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Interaction of SiO_2 nanoparticles with neuronal cells: ionic mechanisms involved in the perturbation of calcium homeostasis

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Abstract

SiO_2 nanoparticles (NPs), in addition to their widespread utilization in consumer goods, are also being engineered for clinical use. They are considered to exert low toxicity both in vivo and in vitro, but the mechanisms involved in the cellular responses activated by these nanoobjects, even at non toxic doses, have not been characterized in detail.This is of particular relevance for their interaction with the nervous system: silica NPs are good candidates for nanoneuromedicine applications. Here, by using the GT1-7 neuronal cell line, derived from gonadotropin hormone releasing hormone (GnRH) neurons, we describe the mechanisms involved in the perturbation of calcium signaling, a key controller of neuronal function. At the non toxic dose of 20 µg mL^{-1}, 50 nm SiO_2 NPs induce long lasting but reversible calcium signals, that in most cases show a complex oscillatory behavior. Using fluorescent NPs, we show that these signals do not depend on NPs internalization, are totally ascribable to calcium influx and are dependent in a complex way from size and surface charge. We provide evidence of the involvement of voltage-dependent and transient receptor potential-vanilloid 4 (TRPV4) TRPV4 channels.

Keywords: Silica nanoparticles; Neurons; Calcium signaling/homeostasis; Voltage dependent channels; TRPV4 channels.

Abbreviations

$[Ca^{2+}]_i$	cytosolic free calcium concentration MANCHEREBBE
DLS	dynamic light scattering
DMEM	Dulbecco'smodified Eagle's medium
EGTA	ethylene glycol tetraacetic acid
ELS	electrophoretic light scattering
FBS	fetal bovine serum
GnRH	gonadotropin hormone releasing hormone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid SERVE?
NPs	nanoparticles
PM	particulate matter
QDs	quantum dots
RR	ruthenium red
SOCE	store-operated calcium entry
TRPC	transient receptor potential canonical
TRPV	transient receptor potential-vanilloid
VDCCs	voltage dependent calcium channels

Introduction

SiO2 nanoparticles (NPs), in addition to their widespread utilization in consumer goods, are also being developed and engineered for clinical use (van Schooneveld et al., 2008). While they are considered to be among the most biocompatible NPs (Huang et al., 2005; Izak-Nau et al., 2014; Kim et al., 2006), extensive knowledge of the cellular responses elicited by their interaction is still incomplete. This is of particular relevance for the nervous system (NS): nanoneuromedicine is rapidly gaining momentum (Gendelman et al., 2014),and silica NPs are good candidates for applications in this field, ranging from drug delivery (Rosenholmet al., 2011) to cell tracking and subcellular imaging (Montalti et al., 2014). They can pass the blood brain barrier (Kim et al., 2006); moreover, protocols for direct access of NPs to the cerebrospinal fluid have been proposed (Papisov et al., 2013). On the other hand, neurons are particularly delicate and sensitive cells, and even small functional perturbations that can be noninfluential in other cell types may have severe outcomes in the nervous tissue. In this perspective, the understanding of the mechanisms activated by the interaction between NPs and neuronal cells is of primary relevance for the rational design of better and safer nanoparticles, particularly for long-term administration to the nervous system.

Cytosolic free calcium concentration, $[Ca^{2+}]_i$, is a highly relevant parameter, influencing both life and death of cells, and neurons are particularly sensitive to its changes, even subtle ones. These changes are involved in normal neuronal function, such as information coding; loss of control of $[Ca^{2+}]_i$ can lead to neuronal death (Arundine and Tymianski, 2003). During development, its changes are crucial in controlling proper differentiation and the correct establishment of neuronal connections. By interacting with both plasmamembranes and membranes of intracellular organelles, NPs can alter the delicate set of mechanisms controlling influx, efflux and sequestration of calcium ions. Apart from silica NPs (Ariano et al., 2011), data on the perturbations of calcium signaling mechanisms induced by NPs are still scarce (see e.g. Huang et al., 2010; Koeneman et al., 2010) and this is particularly true for neuronal cells (Guo et al., 2013; Haase et al., 2012; Nyitrai et al., 2013; Tang et al., 2008a, 2008b), as recently reviewed (Lovisolo et al., 2014).

