Dopamine induces $\text{Ca}^{2+}$ signalling in astrocytes through reactive oxygen species generated by monoamine oxidase.

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Running title: ROS-induced $\text{Ca}^{2+}$ signal in astrocytes

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Dopamine (DA) is a neurotransmitter that plays a major role in a variety of brain functions, as well as in diseases such as Parkinson’s disease and schizophrenia. In cultured astrocytes, we have found that dopamine induces sporadic cytoplasmic calcium ([Ca$^{2+}$]$_{c}$) signals. Importantly, we show that the dopamine-induced calcium signalling is receptor independent in midbrain, cortical and hippocampal astrocytes. We demonstrate that the calcium signal is initiated by the metabolism of DA by monoamine oxidase, which produces reactive oxygen species and induces lipid peroxidation. This stimulates the activation of phospholipase C, and subsequent release of calcium from the endoplasmic reticulum via the IP3-receptor mechanism. These findings have major implications on the function of astrocytes that are exposed to dopamine, and may contribute to understanding the physiological role of dopamine.

INTRODUCTION

Dopamine (DA) is the predominant catecholamine neurotransmitter in the mammalian brain and controls a variety of functions including locomotor activity, cognition, emotion, positive reinforcement, food intake, and endocrine regulation. The neurotransmitter DA is a monoamine which is synthesised in dopaminergic neurons in the substantia nigra in the midbrain and transferred to the striatum through very fine C-fibres (1,2). Dopaminergic terminals constitute approximately 21% of total axon terminals in the striatum and contact mostly dendritic spines and dendritic shafts. The actions of DA are mediated by specific G-protein coupled receptors, which are divided into two major families based on their ability to stimulate (D1-like) or inhibit adenylate cyclase (D2-like). Three human D2-like receptors have been cloned: D2, D3 and D4 (3). Dopamnergic receptors are mostly distributed in the striatum, and to a lesser degree in other parts of the brain. The signalling pathways of DA in different parts of the brain are of broad clinical and scientific interest. The neurodegenerative disorder, Parkinson’s disease, is caused by a loss of dopamine-secreting neurons from the midbrain; this leads to rigidity, tremor and the characteristic slowness of movement.
Impairment of DA signalling is thought to play a role not only in Parkinson’s disease but also Alzheimer’s disease and psychomotor syndromes such as schizophrenia (4-6).

DA is catabolised by monoamine oxidase (MAO), which breaks down monoamines using FAD, producing aldehydes and hydrogen peroxide. Astrocytes express both forms of MAO: MAO-A and MAO-B (7).

Activation of D-1 and D-2 receptors is believed to modulate intracellular calcium levels by a single mechanism, that is, the stimulation of phosphatidylinositol (PI) hydrolysis by phospholipase C (PLC), resulting in the production of inositol 1,4,5-trisphosphate, which mobilizes intracellular calcium stores (2,3). Other mechanisms of release of Ca2+ from internal stores have also been proposed. DA increases cAMP levels (8-10). DA appears to affect the activity of calcium channels. In neurons and PC12 cells, DA reduced calcium currents by L-, N- and P-type calcium channels (3,9,11). Reports on the effects of DA on intracellular calcium in astrocytes from different areas of the brain are controversial and many of the effects cannot be explained solely by the interaction of dopamine with D1/2-receptors (3). The effect of DA on calcium homeostasis in astrocytes is potentially very important, in light of the interplay between neuronal and glial signals in physiology and pathology reported recently (12-14).

**EXPERIMENTAL PROCEDURE**

**Cell culture**

Mixed cultures of hippocampal, cortical or midbrain neurones and glial cells were prepared as described previously (13) with modifications, from Sprague-Dawley rat pups 2-4 days post-partum (UCL breeding colony). Hippocampi, cortex and midbrain were removed into ice-cold HBSS (Ca2+, Mg2+-free, Gibco-Invitrogen, Paisley, UK). The tissue was minced and trypsinised (0.1% for 15 min at 37°C), triturated and plated on poly-D-lysine-coated coverslips and cultured in Neurobasal A medium (Gibco-Invitrogen, Paisley, UK) supplemented with B-27 (Gibco-Invitrogen, Paisley, UK) and 2 mM L-glutamine. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air for a minimum of 12 days before experimental use to ensure the expression of receptors. Neurons were easily distinguishable from glia: they appeared phase bright, had smooth rounded somata and distinct processes, and lay just above the focal plane of the glial layer. Cells were used at 12-15 days in vitro unless differently stated.

