Role of transient receptor potential canonical 6 (TRPC6) in non-transferrin-bound iron uptake in neuronal phenotype PC12 cells

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Cells take up transferrin-bound iron or NTBI (non-transferrin-bound iron). After treatment with NGF (nerve growth factor), PC12 cells exhibited a neuronal phenotype and an increase in the NTBI uptake ($^{55}Fe^{2+}$ or $^{55}Fe^{3+}$). We loaded the cells with the dye calcein, whose fluorescence increases in the presence of Ca^{2+} but is quenched with Fe^{2+} or Fe^{3+}. When examined using calcein fluorescence or radioactive iron, DAG (diacylglycerol)-stimulated NTBI entry was more in NGF-treated PC12 cells compared with untreated cells. All experiments were performed at 1.5 mM extracellular Ca^{2+}. Nramp2 (natural-resistance-associated macrophage protein 2) mRNA expression did not change after the NGF treatment. Expression of the bivalent cation entry protein TRPC6 (transient receptor potential canonical 6) was detected only in the NGF-treated cells. To verify that increased NTBI uptake depended on TRPC6, we examined whether transfecting HEK-293 (human embryonic kidney 293) cells with TRPC6 also increased the NTBI (^{55}Fe) uptake. We also cotransfected HEK-293 cells with two plasmids, one expressing TRPC6 and the other expressing the fluorescent protein DsRED2 to identify the transfected cells. Challenging the calcein-loaded HEK-293 cells (which intrinsically express the α_1-adrenergic receptors) with phenylephrine or a cell-permeant DAG increased the fluorescence signal more rapidly in transfected cells compared with untransfected cells. However, when iron (Fe^{2+} and Fe^{3+}) was added before adding phenylephrine or DAG, the fluorescence intensity decreased more rapidly in transfected cells compared with untransfected cells, thereby indicating a greater stimulation of the NTBI uptake in cells expressing TRPC6. We postulate that the increase in the NTBI entry into neuronal PC12 cells is through TRPC6, a pathway that is unique since it is receptor-stimulated. Since neuronal cells express TRPC6, this pathway may have a role in neurotoxicity.

Key words: calcium channel, nerve growth factor, non-transferrin-bound iron, Parkinson's disease, PC12 cell, transient receptor potential canonical 6 (TRPC6).

INTRODUCTION

Ca^{2+} is a key second messenger and, hence, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is regulated in response to various stimuli. Ca^{2+} sequestration into the endoplasmic reticulum by SERCA (sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase) pumps and removal of Ca^{2+} from cells by the plasma membrane Ca^{2+} pumps and the Na^+–Ca^{2+} exchanger decrease the $[Ca^{2+}]_i$ to basal levels in excited cells [1]. An increase in $[Ca^{2+}]_i$ may occur through the release of Ca^{2+} from the endoplasmic reticulum via IP_3-dependent channels or by Ca^{2+}-induced Ca^{2+} release (ryanodine-sensitive Ca^{2+} channels). Ca^{2+} entry into cells occurs via voltage-, store- and receptor-operated Ca^{2+} channels. Together with the well-characterized voltage-operated Ca^{2+} channels, the store- and receptor-operated Ca^{2+} channels provide a diversity by which $[Ca^{2+}]_i$ can be altered to achieve different responses to a variety of physiological stimuli.

Ca^{2+} can enter into *Drosophila* photocells via a TRP (transient receptor potential) voltage-independent pathway [2]. Mammalian homologues of the *Drosophila* TRP channels have been linked to capacitative entry of Ca^{2+} and to G-protein-coupled-receptor-operated influx of Ca^{2+} and are, thus, potential candidates for store- and receptor-operated Ca^{2+} channels [3,4]. The TRP family has been further subdivided into three major subfamilies, namely the canonical or short subfamily [TRPC (transient receptor potential canonical)], the melastatin or long subfamily (TRPM) and the vanilloid subfamily (TRPV) [5]. TRPC subfamily members share highest similarity with the *Drosophila* TRP or TRP-like proteins. When activated, TRPCs allow Ca^{2+} entry and may be linked to stimulation of the phospholipase C pathway and/or depletion of internal Ca^{2+} stores [4,6–8]. The superfamily contains up to seven members of which TRPC3 and TRPC6 mRNA have been shown to be more abundant in the brain, and it has been suggested that they are mainly receptor-operated Ca^{2+} channels [9–11]. TRPC3 and TRPC6 have also been shown to be activated by DAG (diacylglycerol), independent of protein kinase C activation. Alternative splicing and formation of complexes between heterologous TRPC molecules may yield additional channels [12]. The TRPC channels are less selective for Ca^{2+} compared with voltage-operated Ca^{2+} channels and are therefore termed non-selective cation channels [5,12–14].

