessed with pharmacological tools. However, with antisense technology, we demonstrate that L-type Ca^{2+} channels contribute to D_1 receptor-mediated CREB phosphorylation. We conclude that the D_1 receptor signal transduction pathway depends on L-type Ca^{2+} channels to mediate CREB phosphorylation.

Keywords

D_1 receptor; L-type Ca^{2+} channels; Phosphorylation; Nifedipine; Dopamine; Striatum

1. Introduction

The dopaminergic neurotransmitter system plays a central role in numerous neurologic and psychiatric disorders such as Parkinson's disease, schizophrenia and drug addiction. Changes in dopamine (DA) release or DA receptor sensitivity cause long-term adaptations that contribute to the clinical manifestations of these disorders [1,4,11,12,29,31]. The

biological substrate for these adaptations is presented in DA receptor second messenger pathways, which regulate the activity of various transcription factors and the expression of genes and proteins under the control of these transcription factors [17,24]. One such transcription factor is the Ca^{2+} and cyclic AMP response element-binding protein (CREB), which is activated by D_1 DA receptors [26], and which induces the immediate early gene, *c-fos* [33,36]. CREB, which has been shown to be involved in mechanisms of memory formation and drug addiction [3,15,30,37], is activated via phosphorylation of Ser^{133} [18]. We chose CREB phosphorylation as target in the cell nucleus to study D_1 receptor-mediated signal transduction in striatal neurons.

Any element of the second messenger pathway that leads from DA receptor activation at the cell surface to altered CREB phosphorylation in the nucleus is a possible target for pharmacological intervention. We have shown that the *N*-methyl-D-aspartate (NMDA) receptor is an important component of D_1 receptor-mediated gene expression, and that inhibition of NMDA receptors blocks the D_1 receptor-mediated signal transduction pathway [27]. The molecular findings are supported by electrophysiology, which showed in rat striatum and in striatal culture an enhancement of NMDA currents by DA [6,7,14]. Electrophysiology showed furthermore an interdependence between DA, NMDA receptors and L-type Ca^{2+} channels [8]. In our initial molecular studies, Ca^{2+} channel blockers did not block *c-fos* expression by DA receptors, suggesting that the signal transduction pathway from D_1 receptors to *c-fos* expression is independent of L-type Ca^{2+} channels [27]. However, upon further examination, a potentiation of D_1 receptor-mediated *c-fos* expression by Ca^{2+} channel blockers was observed.

Here we investigate this phenomenon and show that in D_1 receptor-stimulated neurons, L-type Ca^{2+} channel antagonists *facilitate* Ca^{2+} accumulation. This facilitation is protein kinase A (PKA)-dependent, and enables L-type Ca^{2+} channel blockers to *promote* CREB phosphorylation, an action contrary to their usual properties. Furthermore, we show in mRNA antisense knockdown experiments that a reduction in the expression of L-type Ca^{2+} channels blocks DA receptor-mediated CREB phosphorylation. Thus, L-type Ca^{2+} channels and D_1 receptors interact closely in the striatum.

2. Materials and methods

2.1. Drugs and antibodies

Dopamine, SKF 38393, SKF 82958, NMDA, glutamate, dizocilpine maleate [(+)-MK 801 hydrogen maleate], nifedipine, verapamil and forskolin were purchased from Sigma (St. Louis, MO). H89 (*N*-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, HCl) was purchased from Calbiochem (San Diego, CA). All drug concentrations were chosen based on the smallest concentration of drug that gave persistent results, as established in preliminary experiments with drug concentrations ranging from nanomolar to micromolar ranges. Polyclonal antisera against Ser^{133} CREB and CREB were purchased from Upstate (Charlottesville, VA), and the antiserum against actin was purchased from Sigma. A monoclonal Ser^{133} CREB antibody from Cell Signaling Technology (Beverly, MA) was used for comparison purposes in immunoblots, and gave identical results to the polyclonal

antiserum. Treatment with antagonists was initiated 1 h before harvest, treatment with agonists was initiated 15 min before harvest.