Isolated cortical astrocytes were prepared as previously described (15). Cerebra taken from 2-5 day old Sprague-Dawley rats (UCL breeding colony). The cerebra were chopped and triturated until homogenous and trypsinised (50000 U/ml -1 porcine pancreas, Sigma, Gillingham, UK) with 336 U/ml -1 DNAse 1 (bovine pancreas, Sigma, Gillingham, UK), and collagenase 1.033 U/ml -1 (Sigma, Gillingham, UK) at 37°C for 15 minutes. After addition of foetal bovine serum (10% of final volume) and filtering through 140 μM mesh, the tissue was centrifuged through 0.4 M sucrose (400 g, 10 minutes), and the resulting pellet transferred to Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 5% foetal calf serum, 2 mM glutamine and 1 mM malate in tissue culture flasks pre-coated with 0.01% poly-D-lysine. The cells reached confluency at 12-14 days in vitro, and were harvested and reseeded onto 24-mm-diameter glass coverslips, precoated with 0.01% poly-D-lysine for fluorescence measurements and used for 2-4 days.

For the MAO-B knockdown experiment, cells were transiently transfected on second day in vitro using Lipofectamine LTX transfection reagent (Invitrogen). Empty shRNAmir-GFP or MAO-B containing lentiviral constructs were purchased from Open Biosystems, UK. Cortical explant cultures were generously provided by Drs A.V. Gourine and V. Kasymov and prepared as described in (16).

**Imaging [Ca2+], and lipid peroxidation**
For measurement of $[\text{Ca}^{2+}]_c$, primary astrocytes were loaded for 30 min at room temperature with $5\mu M$ fura-2 AM or fluo-4 AM with/without Rhod-2 and 0.005% Pluronic in a HEPES-buffered salt solution (HBSS) composed (mM): 156 NaCl, 3 KCl, 2 MgSO$_4$, 1.25 KH$_2$PO$_4$, 2 CaCl$_2$, 10 glucose and 10 HEPES, pH adjusted to 7.35 with NaOH. Ca$^{2+}$-free HBSS also contained 0.5mM EGTA. For measurement of the lipid peroxidation rate, astrocytes were loaded with 5 µM C11-BODIPY (581/591) for 30 min.

Fluorescence measurements were obtained on an epifluorescence inverted microscope equipped with a 20x fluorite objective. $[\text{Ca}^{2+}]_c$ and plasmalemmal membrane potential were monitored in single cells using excitation light provided by a Xenon arc lamp, the beam passing monochromator at 340, 380 and 490 nm (Cairn Research, Kent, UK). Emitted fluorescence light was reflected through a 515 nm long-pass filter to a cooled CCD camera (Retiga, QImaging, Canada) and digitised to 12 bit resolution. All imaging data were collected and analysed using software from Andor (Belfast, UK). Traces, obtained using the cooled CCD imaging system, are presented as the ratio of excitation at 340 and 380 nm, both with emission at $>515$ nm. For some measurements, $[\text{Ca}^{2+}]_c$ was calculated using the equation (17): $[\text{Ca}^{2+}]_c = K(R - R_{\text{min}})/(R_{\text{max}} - R)$; where $R$ is the fluorescence ratio (340 nm/380 nm) and $K$ is the effective dissociation constant of fura-2. $R_{\text{max}}$ and $R_{\text{min}}$ were determined by application of 50 µM digitonin followed by 1mM MnCl$_2$.

Confocal images were obtained using a Zeiss 510 uv-vis CLSM equipped with a META detection system and a 40x oil immersion objective. The 488nm Argon laser line was used to excite fluo-4 which was measured using a bandpass filter from 505-550nm. For Rhod-2 measurements the 543 nm laser line and 560nm longpass filter were used. Illumination intensity was kept to a minimum (at 0.1-0.2% of laser output) to avoid phototoxicity and the pinhole set to give an optical slice of ~2µm. C11-BODIPY (581/591) was excited using the 488 and 543nm laser line and fluorescence measured using a bandpass filter from 505-550nm and 560nm longpass filter. All data presented were obtained from at least 5 coverslips and 2-3 different cell preparations. Where indicated, solvent-only control or DA-free controls were used.