Iron is essential for the survival of all cells, although excess iron can be toxic [15,16]. Iron entry into cells may occur as TBI (transferrin-bound iron) or as NTBI (non-transferrin-bound iron). TBI entry involves binding of Fe^{3+}–transferrin complexes to cell-surface receptors. NTBI uptake (as Fe^{2+}) has been reported to occur through Nramp1 (natural-resistance-associated macrophage protein 1) in macrophages and through Nramp2 in intestinal epithelium, erythroid and several other cell types [17–21]. It has been suggested that Fe^{3+} transport may also involve reduction by a ferric reductase [22]. Recently, additional pathways

Abbreviations used: DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; NGF, nerve growth factor; Nramp2, natural-resistance-associated macrophage protein 2; NTA, nitrilotriacetate; NTBI, non-transferrin-bound iron; PSS, physiological saline solution; RT, reverse transcriptase; TBI, transferrin-bound iron; TRP, transient receptor potential; TRPC, transient receptor potential canonical (subfamily).

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for NTBI transport involving $\alpha_v\beta_3$-integrin and mobiliferrin have also been proposed [15,23]. TRPCs are non-selective cation channels that allow the entry of many ions, including Mn^{2+} [14]. However, iron entry through these channels has not been reported, probably because the presence of very low concentrations of Fe^{2+} or Fe^{3+} in solution makes electrophysiological measurements very difficult. Neuronal cells show entry of TBI and NTBI uptake [16]. Brain tissues express TRPC6 at high levels where it could contribute to excessive iron accumulation, leading to neurotoxicity. We show the relevance of this pathway using rat adrenal medullary tumour pheochromocytoma PC12 cells as a neuronal model, since they show higher NTBI uptake when differentiated into neuronal cells after treatment with NGF (nerve growth factor) [24–27]. We further support this model using HEK-293 (human embryonic kidney 293) cells transfected with TRPC6.

EXPERIMENTAL

Cell cultures

PC12 cells in passages 12–30 were cultured and treated with NGF (40 ng/ml of 2.5 S NGF) on days 2, 5 and 7 after the initial plating as described previously [28]. The cells were harvested 9 days after the initial plating in PSS (physiological saline solution), which contained 10 mM Hepes, 140 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$ and 1.5 mM $CaCl_2$ at pH 7.4 (pH at 37 °C). HEK-293 cells were cultured to confluence in DMEM (Dulbecco's modified Eagle's medium), supplemented with 10 % (v/v) foetal calf serum, 2 mM glutamine, 50 μg/ml gentamicin and 0.5 mM Hepes. The cells were transfected with either the control plasmid (pcDNA3; Invitrogen) or the plasmid containing the TRPC6 cDNA cloned in the *Spe*I–*Eco*RI sites of pcDNA3 [9]. For the radioactive uptake experiments, 3×10^6 cells were plated per 100 mm dish, and the cells were transfected, after 24 h, with 6 μg of plasmid DNA and 25 μl of LIPOFECTAMINE™ 2000 (Gibco-BRL) in 4 ml of serum-free DMEM. After 2 h, the medium was replaced with DMEM containing 10 % foetal calf serum. For use in the calcein-quenching experiments, 1×10^6 HEK-293 cells were seeded in a 60 mm Petri dish with a 40 mm round glass coverslip attached to the bottom. After 24 h, the cells were co-transfected with TRPC6 contained in pcDNA3 plasmid (2 μg) and the DsRED2 vector plasmid (0.5 μg; Clontech, Palo Alto, CA, U.S.A.) to identify the transfected cells.

NTBI uptake experiments using ^{55}Fe

Initial velocity measurements of NTBI uptake using Fe^{2+} were performed for 2 min at a cell protein concentration of 0.5–0.8 mg/ml, using 1 mM ascorbate to reduce the iron and 0.43 μM of total iron chelated with 10 μM NTA (nitrilotriacetate) to obtain a free Fe^{2+} concentration of 0.1 μM as described previously [28]. Modifications of this method for Fe^{3+} uptake and for using citrate instead of NTA have also been described earlier [28]. The uptake was performed at 0 and 37 °C and the difference between the uptakes at the two temperatures was considered to be NTBI uptake.

NTBI uptake experiments using calcein quenching

NTBI entry into cells was determined by monitoring the quenching of calcein fluorescence as described previously [29,30]. NGF-treated or control PC12 cells cultured in 60 mm dishes were incubated with calcein/AM (Molecular Probes, Eugene, OR, U.S.A.) in PSS for 40 min at 37 °C. The cells were then washed and harvested with PSS and placed in a conical test tube where they were incubated for 30 min at 22–24 °C. The cells were centrifuged at 200 ***g*** for 5 min and then resuspended in 1 ml of PSS. The cells were kept at 22–24 °C and used within 2 h. To measure the fluorescence, 50 μl of the cells were added to 2 ml of PSS in a stirred cuvette and calcein fluorescence was measured using a Spex Fluorolog 112 at 37 °C at an excitation wavelength of 490 nm and emission wavelength of 530 nm. An experiment was also conducted using radioactive iron to verify these results. In this experiment, calcein was not used, but the NGF-treated PC12 cells were incubated at 37 °C for 10 min in the presence of 1 μM unlabelled Fe^{2+} or Fe^{3+} and 10 μM NTA before adding 1 μM $^{55}Fe^{2+}$ or $^{55}Fe^{3+}$, 10 μM NTA and 0 or 100 μM DAG. The reaction was terminated by filtration after another 2 min. HEK-293 cells were loaded 48 h after the transfection, while still attached to the coverslips, for 15 min with 2 μM calcein/AM. The coverslips were then mounted on a perfused heated chamber. The transfected cells were identified by DsRED2 fluorescence using a Zeiss microscope attached to an M series dual-wavelength excitation system from Photon Technologies (Monmouth Junction, NJ, U.S.A.). The excitation and emission wavelength pair used for DsRED2 was 553 and 588 nm. Calcein fluorescence was measured at the excitation and emission wavelength pair, 488 and 525 nm. Fluorescence intensity was measured for 5 min while perfusing with PSS to obtain the basal levels, at which time the perfusion solution was changed to PSS containing 1 μM total Fe^{2+} or Fe^{3+}, 10 μM NTA and 1 mM ascorbate when Fe^{2+} was used. The fluorescence signals were processed using ImageMaster software and the change in fluorescence intensity in single cells was determined by defining regions of interest. A total of at least 12 cells were used in one such field obtained in each experiment with a total of three experiments.