2.2. Primary striatal cultures

Striata were dissected under a stereomicroscope from 18-day-old Sprague–Dawley rat fetuses (Taconic Farms, Germantown, NY), dissected and plated on polyethylenimine-treated plates at a concentration of 1.2×10^6 cells/ml as described previously [28]. All protein and RNA experiments were performed in duplicates with cells 6–8 days in culture and repeated at least once in an independent dissection. For Ca^{2+} imaging (described below), cells were grown for 12 to 13 days on PEI-coated two-well borosilicate chambered glass cover slips (Nalge Nunc, Rochester, NY) in defined medium at 2×10^5 cells/chamber. Because neurons can be easily distinguished from glia under the microscope, 0.5% fetal bovine serum (Sigma), which can promote glial growth, was added to the medium to support neuronal viability in these low-density cultures. The cultures were re-fed with defined medium containing additives plus 0.25% FBS on the 9th day in vitro.

2.3. Immunoblots

Immunoblotting was carried out as described previously [32]. Primary rat striatal neurons were harvested in Leammli buffer as described, and proteins were separated on 12% polyacrylamide gels with 4% stacking area. Concentrations for primary antisera were 1:2000 dilution of anti-Ser¹³³ phospho-CREB, 1:20,000 dilution of anti-CREB, and 1:10,000 dilution for actin. Secondary antibody was goat anti-rabbit horseradish peroxidase-linked IgG (Vector Laboratories, Burlingame, CA) at a dilution of 1:3000. Blots were developed with Western Lightning Chemiluminescence Reagent PLUS (PerkinElmer, Boston, MA), and exposed to autoradiographic film (Kodak, Rochester, NY). Kaleidoscope-prestained standards (Bio-Rad, Hercules, CA) were used for protein size determination. Ser¹³³ phospho-CREB, CREB and actin bands were detected around 40 kD. All blots were exposed to the Ser¹³³ CREB antibody and the CREB antibody, and some blots were also exposed to the actin antibody. We did not see any changes in total CREB levels. Total CREB and actin were used as loading controls.

2.4. Antisense experiments

Antisense sequences were targeted to the translation initiation sites. Both antisense and scrambled antisense oligonucleotides were synthesized by the MGH core DNA synthesis facility. The first and last three nucleotides were phosphorothioate modified, to avoid degradation of the oligonucleotides on one hand, and to reduce the cytotoxic effect observed with fully phosphorothioate modified oligonucleotides, on the other. Striatal cultures were treated as previously described [32], with a mixture of 5 μM $\alpha 1\text{C}$ (GGCCCGAATCATTGTGACTCCAGT) and 5 μM $\alpha 1\text{D}$ (TCATCATCATCATCATCCACG) antisense oligonucleotides. Oligonucleotides were added 30 min after cells were plated and daily thereafter until 4 DIV. Twenty four hours after the last addition, cells were treated with DA or FPL 64176. For scrambled antisense DNA controls, every 3rd and 4th nucleotide of the antisense constructs were exchanged.

2.5. Ca²⁺ imaging

All drug treatments and imaging were performed on DIV 12 or DIV 13 with the cell-permeable Ca²⁺ indicator, fluo-3 acetoxymethyl (AM) ester (Molecular Probes, Eugene, OR). Cultures were rinsed with Locke's buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl₂, 1.0 mM MgCl₂, 3.6 mM NaHCO₃, 5.6 mM D-Glucose, 5 mM HEPES; pH 7.3) and loaded for 30 min with 2.5 μM fluo-3 AM stock, in Locke's buffer. The cells were washed twice with Sodium Locke's Buffer followed by a 15-min incubation to allow for deesterification. All fluorescence recordings were made at 40× magnification using an inverted Leica TCS NT confocal microscope (Leica Microsystems, Bannockburn, IL). Pharmacological compounds were added directly to the cells under the microscope in Locke's Buffer after several baseline frames were taken. A 488-nm argon laser, filtered at 530±30 nm was used. The total imaging session consisted of 250 frames, collected with approximately 3.4 s between frames. Numerical data were acquired from the series using the Leica TCS NT software physiology package (Leica Microsystems). Data are presented as average fluorescence intensity in the soma of all identified neurons in the frame. Glial cells were occasionally found in the cultures but were easily identified and excluded from analysis. In a series of preliminary experiments, dual imaging of Fluo-3 and Fura Red was compared to imaging of Fluo-3 alone (data not shown). Fura Red was used to control for photobleaching. Because no significant photobleaching was observed in our paradigm, neurons for the experiments shown were loaded with and imaged for Fluo-3 only.