**Cell viability experiment**

In order to measure cell viability, cells were loaded simultaneously with 20µM propidium iodide (PI), (which is excluded from viable cells but exhibits a red fluorescence following a loss of membrane integrity in non-viable cells) and 4.5µM Hoechst 33342 (Molecular Probes, Eugene, OR), which labels nuclei blue, to count the total number of cells. Each experiment was repeated four times using independent cultures.

**Statistical analysis**

Statistical analysis was performed with the aid of Origin 8 (Microcal Software Inc., Northampton, MA, USA) software. Means expressed ± the standard error of the mean (S.E.M.).

**RESULTS**

**DA induces a Ca$^{2+}$ signal in astrocytes from midbrain, cortex and hippocampus**

Application of DA (20µM) to astrocytes in culture produced a complex fluctuation in $[\text{Ca}^{2+}]_c$ shown in Fig 1A. There was no observable difference in the DA-induced Ca$^{2+}$ signal in primary cortical, midbrain or hippocampal astrocyte cultures, and the data shown is from experiments using cortical astrocytes, except where stated differently. In order to investigate the concentration of DA required to induce a Ca$^{2+}$ signal, DA was applied in the concentration range of 0.1 to 100µM to cultured astrocytes (Fig 1B). Low concentrations of DA were sufficient to induce an increase in the $[\text{Ca}^{2+}]_c$, and the amplitude of the DA-induced Ca$^{2+}$ signal (50 – 400nM) and was dependent on the concentration of DA applied (Fig 1B).
We observed and characterised three major types of DA-induced Ca^{2+} responses in astrocytes: (1) single spikes with amplitude in the peak 200-300 nM (Fig 1Di), (2) low amplitude 150-250 nM oscillations (Fig 1Dii), (3) broad single peak of 400-500 nM (Fig 1Diii). We quantified the proportion of astrocytes exhibiting each type of DA-induced Ca^{2+} signal (Fig 1C). In the majority of astrocytes respond to DA (20 μM) either with single spikes (46.8±4.5% astrocytes) or low amplitude oscillations (40.7±3.9% astrocytes). Less than one fifth showed a broad single peak (12.5±3.9% astrocytes (Fig.1C). After washing the cells with DA-free saline, the Ca^{2+} responses persisted for up to 5 minutes (n=38; data not shown).

The DA-induced increase in [Ca^{2+}]_c (measured by the fluo-4 fluorescence) stimulates transient Ca^{2+} uptake in mitochondria, resulting in an increase in mitochondrial [Ca^{2+}] as measured by Rhod-2 fluorescence (n=29; Fig. 1E). This mitochondrial calcium uptake is typical for physiological calcium stimuli in astrocytes. As responses like these have not been described previously, we were concerned that the properties of cells might be dictated by our culture conditions. We therefore repeated the experiments using cortical explant cultures, in which the properties of the tissue in vivo are well retained. Confocal imaging of explant cultures loaded with fluo-4 demonstrated that exposure to DA (20μM) provoked an increase in Ca^{2+} signalling in glial cells within the culture (Fig.1F). (The neurons in the explants culture were identified as the only cells in the culture to show a rise in [Ca^{2+}]_c with 50mM KCl (data not shown.).)

The DA-induced Ca^{2+} signal is receptor independent

We sought to investigate the mechanism of the DA-induced Ca^{2+} signal. It has been shown previously that the signalling effects of DA on astrocytes are mediated by D1- and D2-like receptors (18,19). We therefore measured the DA induced Ca^{2+} signal in cells loaded with fura-2 in the presence and absence of DA receptor antagonists. Application of the antagonist for D2-like receptors (20 μM sulpiride) did not prevent the DA-induced Ca^{2+} response in astrocytes (n=157, Fig.2A). Similarly, application of the D1/D5 antagonist (20 μM SCH-23390) did not block the DA induced Ca^{2+} response (n=171, Fig.2B). D1/D5 or D2 antagonists did not significantly change amplitude of the DA-induced Ca^{2+} signal in astrocytes. All three classes of DA-induced Ca^{2+} responses were observed in the presence of receptor antagonists. Furthermore application of the receptor antagonists did not alter the proportion of cells exhibiting each type of DA-induced Ca^{2+} response. (Fig. 2C).