RT (reverse transcriptase)–PCR

RNA was isolated from PC12 or HEK-293 cells using TRIzol® reagent (Gibco-BRL) according to the manufacturer's instructions. The RNA samples were treated with DNase so as to exclude the possibility that the PCR products were amplified from contaminating genomic DNA. As described previously, first-strand cDNA was synthesized from 1 μg of DNase-treated total RNA using the primer $d(T)_{20}$. PCR was performed for 30 cycles as described previously using primers based on sequences with GenBank® accession numbers AF061266, AF136401, AB008889, AF106860, AF008439 and BC029618 [11]. As a negative control, PCR was also performed on RNA samples without RT. The size of the PCR products was determined by using a 100 bp molecular-mass ladder and the expected sizes are given below. We had verified all the PCR products earlier by sequencing: TRPC1 (TGCTGTTGGCTGTGAATGCACGCT, ACTTCCAGTTCACGAGAATTCCGA; 280 bp), TRPC2 (GAAACGTTCCAGTTTCTCTTCTGG, CTTGGAGCGAGCAAACTTCCACTC; 220 bp), TRPC3 (CCTGAGCGAAGTCACACTCCCAC, CCACTCTACATCACTGTCATCC; 525 bp), TRPC4 (CTCTGCAGATATCTCTGGGAAG, AAGCTTTGTTCGAGCAAATTTCCA; 490 bp), TRPC5 (CTATGAGACCAGAGCTATTGATG, CTACCAGGGAGATGACGTTGTATG; 220 bp), TRPC6 (GTGCCAAGTCCAAAGTCCCTGC, CTGGGCCTGCAGTACGTATC; 305 bp), rat G3PDH (glyceraldehyde-3-phosphate dehydrogenase) (CACGGTCAAGGCTGAGAAC, CGACCTGGTCCTCGGTGT; 671 bp), human G3PDH (GCTGAGTACGTCGTGGAGTC, GTCGCTGTTGAAGTCAGAGG; 593 bp), Nramp2-a (TGCGGCCAGTGATGAGTGAGTT,

ATTGCCACCGCTGGTATCTTCG; 289 bp) and Nramp2-b (GACCAGGTCTATTGCCATCATC, TCCGTTGGAGAACTC-ACTCATC; 177 bp).

In some experiments, we performed co-PCR as specified in the Results section. All PCR products were analysed by PAGE and the gels were stained with ethidium bromide to visualize the bands and the molecular-mass standards.

Western-blot analysis

NGF-treated and control PC12 cells were harvested and used for preparing crude membranes, which were used for SDS/PAGE under reducing conditions for Western-blot analysis. The TRPC6 protein was detected using anti-TRPC6 antibody from Alomone Laboratories (Jerusalem) and a chemiluminescence kit from Amersham Biosciences (Piscataway, NJ, U.S.A.). For Nramp2, a rabbit polyclonal serum was used against a peptide segment derived from the N-terminal region of Nramp2 (residues 1–71) fused to a glutathione S-transferase [31].

Statistical analysis

All experiments were replicated 3–6 times. Student's t test was used where applicable and $P < 0.05$ was considered to be statistically significant.

RESULTS

^{55}Fe uptake in PC12 cells

Differentiation of PC12 cells into neuronal cells after NGF treatment has been well-characterized [24,25,27]. PC12 cells can proliferate rapidly, but NGF treatment transforms them into a phenotype that exhibits neurite outgrowth and electrical properties of sympathetic neurons. They express cholinergic receptors, Na^+ channels, N-type voltage-operated Ca^{2+} channels and neuronal nitric oxide synthase. The initial velocity of NTBI uptake by NGF-treated PC12 cells was significantly higher compared with control cells regardless of whether one examined the uptake with Fe^{2+} or Fe^{3+} and whether the chelator NTA or citrate was used (Figure 1, $P < 0.05$). For the NGF-treated cells, there was no significant difference between the uptake with Fe^{2+} or Fe^{3+} or between values obtained using the chelator NTA or citrate. This was also true for the control cells.