2.6. Statistical analyses

Blots were developed on a Kodak Imaging station 440, or on film. Autoradiographic films were scanned with the Microtek Scanmaker 4. Data were analyzed with one-way ANOVAs. The Tukey–Kramer HSD (honestly significant difference) was used to analyze differences between the groups. The JMP computer program (SAS Institute, Cary, NC) was used for data analysis.

3. Results

3.1. D₁ receptor-mediated CREB phosphorylation is blocked by NMDA antagonists, but not by L-type Ca²⁺ channel blockers

DA and the D₁ agonist SKF 38393 induced CREB phosphorylation in primary striatal culture, which was blocked by the NMDA antagonists MK 801 (Fig. 1A–D) and APV (not shown). No change was observed in total CREB levels under either treatment. These data are in line with *c-fos* mRNA expression after D₁ receptor stimulation, which was also blocked by NMDA antagonists [27]. Because D₁ receptor-mediated signal transduction depends on functional NMDA receptors [13,27], we examined how NMDA receptors mediate CREB phosphorylation in primary striatal culture. In agreement with previous observations [32], the L-type Ca²⁺ channel blocker nifedipine inhibited glutamate- and NMDA-mediated CREB phosphorylation in primary striatal culture (Fig. 2A–D). The amount of nifedipine (and verapamil, not shown) needed for a full inhibition of CREB phosphorylation was confirmed with the L-type Ca²⁺ channel agonist FPL 64176 (Fig. 2E), and was verified with NMDA (Fig. 2F) and glutamate (not shown). Interestingly, a previous study that did not find an inhibition of NMDA receptor-mediated CREB phosphorylation by

nifedipine, used a concentration of nifedipine, 10 μ M, that in our experiments does not consistently block [9]. In contrast to NMDA receptors, D₁ receptor-mediated CREB phosphorylation seemed independent of L-type Ca²⁺ channels, since nifedipine and another L-type Ca²⁺ channel blocker, verapamil, failed to inhibit (Fig. 3A–D,G). The effectiveness of L-type Ca²⁺ channel blockers was verified in parallel experiments in the same cultures with the Ca²⁺ channel activator FPL 64176 (Fig. 3E–F). Although it was puzzling that CREB phosphorylation by D₁ receptors depends on NMDA receptors but not on L-type Ca²⁺ channels, these data were in line with previous studies of *c-fos* expression in our laboratory [27]. Neither DA-, SKF38393- nor SKF82958-mediated CREB phosphorylation was blocked with either nifedipine, or verapamil as L-type Ca²⁺ channel blockers. Both nifedipine and verapamil were examined at concentrations ranging from 10 nM to 100 μ M, but no inhibition was observed at any combination of D₁ receptor agonist and L-type Ca²⁺ channel antagonist (not shown, for *c-fos*, see Ref. [27]). Interestingly, nifedipine, at concentrations that blocked FPL 64176-mediated CREB phosphorylation, even *facilitated* D₁ receptor-mediated CREB phosphorylation (Fig. 3A–D). Similar observations were also made for *c-fos* (not shown, data similar to Ref. [27]).

3.2. Activation of D₁ receptors alters the properties of L-type Ca²⁺ channel blockers

Since we had previously established that D₁ receptors depend on the NMDA receptor pathway for gene expression [27], and that NMDA receptors depend on L-type Ca²⁺ channels for gene expression [32], we were trying to find an explanation for the apparent dissociation of the D₁ receptor pathway and the L-type Ca²⁺ channel pathway. We used Ca²⁺ imaging to examine the effect of D₁ receptor agonists and L-type Ca²⁺ channel blockers on Ca²⁺ levels in striatal neurons. SKF 38393 or nifedipine, when given alone, had no effect on levels of free Ca²⁺ inside neurons (Fig. 4A) in the presence of the NMDA antagonists MK 801 and the sodium channel blocker tetrodotoxin. MK 801 was added to prevent DA-mediated activation of NMDA receptors [25,27], and tetrodotoxin was added to prevent depolarization and secondary Ca²⁺ influx. However, when nifedipine was administered after SKF 38393, Ca²⁺ levels rose in the cytosol as direct consequence of nifedipine addition. The PKA-stimulating agent, forskolin, had effects similar to SKF 38393 (Fig. 4B). These data suggested that DA receptors and PKA altered the properties of one or more proteins that interact with L-type Ca²⁺ channel blockers and that monitor Ca²⁺ flux into the cytosol.