Moreover, for potential applications in nanomedicine, it is relevant to investigate these mechanisms at non toxic doses. To this purpose, we have taken advantage of the properties of silica NPs (both bare and hybridized in the bulk with organic fluorophores) previously shown (Miletto et al., 2010) to be non toxic at the same size and dose used in a preliminary paper on calcium signals, performed with similar silica NPs (Ariano et al., 2011). They can be safely incorporated into neuronal (Miletto et al., 2010) and other cell types (Accomasso et al., 2012), and, because of their high fluorescence efficiency are quite suited to be used in cell tracking and subcellular imaging. By means of this tool, we have correlated their interaction and internalization in neuronal cells with the

time course of the calcium signals they elicit and investigated the molecular targets involved in the perturbation of calcium homeostasis.

Methods

Materials: Both lab-made SiO_2 NPs and spherical commercial SiO_2 micro- and nanoparticles were used (Corpuscular Inc., www.microspheresnanosphere.com). These latter will be hereafter referred as 2000-SiO_2 and 500-SiO_2, because of their diameter of 2.0 or 0.5 μm, i.e. 2000 and 500 nm (see below). Lab-made silica NPs were prepared using the reverse microemulsion technique, following the procedure previously reported (Alberto et al., 2009; Miletto et al., 2010). Details are in Supplementary Materials (hereafter SM), Methods. These NPs exhibit a mean diameter close to 50 nm and then they will be referred to as 50-SiO_2.

Lab-made silica NPs carrying at their surface amine groups were also prepared, with mean diameter 55 nm (see below) and then referred to as NH_2-55-SiO_2. For preparation protocol and data dealing with sample characterization see SM, Methods and Figure S1.

A third type of lab-made SiO_2 NPs was constituted by fluorescent nanoparticles (hereafter referred to as FL-50-SiO_2) resulting from the hybridization of silica with a fluorescent cyanine dye. The cyanine-silane derivative was used together with TEOS for the preparation of hybrid dye-SiO_2 NPs following the same procedure reported above (see SM, Methods and Figure S2). Previous studies (Alberto et al., 2009) indicated that the procedure adopted resulted in the presence in each NP of ca. 110 cyanine molecules, all entrapped within the silica matrix.

All reagents and solvents used for the preparation of nanoparticles and for cell cultures were highly pure Sigma-Aldrich products and were used as received, unless otherwise mentioned.

Physicochemical characterization of NPs: Transmission electron microscopy images were obtained with a 3010 Jeol instrument operated at 300kV. For the measurements, a droplet of the suspensions of lab-made silica nanoparticles was spread on a copper grid coated with a lacey carbon film, and then the liquid was allowed to evaporate. Histograms of the size distribution of NPs were obtained by measuring ca. 200 particles, and the mean particle diameter (d_m) was calculated as $d_m=\Sigma d_i n_i/\Sigma n_i$, where n_i was the number of particles of diameter d_i. The results are indicated as $d_m \pm$standard deviation.

For dynamic light scattering (DLS) measurements (90Plus Particle Size Analyzer, laser wavelength 660 nm, detection angle 90°, T = 20° C), the materials were suspended (0.1 mg mL^{-1}) in water, DMEM and Tyrode solution (see below for the composition); for each sample three measurements were performed. Large agglomeration, typically in the range of microns, resulted in

significant differences among repeated measurements, because of the much more complex scattering behaviour (Orts-Gil et al., 2011).

Hydrodynamic diameters are reported as mass distributions (SM, Figure S3). The same samples were used for ζ-potential measurements by electrophoretic light scattering (ELS), using a Zetasizer Nano-ZS (Malvern Instruments). Results are reported as the mean value of five separate measurements each resulting from 10 runs ± standard deviation.