The responses to DA were also not significantly affected by inhibitors of either ionotropic or metabotropic glutamate receptors, including 10μM MK-801 (n=29 cells; Fig. 2D), 20μM CNQX (n=34 cells, Fig.2E), or 50μM (S)-MCPG (n=32 cells, data not shown), suggesting that the responses do not reflect glutamate release into the culture.

We investigated the source of the Ca^{2+} in the DA-induced Ca^{2+} signal in astrocytes. Application of DA to primary cortical cell co-cultures in Ca^{2+}-free medium did not prevent the Ca^{2+} signal in astrocytes (Fig.3A). This suggests that the DA-induced Ca^{2+} signal in astrocytes is dependent on intracellular Ca^{2+}. However, the absence of extracellular Ca^{2+} delayed the onset of the signal in astrocytes (n=80), time from application of dopamine to appearance of Ca^{2+} signal was 120-180 seconds (Fig 3A, compared to immediate onset of Ca^{2+} signal in Figure 1A). Incubation of primary co-cultures with 0.5 µM thapsigargin (an inhibitor of endoplasmic reticulum (ER) Ca^{2+} pumps) depleted Ca^{2+} from the ER and completely prevented the DA-induced [Ca^{2+}]_c changes in astrocytes (n=52 astrocytes, Fig.3B). This confirmed that the astrocytic DA-induced Ca^{2+} signal is dependent on intracellular stores. U73122 is an inhibitor of phospholipase C (PLC). Application of U73122 completely prevented the astrocytic DA–induced Ca^{2+} signal (n=73; Fig.3C). Furthermore, application
of 2-APB (n=27 cells), an inhibitor of IP$_3$-dependent ER Ca$^{2+}$ release, also blocked the DA-induced Ca$^{2+}$ signal in astrocytes (Fig.3D). Although the expression and role of ryanodine receptors (RyR) in astrocytes is controversial (20), we tested the effect of the RyR inhibitor dantrolene (10μm), which produced no significant effect on the DA-induced Ca$^{2+}$ signal (n=39; data not shown). Taken together, these experiments suggest that in astrocytes, DA activates PLC, inducing IP$_3$ dependent Ca$^{2+}$ release from ER, resulting in a rise in intracellular Ca$^{2+}$.

It has been reported that activation of PLC and IP$_3$ dependent ER Ca$^{2+}$ release can be initiated by stimulation of P$_2$-purinoreceptors (21). However, incubation of primary cultures of astrocytes with the P$_2$-purinoreceptor antagonist 20μM PPADS (pyridoxalphosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate) did not affect the DA induced [Ca$^{2+}$]$_c$ changes in astrocytes (n=29, data not shown). Therefore DA does not activate PLC via P$_2$-purinergic receptors.

As mentioned earlier, incubation of astrocytes in calcium free medium (Ca$^{2+}$-free HBSS + 0.5mM EGTA) induced a delay in the DA-induced Ca$^{2+}$ signal in astrocytes. To exclude involvement of extracellular Ca$^{2+}$ in the DA-induced Ca$^{2+}$ signal we employed the method of manganese quench of fura-2 fluorescence. Extracellular Mn$^{2+}$ enters cells via open Ca$^{2+}$-permeate channels and quenches the fluorescence of intracellular fura-2. This is most readily seen when the fura-2 is excited at ~360 nm, the Ca$^{2+}$-independent (isosbestic) point of the fura-2 excitation spectrum (whereas the Ca$^{2+}$-dependent change of the 340/380 nm ratio is not altered by Mn$^{2+}$). The DA-induced Ca$^{2+}$ spikes in astrocytes (n=62) (which were recorded as high increases in the fura-2 ratio signal) induced only a small decrease in 360 nm fluorescence in the presence of Mn$^{2+}$ (Fig.3E). This experiment confirmed that extracellular Ca$^{2+}$ does not play a role in the DA-induced [Ca$^{2+}$]$_c$ responses. The small changes in 360 nm signal in astrocytes (Fig 3E) may be explained by the opening of store operated Ca$^{2+}$ channels.