Further molecular analysis showed that the combination of nifedipine with DA resulted in CREB phosphorylation even when the DA-signal transduction pathway was blocked by MK 801 (Fig. 5A,B). The PKA inhibitor H89 blocked CREB phosphorylation by D₁ receptors, and CREB phosphorylation by the combination D₁ receptor-nifedipine (Fig. 5C; SKF 38393; $p < 0.05$ in $n = 4$ samples; H89 also blocked CREB phosphorylation by DA and by DA/nifedipine; $p < 0.05$ in $n = 4$ samples, not shown). These data suggested that although not the entire D₁ signal transduction pathway, which includes the NMDA receptor [13], was needed for CREB phosphorylation by L-type Ca²⁺ channel blockers, PKA, stimulated by D₁ receptors, was essential. Furthermore, because L-type Ca²⁺ channel antagonists cause CREB phosphorylation when D₁ receptors are activated, the role of L-type Ca²⁺ channels in D₁ receptor-mediated CREB phosphorylation cannot be addressed by pharmacological means.

3.3. Knocking down L-type Ca^{2+} channels with antisense oligonucleotides suppresses DA receptor-mediated CREB phosphorylation

In an attempt to understand the role of the L-type Ca^{2+} channel in DA receptor-mediated CREB phosphorylation, we used an antisense knockdown technique, to circumvent the apparent pharmacologic problems. Primary striatal neurons were treated with a mixture of antisense oligonucleotides against the $\alpha_1\text{C}$ and 1D subunits of Ca^{2+} channels, since these two subunits are necessary for the two forms of the L-type Ca^{2+} channel in neurons [23]. In the presence of the antisense oligonucleotides, a significant reduction in DA-mediated CREB phosphorylation was observed (Fig. 6). No reduction in CREB phosphorylation was observed in the presence of scrambled antisense oligonucleotides. CREB phosphorylation by the L-type Ca^{2+} channel agonist FPL 64176 was also significantly reduced.

4. Discussion

The initial premise of the study was to determine, with molecular tools, the role of L-type Ca^{2+} channels in D_1 receptor-mediated CREB phosphorylation and *c-fos* mRNA expression. Past research in our laboratory on striatal signal transduction pathways had shown a strong interaction between D_1 receptors and NMDA receptors [13,27], and between NMDA receptors and L-type Ca^{2+} channels [32]. From these data, a model emerged by which D_1 receptors recruit the NMDA receptor pathway for signal transduction, and the NMDA receptor recruits the L-type Ca^{2+} channel pathway. In this molecular model, L-type Ca^{2+} channel blockers, like NMDA receptor antagonists, should have intercepted D_1 receptor-mediated signal transduction to the cell nucleus. Unexpectedly, this conclusion did not hold true and left us with a series of conflicting findings that could not be reconciled. While NMDA antagonists *inhibited* D_1 receptor-mediated *c-fos* expression [27] and CREB phosphorylation [13], L-type Ca^{2+} channel antagonists appeared to *facilitate* CREB phosphorylation. Elucidation came from Ca^{2+} imaging, which demonstrated that nifedipine led to Ca^{2+} accumulation in striatal neurons when D_1 receptors were activated. Thus, the activation of D_1 receptors leads to some fundamental change in the properties of a protein that controls Ca^{2+} accumulation in the cytosol and that interacts with nifedipine, leading to Ca^{2+} -mediated CREB phosphorylation and *c-fos* gene expression. Studies on CREB phosphorylation reflected the observations from Ca^{2+} imaging and shed some light on the molecular details. For example, only a part of the D_1 signal transduction pathway that is usually necessary for CREB phosphorylation is necessary for CREB phosphorylation by L-type Ca^{2+} channel antagonists; PKA is needed, but the NMDA receptor is superfluous. Furthermore, in the absence of L-type Ca^{2+} channel inhibitors, the D_1 receptor signal transduction pathway depends of L-type Ca^{2+} channels, since a knock-down in Ca^{2+} channels blocks the pathway.

Which protein interacts with L-type Ca^{2+} channel blockers and controls Ca^{2+} accumulation into the cytosol? An obvious candidate is the L-type Ca^{2+} channel itself. It is conceivable that PKA, activated by D_1 receptors, phosphorylates an L-type Ca^{2+} channel subunit that is accessible from inside the cell (e.g. α_1 , β ,), and thereby affects the interaction between the channel and its antagonists.