Calcium imaging: For ratiometric measurements of $[Ca^{2+}]_i$ GT1-7 cells, an immortalized line derived from highly differentiated mouse gonandotropin-hormone releasing hormone (GnRH) neurons (generously donated by Prof. P.L. Mellon), were plated on glass coverslips (32 mm diameter) coated with poly-L-lysine (100 μg mL^{-1}) at densities of 10000 cells cm^{-2}. The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Lonza), gentamycin (50 μg mL^{-1}), and 2 mM glutamine at 37 °C, in a humidified atmosphere of 5% CO_2 in air and then switched for 4-5 days to 0.5% FBS supplemented with B27 (Invitrogen), to improve survival and differentiation. GN11 cells, another cell line derived from GnRH neurons at an early developmental stage (Radovick et al., 1991), were plated with the same protocol as for GT1-7 cells but kept in 10% FBS DMEM and used after 1-2 days in culture. Cells were loaded with the Fura-2 acetoxymethyl ester (2.5 μM, 45 min, 37 °C) and subsequently shifted to a standard physiological Tyrode solution of the following composition (in mM): NaCl, 154; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5; glucose, 5.5; and NaOH (pH 7.35). The NPs were dissolved in the Tyrode solution at the required concentration. The solution was applied with a microperfusion system; for calcium-free conditions, the $CaCl_2$ salt was omitted and the calcium chelator ethylene glycol tetraacetic acid (EGTA; 0.5 mM) was added. Cells were imaged every 3 s at 37 °C using a monochromator system attached to an inverted microscope with a 20× objective (SFluor, Nikon). For experiments lasting several hours, in order to avoid excessive photodamage, after the initial phase of stimulation with the NPs, acquisition time was one every 5 min; control recordings were performed to check the stability of the fluorescence at the isosbestic point. Images were acquired using an enhanced CCD camera (PCO,) and the Metafluor software (Universal Imaging Co.).

Wavelet analysis of calcium signals: To provide a quantitative evaluation of the changes in the oscillatory pattern of calcium signals following administration of SiO_2 NPs and of the calcium channel blockers, an approach based on wavelet analysis was employed, using KYM 0.5 software (http://sourceforge.net/projects/kym/) and according to the formulation given in Ruffinatti et al., 2011. Briefly, we calculated the index J(t) as an estimate of the oscillatory activity for each time interval, as described inSM, Methods.

Internalization tests: GT1-7 cells were seeded on glass microscope coverslips (Marienfeld, ~~Lauda-Königshofen, Germany~~) and maintained in culture according to the same protocol as for calcium imaging experiments at 30000 cells cm^{-2}. The cells were kept in serum-free DMEM medium for 7 min, 30 min and 4 hours in the presence of a 20 $\mu g\ mL^{-1}$ suspension of FL-50-SiO_2. Details are given in SM, Methods. 15-20 cells from 2-4 experiments were analyzed for each protocol.

Quantitative real-time PCR: The protocols are described in SM, Methods.

Statistical analysis: Unless otherwise indicated, 3 to 9 experiments were performed for each protocol. Data are expressed as mean ± standard error of the mean.

Results

Size, surface properties and dispersion in the incubation media of silica particles

Codes and mean particle size of the silica material used are listed in Table 1. Representative TEM and SEM images, as well as histograms of particles size distributions (for TEM data) are displayed in SM Figure S4 and S5, respectively. Diameters of lab-made nanoparticles and of commercial particles are in agreement with previous results (Alberto et al., 2009), and whit data provided by the producer, respectively.

Table 1.Sample codes and means size (nm) of silica particles.