Expression of TRPC1–6 mRNA in NGF-treated and -untreated PC12 cells

We next examined the expression of TRPC1–6 and G3PDH mRNA (as a control) in NGF-treated and control PC12 cells using RT–PCR (Figure 2A). Intensity of the G3PDH control bands in the RT–PCR reaction mixture from RNA isolated from NGF-treated and control cells was similar (Figure 2A). RT–PCR using the RNA isolated from the control cells gave a band corresponding to TRPC3, although further amplification for another 30 cycles of PCR also showed faint bands for other TRPC isoforms (results not shown). RT–PCR of the RNA isolated from NGF-treated cells gave strong bands, corresponding to TRPC3 and TRPC6 (Figure 2A). A co-PCR experiment using primers for TRPC3 and TRPC6 confirmed that TRPC6 expression was virtually absent from the control PC12 cells and was prominent in the NGF-treated cells (Figure 2B). Western-blot analysis using a TRPC6-selective antibody showed that TRPC6 protein was expressed in the NGF-treated cells, but not in the control PC12 cells (Figure 2C). Thus the expression of TRPC6 mRNA and protein

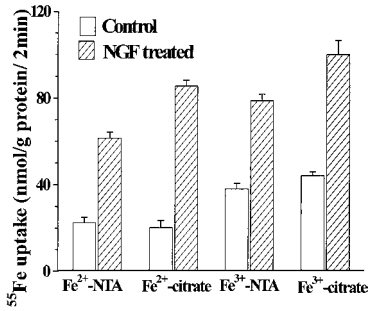

Figure 1 NTBI uptake in NGF-treated and control PC12 cells

Uptake of NTA- or citrate-chelated iron was examined at 1 μM free $^{55}Fe^{2+}$ and $^{55}Fe^{3+}$. The values shown are the means $\pm$ S.E.M. for six replicates. The values for the NGF-treated cells were significantly higher compared with the control ($P < 0.05$). The experiment was repeated three times.

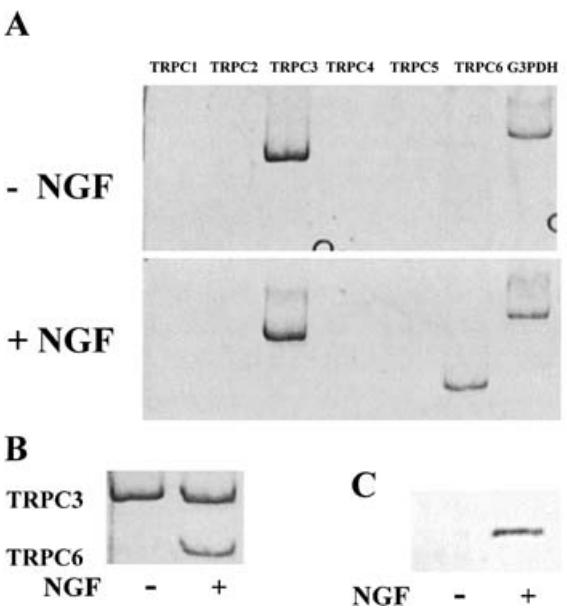

Figure 2 Expression of TRPC1–6 in NGF-treated and control PC12 cells

(**A**) RT–PCR products of TRPC1–6 and control G3PDH. A 100 bp ladder was used for calibration. (**B**) Co-PCR with TRPC3 and TRPC6 primers, showing the increase in TRPC6 when the cells are NGF-treated. (**C**) Western blot of membranes treated with the anti-TRPC6 antibody, showing the increase in TRPC6 protein levels in the differentiated PC12 cells. On the basis of molecular-mass standards, the size of the TRPC6 band was 105 kDa.

increased significantly in PC12 cells of the neuronal phenotype obtained by NGF treatment.

We also examined the expression of the NTBI transport protein Nramp2 in the NGF-treated and control PC12 cells. The Nramp2 protein could not be detected by Western-blot analysis. In RT–PCR experiments using two separate sets of primers to amplify Nramp2, the level of this mRNA did not differ significantly between the NGF-treated and the control PC12 cells (results not shown).

Effect of DAG on NTBI entry into PC12 cells

A large number of neuronal receptors act via the DAG pathway [32–36]. Since DAG can activate TRPC6 channels and