L-type Ca^{2+} channels usually have three physiological states: open/activated, inactive (refractory) and closed/resting [5,22]. L-type Ca^{2+} channel antagonists such as nifedipine bind preferentially during the refractory time, and keep the channel in the inactive conformation. Phosphorylation could change the structure of the channel, allowing antagonists to bind at the open state (like agonists do) and to interfere with the closing of the channel. Because the culture medium as well as the salt solution contains KCl (5 mM), a minor fraction of L-type Ca^{2+} channels should open periodically allowing nifedipine to bind and to pry the channel open. Prolonged Ca^{2+} influx through these channels could slowly depolarize the membrane by opening more and more channels. Our hypothesis is supported by prevailing knowledge on L-type Ca^{2+} channel properties. First, L-type Ca^{2+} channels can be phosphorylated by PKA: at least two subunits of the L-type Ca^{2+} channel, α_1 and β , have PKA phosphorylation sites [2,23,35] and PKA is anchored to the L-type Ca^{2+} channel [19] facilitating its function [10,16,20,23]. Second, G proteins are known to modulate L-type Ca^{2+} channel activity [21,23]. Third, L-type Ca^{2+} currents are potentiated by L-type Ca^{2+} channel antagonists in the presence of GTP- γ -S in cerebellar granule cells [34]. Fourth, many antagonists of the L-type Ca^{2+} channel have similar chemical structures to agonists [22], and the mode of interaction between drug and channel might not only be affected by modulations of the chemical structure of the drugs, but also by modulations of protein conformation of the channel after phosphorylation. Indeed, even single stereoisomers such as the agonist (-)BayK 8644 can have both, agonistic and antagonistic effects under different experimental conditions [22].

Against our hypothesis may count that we found that antagonists of all three known groups of L-type Ca^{2+} channel blockers, dihydropyridines (nifedipine), phenylalkylamines (verapamil) and benzothiazepines (diltiazem; not shown), failed to block D_1 receptor-mediated CREB phosphorylation, although these drugs bind to three separate receptor sites on L-type Ca^{2+} channels [22]. However, these sites are allosterically linked and could be similarly affected by phosphorylation of channel subunits [22]. Moreover, in an electrophysiological study all three groups of channel blockers potentiated L-type Ca^{2+} currents in the presence of GTP- γ -S [34]. Finally, it is entirely possible that phosphorylation by PKA enables another Ca^{2+} controlling protein to bind to L-type Ca^{2+} channel blockers and to promote Ca^{2+} influx from the outside or from internal Ca^{2+} stores.

In addition to the effect of D_1 receptors on the properties of L-type Ca^{2+} channel blockers, we found that L-type Ca^{2+} channels are necessary for DA-mediated CREB phosphorylation. A knock-down of L-type Ca^{2+} channel mRNA with antisense technology prevented DA-mediated CREB phosphorylation. Thus, the present paper supports a role for L-type Ca^{2+} channels in DA receptor-mediated molecular signal transduction, unifying our past findings.

Acknowledgments

This work was supported by DA07134.

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Fig. 1.

The NMDA antagonist MK801 inhibits DA D₁ receptor-mediated CREB phosphorylation and *c-fos* expression. (A) Immunoblot showing that CREB phosphorylation by DA (50 μ M) is blocked by the NMDA antagonist MK801 (1 μ M). (B) Average fold induction of *p*-CREB/CREB, $\pm$ S.E.M., of $n=22$ samples in 11 independent experiments; untreated control levels were arbitrarily set to "1". (C) Immunoblot showing that CREB phosphorylation by the D₁ agonist SKF38393 (50 μ M) is blocked by MK801 (1 μ M). (D) Average fold induction of *p*-CREB/CREB, $\pm$ S.E.M., of $n=10$ samples in five independent experiments; untreated control levels were arbitrarily set to "1". *** p 0.001.

Fig. 2.

Glutamate- and NMDA-mediated CREB phosphorylation is inhibited by nifedipine. (A) Immunoblot showing that CREB phosphorylation by glutamate (50 µM) is blocked by nifedipine (20 µM). (B) Average fold induction of *p*-CREB/CREB, $\pm$ S.E.M., of $n=6$ samples in three independent experiments; untreated control levels were arbitrarily set to "1". (C) Immunoblot showing that CREB phosphorylation by NMDA (50 µM) is blocked by nifedipine (20 µM). (D) Average fold induction of *p*-CREB/CREB, $\pm$ S.E.M., of $n=4$ samples in two independent experiments; untreated control levels were arbitrarily set to "1". (E, F)

Immunoblot showing the inhibitory potential of 10 and 20 μ M nifedipine on FPL 64176-mediated CREB phosphorylation (E) and on NMDA-mediated CREB phosphorylation (F). Similar results were obtained with glutamate and verapamil (not shown). ** $p < 0.01$, *** $p < 0.001$.