Sample code	Mean particle size ± stdv (nm)
50-SiO_2	49 (±2)
FL-50-SiO_2	47 (±4)
NH_2-55-SiO_2	55 (±3)
500-SiO_2	500 (±25)
2000-SiO_2	2000 (±100)

As far as lab-made 50-SiO_2 NPs are concerned, infrared spectroscopy (SM, Figure S1) witnessed for the absence on their surface of molecules remaining from the preparation procedure (which was ended by several washing steps, see the Methods in SM), and then the only process contributing to the appearance of surface charges when suspended in pure water should be the dissociation of protons from surface hydroxy groups (silanols).

The ζ-potential values obtained for the various materials suspended in water and in media used for cell tests, DMEM and Tyrode, are listed in Table 2, as well as the maximum of the distribution of hydrodynamic diameters measured by DLS in the various cases (full set of experimental data in SM, Figure S3).

Table 2. ζ-potential (ζ, mV) and hydrodynamic diameters (HD, nm) values ± standard deviation of the various types of silica nano- and microparticles suspended in water, DMEM and in Tyrode solution.

Suspension media		**Materials**			
		50-SiO_2; FL-50-SiO_2	NH_2-55-SiO_2	500-SiO_2	2000-SiO_2
Water (pH 5.5)	**ζ, mV**	-29.0 (±0.56)	+0.3 (±1.12)	-54.3 (±0.86)	-81.5 (±1.42)
	HD, nm	50 (±1.15)* 152(±24.00)*	602 (±51.00)	501 (±36.59)	3452 (±253.00)
DMEM (pH 7.35)	**ζ, mV**	-20.8 (±0.47)	+5.0 (±0.67)	-9.7 (±0.92)	-22.4 (±0.82)
	HD, nm	120 (±15.01)	4360 (±1111.92)	1930 (±451.75)	4262 (±1347.50)
Tyrode (pH 7.35)	**ζ, mV**	-17.5 (±1.01)	+26.7 (±0.76)	-3.6 (±0.37)	-19.7 (±0.66)
	HD, nm	1680 (±194.42)	3008 (±974.12)	3758 (±1014.82)	3734 (±1586.89)

* in this case a bimodal distribution was obtained

When suspended in water, bare silica materials 50-SiO_2, 500-SiO_2 and 2000-SiO_2 exhibited a negative potential (natural pH of the suspensions 5.5), in agreement with the presence of silanols on their surface. The largely negative ζ-potential values obtained for 500-SiO_2 and 2000-SiO_2 appeared in agreement with data reported in the literature for particles with diameter in this range for a similar pH (Xu et al., 2003). Different absolute values of the ζ-potential of these particles should monitor a different amount of surface silanols. These surface groups should be even less in the case of 50-SiO_2 NP, which exhibit a significantly less negative ζ-potential. In FL-50-SiO_2 all fluorescent molecules were located within the silica bulk (Alberto et al., 2009), and then these NP exhibited surface features equivalent to the 50-SiO_2 ones. Passing to NH_2-55-SiO_2 NPs in water, the ζ-potential resulted slightly positive (basically approaching 0) due to the presence of protonated

amino terminations which compensate the negative charge of silanols still present after partial surface functionalization (Figure S1 in SM).

Both culture media were buffered at a slightly basic pH, definitely higher than that of the suspensions in pure water, and then this condition should furthermore promote the deprotonation of surface silanols (and of NH_3^+ moieties in NH_2-55-SiO_2), and then more negative ζ-potential values were expected for all types of silica (nano)particles. Conversely, the opposite occurred in all cases, clearly indicating that additional phenomena occurred. These media are saline solutions and electrolytes are known to affect significantly the ζ-potential of suspended particles (Israelachvili, 1996). In fact cations can adsorb on the negatively charged surface sites of particles (≡Si-O^- resulted from the deprotonation of silanols) and, for NH_2-55-SiO_2, on $–NH_2$ moieties through coordination by the lone pair of the nitrogen atom (Liu et al. 2014, Kim et al. 2011, Sukhorukov et al. 1998). Moreover, aminoacids and vitamins present in DMEM and glucose present in both DMEM and Tyrode could adsorb on the suspended particles contributing to the actual state of their surface.