**The DA-induced Ca$^{2+}$ signal in astrocytes is mediated by lipid peroxidation**

We tested the role of DA metabolism on the DA-induced Ca$^{2+}$ signal. Monoamine oxidase is the enzyme responsible for utilization of DA in the brain (7). Pre-incubation (5 min) of astrocytes with the inhibitor of MAO, selegiline (20μM inhibits both isoforms MAO-A and MAO-B) completely blocked the DA-induced Ca$^{2+}$ signal in astrocytes (n=142 astrocytes; Fig.4A). Tyramine may be used as another substrate for MAO. Addition of 50μM tyramine induced DA-like changes in the astrocyte Ca$^{2+}$ signal (n=29 cells, Fig.4B) suggesting that the activity of MAO is necessary for the DA-induced Ca$^{2+}$ signal.

To confirm the involvement of MAO in the DA-induced Ca$^{2+}$ signal in astrocytes we performed MAO-shRNA in midbrain cocultures. Application of DA (20μM) to cells with MAO knockdown did not produce a Ca$^{2+}$ signal in astrocytes (n=21; Fig.4C). MAO catalyses DA using FAD and produces H$_2$O$_2$ and aldehyde. To test whether the Ca$^{2+}$ signal was due to reactive oxygen species (ROS) production by MAO, we applied MnTBAP, a scavenger of ROS, to astrocytes in the presence of DA. Pre-incubation of primary co-cultures with 50μM MnTBAP (a superoxide dismutase mimic and hydrogen peroxide (H$_2$O$_2$) scavenger) had no effect on the DA-induced [Ca$^{2+}$]$_c$ responses in astrocytes (n=67 cells, Fig.4D).

However, incubation of cells with an antioxidant that inhibits lipid peroxidation, 100μM Trolox (water soluble vitamin E analogue), was effective in inhibiting the DA-induced Ca$^{2+}$ signal in astrocytes (n=81 cells; Fig.4E). It should be noted that Trolox is a water soluble antioxidant and can be effective at ROS scavenging in the cytosol. 50 min preincubation of the cells with 100μM vitamin E (α-tocopherol) also completely prevented the
DA-induced Ca\(^{2+}\) signal in astrocytes (n=49; data not shown).

In order to test whether DA affects lipid peroxidation, we employed the specific indicator C11-BODIPY (581/591), which allows live changes in the rate of lipid peroxidation in live cells to be measured. Addition of 20 µM DA induced a rapid increase in the rate of lipid peroxidation in astrocytes (signal rose to 315.3±26% of basal rate, p<0.001; n=36 cells; Fig.4F). The effect of DA on lipid peroxidation was induced by the activation of MAO as it could be blocked by application of the MAO inhibitor selegiline 20µM (rate of C11-BODIPY (581/591) oxidation dropped from 315.3±26% to 95.3±6.2% of basal rate; Fig.4F). Considering the differences in the effect of MnTBAP and vitamin E on DA-induced Ca\(^{2+}\) signalling we also tested the effect of these two antioxidants on DA-stimulated lipid peroxidation. Pre-treatment of astrocytes with α-tocopherol (100µM, 20 min) completely prevented the increase in the rate of DA-induced lipid peroxidation (108.6±6.8% of basal rate C11-BODIPY (581/591) oxidation, n=27; Fig.4F). Exposure of astrocytes with 50µM MnTBAP (20 min before and during the time of experiment) did not abolish DA-induced lipid peroxidation, but did result in a reduction in the rate of peroxidation. The rate of C11-BODIPY (581/591) oxidation in cells treated with MnTBAP rose to 231.4±28.3% in response to DA (n=34; p<0.001; Fig.4F). Thus, MnTBAP is not as effective against DA-induced lipid peroxidation as α-tocopherol and this may underlie the difference in the of vitamin E and MnTBAP on the DA-induced Ca\(^{2+}\) signal. Thus, activation of MAO in astrocytes by DA induces lipid peroxidation which leads to the stimulation of PLC and IP\(_3\) induced calcium release from ER.

DA-induced Ca\(^{2+}\) signalling occurs at DA concentrations that do not affect cell viability.