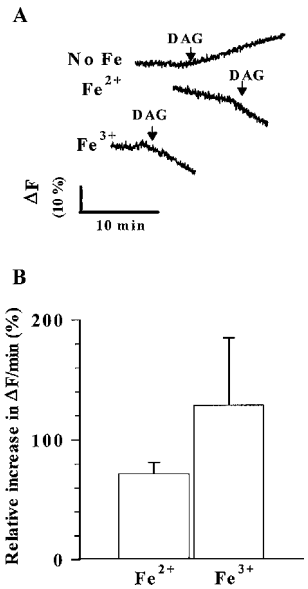

Figure 3 Effects of DAG on Ca^{2+} and NTBI entry into calcein-loaded PC12 cells

(**A**) NGF-treated PC12 cells. DAG was added at the time indicated by the arrow. Cells were placed in a solution without iron to examine the increase in calcein fluorescence representing Ca^{2+} entry or placed in a solution containing either Fe^{2+} or Fe^{3+} for several minutes to examine NTBI entry by quenching of calcein fluorescence. For each tracing, the value of the fluorescence intensity at the time of adding DAG was taken as 100 % and intensities at other times were represented as a percentage of this value. ΔF, change in relative fluorescence. (**B**) From tracings such as those shown in (**A**), ΔF/min was determined by linear regression first before and then after adding DAG to determine the increase in initial velocity resulting from the addition of DAG. Mean value of ΔF/min for control cells was taken as 100 % and the increase in ΔF/min for NGF-treated cells relative to this value is shown. The values shown are from experiments performed on three different days and represent the means ± S.E.M. for 3–7 cells in each group. The NGF treatment was expressed in terms of ΔF/min using Fe^{2+} or Fe^{3+} ($P < 0.05$). Neither the control cells nor the NGF-treated cells showed a significant difference in the ΔF/min value between Fe^{2+} and Fe^{3+} ($P > 0.05$).

NGF-treated PC12 cells express TRPC6, we examined the effect of DAG on NTBI uptake in PC12 cells. The NGF-treated PC12 cells were loaded with calcein. In the absence of iron, DAG caused a time-dependent increase in calcein fluorescence, an indication of Ca^{2+} entry into the cells (Figure 3A). We added Fe^{2+} or Fe^{3+} to the cell suspension, monitored the calcein fluorescence for several minutes and then added DAG. The addition of Fe^{2+} or Fe^{3+} resulted in an initial very rapid decrease in fluorescence (results not shown), presumably due to extracellular calcein; subsequently, there was a gradual decrease in the fluorescence, corresponding to a basal entry of Fe^{2+} or Fe^{3+} into these cells. After the addition of DAG, the decrease in calcein fluorescence became more rapid within seconds (Figure 3A), indicating that DAG further activated NTBI entry into the NGF-treated PC12 cells. The rate of decrease in fluorescence did not differ significantly whether Fe^{2+} or Fe^{3+} was used. As a control, we first tested if NTA would produce any change. In the absence of iron, the presence of NTA had no effect (results not shown), i.e. there was no initial rapid decrease or subsequent basal decrease in fluorescence, and the addition of DAG gave an increase in the fluorescence intensity. Thus the decrease did not occur as an artifact from the NTA used as chelator. As an additional control, we used citrate to chelate iron and obtained similar results as with NTA (results not shown). Next, we compared the DAG-activated decrease in calcein fluorescence with Fe^{2+} or Fe^{3+} in NGF-treated and control PC12 cells. The quenching was significantly higher

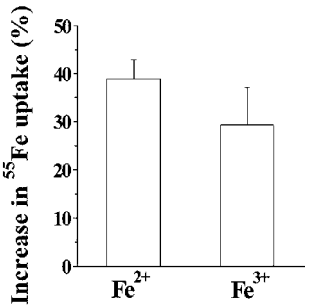

Figure 4 Effects of DAG on NTBI entry into NGF-treated PC12 cells using radioactive iron

The procedure of this experiment simulated the conditions of Figure 3(A) except that no calcein was used, as described in the Experimental section. The values shown are means ± S.E.M. for 5 or 6 replicates. The experiment was repeated three times and the uptake in the presence of DAG was significantly higher ($P < 0.05$) in each experiment.

in NGF-treated cells compared with control cells (Figure 3B), indicating a higher DAG-activated NTBI uptake by the neuronal phenotype.

To exclude the possibility that the DAG-stimulated NTBI uptake observed in NGF-treated PC12 cells resulted from an artifact owing to the use of calcein, we verified this effect of DAG using radioactive iron ($^{55}Fe^{2+}$ or $^{55}Fe^{3+}$; Figure 4). In Figure 3(A), cells were incubated for 10 min and then stimulated with DAG to differentiate the effects of adding iron versus stimulation with DAG. To mimic this experiment, the procedure of the experiment using radioactive iron was identical to that in Figure 3(A), except that the cells were not loaded with calcein.

$^{55}Fe^{2+}$ uptake in TRPC6-transfected HEK-293 cells

Experiments to this point identify a strong association between a striking increase in TRPC6 in PC12 cells after NGF treatment and a less striking, but still considerable, increase in NTBI uptake. To test if TRPC6 was directly responsible for the increased uptake, HEK-293 cells were transfected with the plasmid pcDNA3 containing TRPC6 cDNA or were mock-transfected with pcDNA3 alone. A co-PCR experiment using primers for G3PDH and TRPC6 showed that the TRPC6-transfected cells expressed both mRNAs, whereas the mock-transfected cells did not express the TRPC6 mRNA (Figure 5A). Using the methods for chelation and reduction of iron under our experimental conditions, we examined the initial velocity of the $^{55}Fe^{2+}$ uptake as described for PC12 cells. The TRPC6-transfected cells showed a small but statistically significant ($P < 0.05$) increase in $^{55}Fe^{2+}$ uptake, thus demonstrating that TRPC6 allowed NTBI entry into HEK-293 cells (Figure 5B).