This complexity should have played a role also in determining the dispersion vs agglomeration of the various types of silica (nano)particles in the different media, that in a significant number of cases was not simply related to the surface charge. In fact, the collection of DLS data (Table 2 and Figure S3 in SM) indicated that, by considering water, DMEM and Tyrode, in that order:

i) the hydrodynamic diameter (HD) of 50-SiO_2/FL-SiO_2 moved from a bimodal distribution (monodispersed, HD=52 nm; small agglomerates, HD=152 nm) to a monomodal distribution of small agglomerates (HD=120 nm) and then underwent an extended heterogeneous agglomeration (HD=1680 nm, stdv ca. ± 195), despite a similar ζ-potential in these two media;

ii) the NH_2-55-SiO_2 exhibited a significant agglomeration in water (HD=602 nm, stdv of the order of the size of primary particles), and this can be the result of a ζ-potential value close to zero, but the agglomerates became much larger and heterogeneous in DMEM (HD=4260 nm, stdv ca. ±1110 nm), where, conversely a ζ-potential slightly different from 0 was measured, and large and heterogeneous agglomerates were formed also in Tyrode (HD=3200 nm, stdv ca. ±975), where the ζ-potential increased significantly;

iii) 500-SiO_2 are monodispersed in water (HD=501 nm), agglomerated in DMEM (HD=1930 nm), with heterogeneity in size of the order of the diameter of primary particles (stdv ca. ± 451 nm) and formed even larger and heterogeneous agglomerates in Tyrode (HD=3758 nm, stdv ca. ±1115 nm), this being the only case where the agglomeration trend appeared similar to the trend of the absolute values of ζ-potential;

iv) 2000-SiO_2 slightly agglomerated in water (HD=3452 nm), their agglomeration became more pronounced in DMEM (HD=4360 nm), where a significant decrease in the absolute value of ζ-

potential occurred, while their dispersion in Tyrode appeared similar to that in water (HD=3750 nm) although the ζ-potential remained similar to the suspension in DMEM. In these three cases the profile of HD distribution exhibits a broad tail (see Figure S3 in the SM) down to 2000 nm, indicating also the presence of minor fraction of less agglomerated and even monodispersed particles.

Long lasting calcium signals elicited by NPs

As a first approach, calcium imaging experiments were performed in order to analyze the perturbations in $[Ca^{2+}]_i$ induced by the interaction of the NPs with the GT1-7 cells at the non toxic concentration of 20 μg mL^{-1} (Miletto et al., 2010).

Perfusion of GT1-7 cells with the 50-SiO_2 NPs induced changes in $[Ca^{2+}]_i$: a high percentage of cells (77.8% out of 518 cells from 9 experiments) responded with latencies ranging from 2 to about 7 min, with a strong and long lasting increase in $[Ca^{2+}]_i$, that in general persisted even after washout of the NPs from the extracellular solution. These responses were in agreement with previous preliminary observations, limited to shorter times, obtained with similar NPs (Ariano et al., 2011). A relevant feature is that many responses showed a marked oscillatory behaviour. Typical traces obtained from three cells from a single experiment are shown in Figure 1A; Figure 1B shows the averaged response from all 64 cells of this experiment. In the averaged trace, the oscillatory pattern is not evident, pointing to a random, non-synchronized behaviour of the individual cells. For recordings up to 1h, the reversibility was not complete for most cells.

The specificity of the response was highlighted by the observation that in some cases the activation showed spatiotemporal selectivity at the single cell level. In Fig. 1C, the response to NPs administration was seen first at a very localized region of a neurite, subsequently at the soma and still later at the opposite neurite. Fig. 1D shows the recordings corresponding to the three Regions Of Interest (ROIs) marked in C.

As shown in Figure 1, in all experiments cells were challenged with an external solution containing 40 mM KCl prior to perfusion with the NPs. GT1-7 cells are considered to express several types of voltage-dependent calcium channels, VDCCs (Hoddah et al., 2009; Martinez-Fuentes et al., 2004). Depolarization with the high-K^+ solution, that induces calcium influx through these channels, was used as a control of cell functionality; cultures that did not respond at all to KCl were not taken into account.