The in vivo concentration of dopamine is not well established, and different authors report physiological ranges of dopamine concentration in the nanomolar to millimolar range. In order to establish whether the concentration of dopamine that induces a Ca\(^{2+}\) signal in astrocytes is pathological, we studied the effect of different concentrations of DA (0-500µM) on the viability of astrocytes. Prolonged (24 hours) exposure of 5-50µM DA did not significantly increase the number of cell death in primary cultures of cortical astrocytes (Fig.5) compared to control. Only high concentrations of DA (100µM and 500µM) produced a significant increase in cell death (from 14.3±2.9% in control to 27.8±3.9% for 100µM of DA; p<0.01; and to 73.6±5.5% for 500µM; p<0.001; n=3, Fig.5). Thus, the changes in [Ca\(^{2+}\)]c in astrocytes in response to the concentrations of DA used in these experiments are not reflected in pathological processes in cells.

DISCUSSION

Our findings suggest novel effects of DA on calcium signalling in astrocytes. Astrocytes and neurons are likely to communicate both physiologically and in disease. Physiologically, calcium signals in astrocytes cause cerebrovascular constrictions (22,23). Neurotoxicity in neurodegenerative disease may be mediated by astrocyte-neuronal interactions. For example, beta-amyloid induces a calcium signal in astrocytes which results in overproduction of ROS, reduction of antioxidant levels, and cell death in neurons (12-14). In this study, we have demonstrated that metabolism of non-toxic concentrations of DA by MAO in astrocytes produces hydrogen peroxide, which activates lipid peroxidation in the neighbouring membranes. Lipid peroxidation activates PLC, releasing IP\(_3\) and inducing a Ca\(^{2+}\) signal. While ROS have been extensively implicated in causing oxidative damage in neurodegenerative disease, there is emerging evidence that ROS also acts as a...
physiological mediator of normal cellular function. The cellular redox state and ROS may stimulate as well as inhibit Ca\(^{2+}\) channels and Ca\(^{2+}\) pumps, and thus modulate Ca\(^{2+}\) signalling (24). To our knowledge this is a first evidence of the involvement of ROS in a calcium signalling pathway in response to a physiological stimulus such as dopamine transmission in the brain.

Increased levels of oxidative stress in the brain may be critical for the initiation of glutamate or ATP release, which acts as a trigger for Ca\(^{2+}\)-signalling (25-27). In skeletal and cardiac muscle ROS is known to induce a calcium signal via activation of the ryanodine receptor (28). However, we have shown that this mechanism is not involved in DA-induced \([\text{Ca}^{2+}]_c\) changes because the astrocytic calcium signal was insensitive to inhibitors of glutamate, purino- or ryanadine receptors (see above). DA can produce ROS via both enzymatic (H\(_2\)O\(_2\), superoxide) (29). However the effect of the MAO inhibitor selegiline and the effect of MAO-shRNA confirmed the role of enzymatic ROS production on lipid peroxidation and the induction of calcium signalling in astrocytes.

We have shown for the first time that DA produces effects on Ca\(^{2+}\) signalling that are not mediated by the known receptors. Of note, this is the first demonstration of Ca\(^{2+}\) signalling in astrocytes mediated by ROS. We believe that this DA induced Ca\(^{2+}\) signal may play an important role in dopamine signalling in human brain.

**Acknowledgements**

We thank Parkinson’s UK for support and Dr. Vitaliy Kasimov and Dr. Alexander V. Gourine for providing cortical explant cultures. We are also grateful to Miss Victoria Burchell for her assistance.

**Reference List**


**Figure legends**

Figure 1. Dopamine induces elevation of intracellular Ca^{2+} in astrocytes.
Application of DA (20µM) to astrocytes induces an immediate fluctuation in [Ca\(^{2+}\)]\(_{c}\) (A). The amplitude of the intracellular calcium change, measured by the peak of the signal or maximal peak in the oscillations, is dependent on the concentration of DA applied (B). Three different types of DA-induced Ca\(^{2+}\) response were observed: (i) single sharp spikes of Ca\(^{2+}\) flux (Di), (ii) sporadic increases in [Ca\(^{2+}\)]\(_{c}\) as oscillations (Dii) and (iii) large increase in [Ca\(^{2+}\)]\(_{c}\), seen as broad single peak followed by slow downwards trend to baseline (Diii). The proportion of astrocytes displaying the different types of responses are demonstrated in C. Application of DA induces a rise in [Ca\(^{2+}\)]\(_{c}\) (measured by fluo-4, black) with a concomitant transient increase in [Ca\(^{2+}\)]\(_{m}\), mitochondrial calcium (measured by rhod-2, gray) in astrocytes (E). Application of DA (20µM) induces changes in [Ca\(^{2+}\)]\(_{c}\), measured by fluo-4 fluorescence, in astrocytes from cortical explant culture (F).