Effects of phenylephrine and DAG on NTBI uptake in TRPC6-transfected HEK-293 cells

The increase in the $^{55}Fe^{2+}$ uptake observed on transfection with TRPC6 may have been small, since only 10–20 % of the cells were transfected. Hence, we used an alternative approach based on the principle that when cells are co-transfected with a test plasmid and a plasmid encoding a reporter protein, cells expressing the reporter are usually also transfected with the test plasmid [37,38]. HEK-293 cells were co-transfected with plasmids expressing the fluorescent protein DsRED2 and the test plasmid encoding TRPC6 and then loaded with calcein whose fluorescence increases with Ca^{2+}. Identifying the transfected cells with the

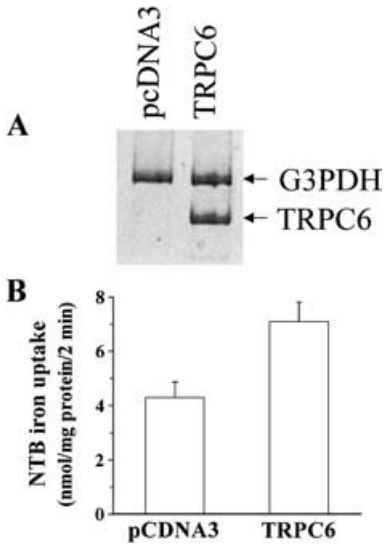

Figure 5 NTBI uptake in TRPC6- and mock-transfected HEK-293 cells

(**A**) Co-RT–PCR using TRPC6 and G3PDH primers, showing that mouse TRPC6 mRNA is expressed by the TRPC6-transfected but not the mock-transfected (pcDNA3) HEK-293 cells. (**B**) NTBI uptake using $^{55}Fe^{2+}$. The values shown are the means ± S.E.M. for six replicates in one experiment. The TRPC6-transfected cells showed significantly higher uptake compared with the pcDNA3-transfected cells ($P < 0.05$). The experiment was repeated four times.

DsRED2 fluorescence allowed a comparison between transfected and untransfected cells in the same microscopic field that had been treated identically in all other respects (Figures 6A and 6B). Since HEK-293 cells are known to contain α_1-adrenergic receptors, we first tested whether the transfected cells showed an increased Ca^{2+} entry on challenge with the α_1-adrenergic receptor agonist phenylephrine. On treatment with phenylephrine, the transfected cells showed a greater increase in calcein fluorescence, which was consistent with the idea that Ca^{2+} entry was caused by TRPC6 acting as an α_1-adrenergic-receptor-operated Ca^{2+} channel (Figure 6C). Thus the experiment validated the method used.

Fe^{2+} or Fe^{3+} can quench calcein fluorescence and bind it considerably more tightly when compared with Ca^{2+} [29,37]. We used this quenching to investigate NTBI entry into TRPC6-transfected HEK-293 cells. The transfected cells showed slightly faster basal calcein quenching (NTBI entry) compared with the untransfected cells in the same field. As a control, we used cells co-transfected with DsRed2 and pcDNA3 without TRPC6 (mock transfection control). These did not show any difference in quenching between the transfected and untransfected cells. We next examined the effect of stimulation of α_1-adrenergic receptor with phenylephrine. Since iron (Fe^{2+} or Fe^{3+}) binds calcein considerably more tightly compared with Ca^{2+} and it quenches calcein fluorescence, we predicted that phenylephrine would speed up the quenching of calcein fluorescence more in TRPC6-transfected cells than in the untransfected cells. Phenylephrine, when added after Fe^{2+} or Fe^{3+}, caused a more rapid quenching of calcein fluorescence and this increase in velocity of quenching was greater in transfected cells compared with untransfected cells (Figure 7). We also examined the effects of 1,2-dioleylglycerol, a cell-permeant DAG, on calcein fluorescence in TRPC6-transfected HEK-293 cells. DAG, when added after Fe^{2+} or Fe^{3+}, increased the rate of quenching of calcein fluorescence and this increase was substantially greater in the transfected cells compared with the untransfected cells (Figure 7). These results