Fig. 3.

The L-type Ca^{2+} channel blocker nifedipine does not inhibit D_1 receptor-mediated CREB phosphorylation or *c-fos* gene expression. (A) Immunoblot showing that pretreatment with nifedipine (20 μM) facilitates DA (50 μM)-mediated CREB phosphorylation. (B) Average fold induction of *p*-CREB, $\pm$ S.E.M., of $n=26$ samples in 15 independent experiments; untreated control levels were arbitrarily set to "1". (C) Immunoblot showing that pretreatment with nifedipine (20 μM) facilitates SKF38393 (50 μM)-mediated CREB phosphorylation. (D) Average fold induction of *p*-CREB, $\pm$ S.E.M., of $n=16$ samples in 9

independent experiments; untreated control levels were arbitrarily set to “1”. (E) Nifedipine (20 μ M) does block CREB phosphorylation by the L-type Ca^{2+} channel agonist FPL64176 (20 μ M). (Note: samples from A and E were run on the same gel). (F) Average fold induction of *p*-CREB, $\pm$ S.E.M., of $n=6$ samples in 3 independent gels; untreated control levels were arbitrarily set to “1”. (G) A second L-type Ca^{2+} channel antagonist, verapamil (20 μ M), also does not block DA (50 μ M)-mediated CREB phosphorylation. Average fold induction of *p*-CREB, $\pm$ S.E.M., of $n=4$ samples in 2 independent experiments; untreated control levels were arbitrarily set to “1”. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Fig. 4.

Nifedipine causes cytosolic Ca^{2+} accumulation after stimulation of either D_1 receptors or cyclic AMP pathways. (A) Incubation with SKF38393 (50 μM ; open squares; time of addition at first arrow) or nifedipine (20 μM ; open circles; time of addition at first arrow) has no effect on Ca^{2+} influx. Pretreatment with SKF38393 (filled circles; time of addition at first arrow) and subsequent addition of nifedipine (time of addition second arrow) causes prolonged cytosolic Ca^{2+} accumulation. $N=16-30$ neurons, each experiment was repeated at least once in a independent dissection. (B) Same as A, but SKF38393 was replaced with

forskolin (5 μ M). Experiment was repeated at least three times. Average $\pm$ S.E.M. fluo-3 intensity in all neurons in the microscopic field ($n=16-30$) of a representative experiment is shown. All experiments in the presence of 2 μ M TTX and 1 μ M MK 801. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to nifedipine alone.

Fig. 5.

In the presence of L-type Ca^{2+} channel blockers, DA mediates CREB phosphorylation independently of NMDA receptors. (A) Immunoblot showing that the inhibition of DA-mediated CREB phosphorylation by MK 801 is reversed by nifedipine. (B) Average fold induction of *p*-CREB, $\pm$ S.E.M., of $n=6$ samples in three independent gels; untreated control levels were arbitrarily set to "1". *** $p<0.001$ compared to DA and compared to nifedipine/MK 801/DA. (C) Immunoblot showing that the PKA inhibitor H89 (10 μM) inhibits SKF 38393-mediated CREB phosphorylation as well as the enhancement by nifedipine (20 μM).

Fig. 6.

Antisense knockdown of the $\alpha 1C$ and $\alpha 1D$ subunits of the L-type Ca^{2+} channel reduces DA-mediated CREB phosphorylation. (A) Phosphorylation of CREB by DA ($50 \mu M$) is reduced after cells are exposed to antisense oligonucleotides against $\alpha 1C$ and $\alpha 1D$ subunits of the L-type Ca^{2+} channel. Reduction of p -CREB by the L-type Ca^{2+} channel agonist, FPL 64176 ($20 \mu M$), is also observed. Actin levels are unchanged. AS=exposure to antisense oligonucleotides, SAS=exposure to scrambled antisense oligonucleotides. (B) Average fold change of p -CREB levels $\pm$ S.E.M. of $n=6$ experiments. ** $p<0.01$, * $p<0.05$.