**Figure 2. Dopamine induces a receptor independent [Ca\(^{2+}\)]\(_{c}\) signal in astrocytes.** DA induces changes in [Ca\(^{2+}\)]\(_{c}\) in astrocytes in the presence of the D2-like receptor antagonist (-)-sulpiride (20µM; A) or the D1-like receptor antagonist SCH-23390 (B). The distribution of the patterns of responses in astrocytes in response to DA in control cells, and cells pre-treated with (-)-sulpiride or SCH-23390 is not significantly different (C). Inhibitors of NMDA (10µM MK 801; D) or 20µM CNQX (E) did not block the DA-induced Ca\(^{2+}\) signal.

**Figure 3. [Ca\(^{2+}\)]\(_{c}\) responses to dopamine are dependent on intracellular Ca\(^{2+}\) stores in astrocytes.**

The removal of external Ca\(^{2+}\) (Ca\(^{2+}\)-free HBSS with 0.5mM EDTA) delays the onset of the DA-induced Ca\(^{2+}\) responses in astrocytes, but does not abolish it (A). Depletion of the intracellular Ca\(^{2+}\) pool by application of the inhibitor of ER Ca\(^{2+}\) pump, thapsigargin (0.5µM) abolishes the DA-induced Ca\(^{2+}\) signal in astrocytes (B). Changes in [Ca\(^{2+}\)]\(_{c}\) in response to DA are dependent on the presence of the inhibitor of phospholipase C U73122 (5µM) (C). Application of the inhibitor of IP3 receptor and capacitive calcium entrance 2-APB (20µM) blocked the effect of DA in astrocytes (D). In the presence of external 100µM Mn\(^{2+}\), the fura-2 response excited at 360nm showed no change during the [Ca\(^{2+}\)]\(_{c}\) transients in astrocytes, demonstrating that this is close to the isosbestic [Ca\(^{2+}\)]\(_{c}\) independent excitation wavelength for fura-2, confirming that the DA-induced Ca\(^{2+}\) signal in astrocytes is independent of external Ca\(^{2+}\) (E).

**Figure 4. Dopamine-induced Ca\(^{2+}\) signal in astrocytes is induced by production of ROS from MAO.**

Application of the MAO inhibitor, selegiline (20µM), blocks the DA-induced Ca\(^{2+}\) signal in astrocytes (A). The Ca\(^{2+}\) signal in primary cortical astrocytes could also induced by application of another monoamine, tyramine (50µM) (B). MAO-B shRNA in astrocytes results in inhibition of the DA-induced Ca\(^{2+}\) signal (C). The DA-induced Ca\(^{2+}\) signal was blocked by preincubation of cells with inhibitor of lipid peroxidation, Trolox (100µM) (D), but not with antioxidant MnTBAP (50µM) (E). Application of DA produces a rise in the rate of lipid peroxidation in astrocytes, which is dependent on MAO activity (F). DA-induced lipid peroxidation could be prevented by pre-treatment of astrocytes with α-tocopherol (100µM) (F). Pre-treatment of astrocytes with the antioxidant MnTBAP (50 µM) reduced the rate of lipid peroxidation but did not abolish it. (F).

**Figure 5. The effect of DA on cell viability.**

Low concentrations of DA (0-50µM) has no significant effect on cell viability in pure cultures of cortical astrocytes, while higher concentrations (100, 500µM) significantly increased the percentage of cell death. **p<0.01 and ***p<0.001 vs control. All data were expressed as mean ± S.E.M.
Figure 1
Figure 2
Figure 3.
Figure 4
Figure 5

Dead astrocytes, %

[DA, μM]

0 5 10 50 100 500

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*J. Biol. Chem.* published online June 14, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.111450

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