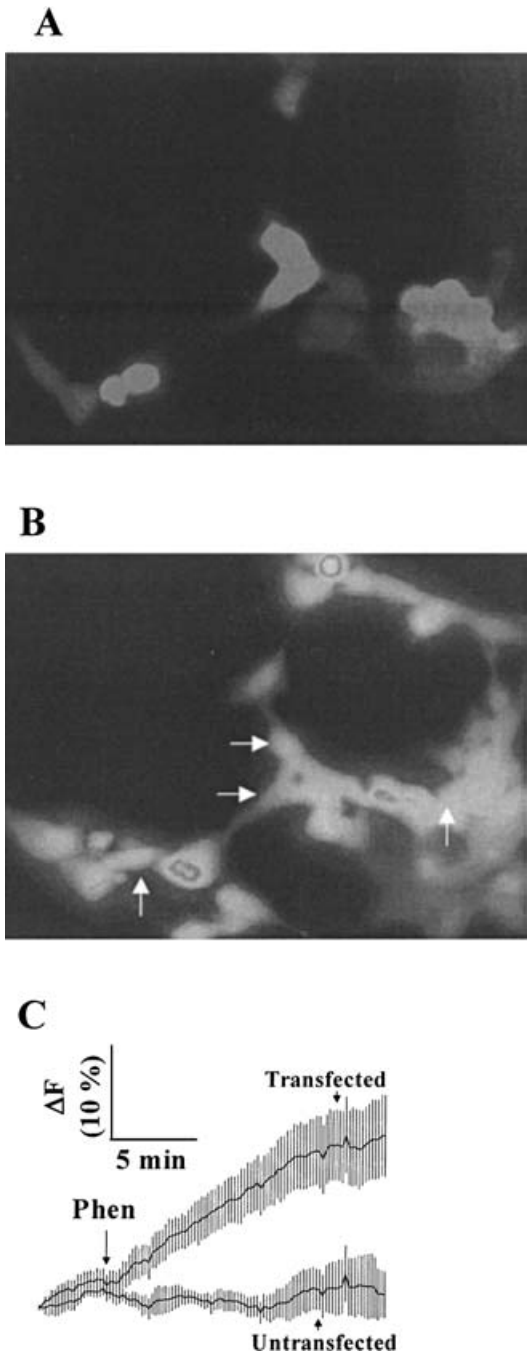

Figure 6 Receptor-stimulated Ca^{2+} entry into TRPC6-transfected HEK-293 cells

In HEK-293 cells co-transfected with DsRed2 and TRPC6 plasmids and loaded with calcein, the transfected cells were identified by DsRed2 fluorescence (**A**) and then the filters were switched to monitor calcein fluorescence (**B**). White arrows indicate the location of the transfected cells. (**C**) Increase in calcein fluorescence representing Ca^{2+} entry was examined. Phenylephrine (100 μM) was added at the time point indicated by the arrow labelled Phen. Background fluorescence determined from an area with no cells was subtracted and this value, at zero time, was taken as 100 %. Fluorescence values at all the other time points were determined relative to this zero time intensity. ΔF, change in relative fluorescence. Values of ΔF shown are the means ± S.E.M. for ten transfected and five untransfected cells.

demonstrate that TRPC6 channels allow NTBI entry via the DAG-mediated receptor-operated pathway. We also tested the effects of 5 μM thapsigargin on the HEK-293 cells.

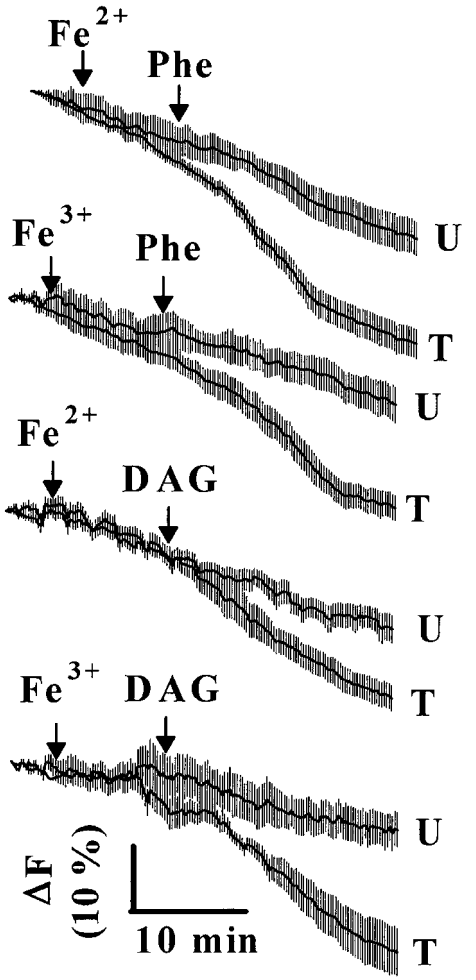

Figure 7 Receptor-stimulated NTBI entry into TRPC6-transfected HEK-293 cells

In HEK-293 cells co-transfected with DsRed2 and TRPC6 plasmids and loaded with calcein, the transfected cells were identified as in Figure 6. Fe^{2+} or Fe^{3+} was added at the time points shown by arrows, and 100 μM phenylephrine (Phe) or DAG was then added at the time points shown by arrows. Values of relative intensity of calcein fluorescence were determined as in Figure 6. ΔF, change in relative fluorescence. Values of ΔF shown are the means $\pm$ S.E.M. for 8–16 transfected (T) and 4–6 untransfected (U) cells.

Thapsigargin, when added after Fe^{2+} or Fe^{3+}, did not increase the rate of quenching of calcein fluorescence (results not shown).

Thus the experiments with HEK-293 cells showed that the population of TRPC6-transfected cells had a small increase in basal NTBI entry. In the absence of added iron, the individual calcein-loaded TRPC6-transfected cells showed an increase in fluorescence in response to phenylephrine; however, in the presence of added iron, they showed a decrease in fluorescence. The same results were not obtained when cells were transfected with the parent plasmid lacking TRPC6, thus establishing that the TRPC6 channel allows NTBI entry.