In order to check the general relevance of these results, we performed a parallel series of experiments on another neuronal cell line, GN11 cells, obtained from GnRH neurons at an early differentiation stage. These cells show quite different functional properties (they have high

proliferative and migratory activity and are electrically inexcitable) and possess very low or non detectable levels of voltage activated calcium channels (Pimpinelli et al., 2003). In these cells, too, perfusion with 50 nm SiO_2 NPs at the concentration of 20 μg mL^{-1} elicited long lasting calcium oscillations in 98% of 110 cells from four experiments (SM, Figure S6A,B).

In routine experiments with GT1-7 cells, non-fluorescent NPs were employed, since their surface properties are virtually identical to the cyanine-doped ones (see Methods). Control experiments with fluorescent NPs gave results fully comparable with those obtained with the plain SiO_2 NPs (SM, Figure S7A). Since in Tyrode solution the NPs form quite large agglomerates, while in DMEM the aggregation is quite limited (Figure 1) we checked if the responses were dependent on the agglomeration state, by challenging the cells with NPs dissolved in DMEM; the same medium was also present in the culture dish. The percentage of responding cells and the time course of the responses (SM, Figure S7B) were identical to what observed in Tyrode solution, providing evidence that the cellular responses are not univocally dependent on the agglomeration state.

Another parameter that can potentially influence the NP-membrane interaction is the electrostatic potential, that in the case of the 50-SiO_2 NPs is strongly negative. Four experiments were performed with NPs of the same size terminated with amino groups. In all cases, no changes in $[Ca^{2+}]_i$ could be recorded (SM, Figure S7C).

A further check was made with silica particles with diameters of 500 nm and 2000 nm, smaller and of the same order of magnitude, respectively, of the agglomerates of 50-SiO_2 NPs, but exhibiting a similar negative surface potential. The 500-SiO_2 NPs elicited transient and rapidly reversible responses in only 42% of 117 cells from 2 experiments. Subsequent perfusion with the 2000-SiO_2 NPs elicited a response in only 12% of cells; amplitude and duration were further reduced (SM, Figure S7D), suggesting that the ability of the NPs to elicit long-lasting calcium signals in GT1-7 cells is dependent in a combined way from both size and surface properties.

NPs-induced calcium oscillations are totally dependent on calcium influx

Next we investigated the mechanisms responsible for the NPs-induced calcium signals. The response was completely abolished in nearly 100% of cells when the standard 2 mM $CaCl_2$ solution was substituted with a calcium free solution containing 0.5 mM EGTA (Figure 2A,B; n = 220 cells, 4 experiments). Reintroduction of 2 mM $CaCl_2$ in the extracellular medium restored the response in 100% of cells tested. In another set of experiments, with the cells bathed in the calcium free solution no response to NPs could be observed. However, when 2 mM $CaCl_2$ was reintroduced into the bath, even after washout of the NPs, an increase in $[Ca^{2+}]_i$ could be observed in a percentage of cells (82.1%; n = 173 cells, 4 experiments) comparable to controls (Figure 2C). Interestingly, also

the calcium responses to 50 nm SiO_2 NPs observed in GN11 cells were totally dependent on influx from the extracellular medium (SM, Figure S6A,B,C).