DISCUSSION

The previous section showed that NGF-treated PC12 cells had a higher NTBI uptake compared with untreated cells and TRPC6 was expressed in NGF-treated PC12 cells and not in control cells. DAG increased NTBI entry into the NGF-treated PC12 cells. This section focuses on properties of the TRPC6 pathway compared with various NTBI entry pathways reported previously [15,16,18–23,42] and implications of these findings with regard to neurotoxicity.

TRPC channels are permeable to Ca^{2+} and they may be linked to Ca^{2+} entry on stimulation of G-protein-coupled receptors acting through phospholipase C and/or depletion of internal Ca^{2+} stores [4,6–8]. Consistent with our study, TRPCs have also been shown to increase cation entry into unstimulated cells. TRPC6 overexpression increases Ca^{2+} entry into cells on stimulation of α_1-adrenergic or M_5-muscarinic receptors in the presence of DAG analogues, but not on inhibition of the SERCA pumps with thapsigargin [8,9,39]. TRPC6 channels have been considered to be non-selective cation channels that allow the entry of Ca^{2+}, Ba^{2+}, Na^{+} and Mn^{2+} and are blocked by SKF 96365 (a receptor-gated Ca^{2+} channel blocker) and La^{3+} [39]. This has been demonstrated by electrophysiology using millimolar concentrations of these ions. Such experiments cannot be conducted with Fe^{2+} or Fe^{3+}, which can exist as ionic species only at submicromolar concentrations under physiological conditions. In the present study, we used radioactive iron and calcein fluorescence quenching to examine NTBI uptake. Using HEK-293 cells, we have shown that TRPC6 overexpression leads to increased basal and receptor-stimulated NTBI entry. The rate of calcein fluorescence quenching obtained with Fe^{2+} and Fe^{3+} in DAG-activated HEK-293 cells was similar. These results may be interpreted either by assuming that TRPC6 channels allow entry of Fe^{2+} and Fe^{3+} or by assuming that only Fe^{2+} goes through these and Fe^{3+} has to be reduced with a ferric reductase as has been proposed for Nramp2 [22]. Owing to uncertainties in concentrations of free Fe^{2+} and Fe^{3+} under physiological conditions and differences in calcein quenching by the two ions, we cannot at present ascertain whether the TRPC6 pathway prefers Fe^{2+} to Fe^{3+} [30]. The DAG pathway is central to neuronal cells as it is involved in the action of several classes of muscarinic, peptidergic and purinergic receptors [32–36]. TRPC3, similar to TRPC6, can also be activated by DAG analogues [12]. Neutrophil gelatinase-associated lipocalin (also called human neutrophil lipocalin, 24p3, uterocalin or neu-related lipocalin) has also been reported to bind bacterial catecholate-type ferric siderophores [40]. It may be an innate immune mechanism; however, unlike TRPC6, it is unlikely to be a receptor-mediated NTBI uptake mechanism in mammalian cells. The NGF-treated PC12 cells express L- and N-type voltage-operated Ca^{2+} channels. NTBI uptake in these cells is not affected by the N-type voltage-operated Ca^{2+} channel inhibitor and is only marginally affected by the L-type voltage-operated Ca^{2+} channel inhibitor protein [26]. Nramp2 is known for its role in NTBI entry into mucosal cells [18], but it is an unlikely cause for the increase in NTBI uptake after NGF treatment of PC12 cells: an increase in Nramp2 mRNA was not observed in RT–PCR experiments with the NGF treatment of PC12 cells and Nramp2 protein was below detection levels in Western blots. Using different antibodies, PC12 cells have been reported to express Nramp2, but the effect of NGF treatment on PC12 cells was not reported [41]. Therefore one cannot fully rule out the role of Nramp2. However, TRPC6 mRNA and protein levels increased in PC12 cells on treatment with NGF and there was a concomitant increase in basal and DAG-stimulated NTBI uptake, suggesting that the increase in the expression of TRPC6 in these cells is associated with the increased NTBI uptake. Whereas the present study establishes a potential role of NTBI uptake in NGF-treated PC12 cells, it leaves open the mechanisms for the basal NTBI uptake in the NGF-treated and control PC12 cells and the receptor-activated NTBI uptake in the control PC12 cells

that do not express TRPC6. It remains to be determined if Nramp2, gelatinase-associated lipocalin or TRPC3 is involved in these roles.

Ideally, one would also show that knocking down TRPC6 expression in NGF-treated cells decreases NTBI entry. However, we were unable to transfect the NGF-treated PC12 cells with green fluorescent protein, DsRed2 or any other plasmid, and reagents used for small interfering RNA transfection altered NTBI entry into the NGF-treated cells with or without the TRPC6 small interfering RNA. Normally, the concentrations of Fe^{2+} and Fe^{3+} in plasma and interstitial fluids are extremely low, but they may increase under pathological conditions [16,43,44]. NTBI uptake may become important under these circumstances. In Parkinson's disease, there is an increase in iron accumulation in the substantia nigra region without a concomitant increase in the expression of transferrin, transferrin receptors or lactoferrin. The mechanism of this increase in iron is not known [43]. Since the brain tissue is rich in TRPC6, NTBI entry via the non-selective cation entry channels such as TRPC6 may play a role in Parkinson's disease and possibly in other neurodegenerative diseases.

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