These findings point to the activation of calcium influx from the extracellular medium as a necessary and sufficient condition for the onset of the response to NPs. A mechanism by which release from intracellular stores can indirectly contribute to influx is store-operated calcium entry (SOCE), by which the depletion of the intracellular stores activates calcium permeable channels at the plasmamembrane (Smyth et al., 2010). To test this hypothesis, cells were first challenged with 10 nM thapsigargin (TG), an irreversible blocker of the sarcoplasmic-endoplasmic reticulum calcium pump SERCA (Thastrup 1990) in the 0 mM calcium, 0.5 mM EGTA extracellular solution. The blocker induced a transient increase in $[Ca^{2+}]_i$ followed, after reintroduction of 2 mM $CaCl_2$ into the extracellular medium, by a strong and steady, non oscillatory signal, ascribable to SOCE (Figure 2D,E); subsequent perfusion with 20 $\mu g\ mL^{-1}$ SiO_2 NPs induced a further and mostly oscillatory increase in $[Ca^{2+}]_i$ in a percentage of cells nearly identical to that observed in control conditions (82.5%; n = 97 cells from 3 experiments), thus providing evidence that release from intracellular stores is not involved in the complex pattern of calcium signals elicited by the interaction with SiO_2 NPs.

Time span and reversibility of NPs-induced oscillations in $[Ca^{2+}]_i$

The lack of reversibility of the responses, at least for times of about 1h, raises the question of how this finding can be reconciled with the absence of cytotoxicity of these NPs at this concentration (Miletto et al., 2010). To clarify this issue, we performed longer (up to 6 h) recordings. In two experiments, the NPs were washed out after 5-10 min, when the response had reached maximum amplitude. $[Ca^{2+}]_i$ levels returned to basal values in 3-4 h; Figure 3A shows the averaged trace from one representative experiment (n = 44 cells). In a second set of experiments, NPs were kept in the bath for the whole duration of the recordings: in two experiments, a nearly complete recovery (about 100%) to basal $[Ca^{2+}]_i$ levels could be observed in about 4 h; in other two the recovery was between 70% and 80%. Thus, the homeostatic mechanisms of these neuronal cells are able to compensate for the massive influx activated by the NPs. Figure 3B shows a representative experiment (averaged trace from 20 cells).

Time course of NPs incorporation

The data presented above support the hypothesis of a mechanism dependent on the interaction of the NPs with the plasmamembrane, and not on their internalization. To provide additional evidence

to this crucial finding, we took advantage of the high photoemission brightness of the cyanine doped FL-50-SiO_2 NPs (Alberto et al., 2009) to perform an evaluation of the time course of their internalization by fixing the cells after different times of exposure to the NPs and analyzing their intracellular localization by means of confocal microscopy. Incubation times were 7 min (corresponding to the longest latency of response to the NPs), 30 min and 4 h (the time at which $[Ca^{2+}]_i$ had returned to basal levels, even in the continued presence of the NPs). As shown in Figure 4, at 7 min the NPs are present on the surface of the cells but are not yet internalized; at 30 min some internalization can be detected, that becomes more evident at 4 h. Therefore, no correlation can apparently be established between activation of calcium influx by the NPs and their internalization.

The ionic channels involved in the NPs-induced calcium influx

Next we addressed a further relevant question: which are the targets that mediate the NPs-induced calcium influx? It has been reported that some NPs, such as uncoated CdSe quantum dots (QDs), can irreversibly disrupt calcium homeostasis in neuronal cells by affecting voltage dependent Na^+ and N-type Ca^{2+} channels (Tang et al., 2008a, 2008b); conversely, CdSe-ZnS QDs can reduce calcium influx through Ca^{2+} VDCCs (Gosso et al., 2011). GT1-7 cells express variable extents of many types of VDCCs (Charles and Hales, 1995; Hoddah et al., 2009; Watanabe et al., 2004). We therefore used a pharmacological approach, by means of specific blockers of voltage dependent calcium channels. In Figure 5 the results of the application of the VDCCs blockers are shown. Remarkably, combined perfusion with L- and T-type VDCCs calcium blockers, respectively nifedipine (10 µM) and $NiCl_2$ (100 µM), abolished nearly 100% of the response to 40 mM KCl (SM, Figure S8): therefore in these cells L- and T-type channels are the major, if not sole, contributors to voltage-dependent calcium influx. Since, as stated above, the responses to high KCl showed a high degree of heterogeneity, we selected for analysis only those cells in which the response to KCl was above a threshold, arbitrarily set at $\Delta R > 0.2$, since in cells with low VDCC expression the signals elicited by NPs could not be significantly dependent on this influx pathway. In Figure 5A a decrease in the oscillatory behaviour following perfusion with the two blockers is clearly detectable on traces from single cells highly responsive to 40 mM KCl. The effect on the averaged response from the same experiment is shown in Figure 5B: on the sustained response to NPs, the two molecules exerted a limited blocking effect (representative of 4 experiments, 54 cells). In order to uncover subtle effects on the oscillatory behaviour that may be difficult to decipher with qualitative observation, we employed a quantitative spectral analysis, based on the wavelet algorithm we have recently developed (Ruffinatti et al., 2011), that enables to perform a spectral

analysis of the signal in both time and frequency domains (see SM, Methods). The results from the same experiment as in B are shown in Figure 5C: in the presence of the two blockers, oscillatory activity, as quantified by the activity index J (see SM, Methods) was significantly reduced. In two control experiments, in the absence of the channel blockers, no significant decrease in the NPs-induced oscillatory activity could be detected for a comparable time interval (SM, Figure S10).
In the light of the limited contribution of VDCCs to the NP- induced calcium influx, the search was extended to other types of calcium permeable channels. We had previously shown that GT1-7 cells express several members of the TRPC (Transient Receptor Potential Canonical) family (Dalmazzo et al., 2008). Interestingly, members of another family of TRP channels, TRPVs (Transient Receptor Potential Vanilloid), have been reported to be activated by particulate in epithelial airway cells and in peripheral neurons (Oortgiesenet al., 2000; Veronesi et al., 2002a). Due to the relevance of these findings, we investigated the presence of transcripts of these two families in GT1-7 cells, by means of real-time quantitative PCR assay. The results are shown in Figure 6A: most members are expressed, with particularly high levels of TRPC2, TRPV2 and TRPV4 channels.

We then looked at the effects of the application of SKF96365 (2 µM) and of Ruthenium Red (RR; 5 µM), blockers respectively of TRPC and TRPV channels (Lovisolo et al., 2012; Patapoutian et al., 2003).

Both from individual cell recordings and averaged traces, in 2 experiments with 94 cells, no clear effect of the TRPC blocker could be detected (SM, Figure 9). On the contrary, when the TRPV blocker RR was added to the medium during the response to the NPs, a reduction in averaged calcium levels and in the oscillatory behaviour of individual traces elicited by NPs could be observed. (Figure 6B,C; one experiment representative of four, for a total of 211 cells). Wavelet analysis provided further evidence of a significant reduction of the oscillatory behaviour. The results from the same experiment as in C are shown in Figure 6D: in the presence of RR, oscillatory activity, as quantified by the mean activity index <J> (see SM, Methods) was significantly and strongly reduced.
Thus, at least two components appear to contribute to the NPs-induced alterations in $[Ca^{2+}]_i$: a calcium influx through TRPV channels that, by causing a depolarization of the membrane potential can lead to activation of VDCCs, thus inducing a further calcium influx.
However, two main issues need more precise investigation: first, RR in this concentration range has been reported to block other members of the TRP superfamily: TRPM6 (Voets et al., 2004) and TRPA1 (McNamara et al., 2007) channels. As for the former, its selectivity is 5-fold higher for Mg^{2+} than for Ca^{2+}, and therefore it is not a likely contributor to the huge increases in $[Ca^{2+}]_i$ induced by the NPs. This was confirmed by the observation that when cells were perfused with a

Mg^{2+}-free extracellular solution during the sustained phase of the response to NPs, no change in the response amplitude, neither in individual responses nor in averaged traces, could be observed (SM, Figure 11A,B; experiment representative of four, for a total of 126 cells). To investigate the potential contribution of TRPA1 channels, we employed the selective antagonist HC 030031 (McNamara et al., 2007) at the concentration of 10 µM: in three experiments (215 cells) no reduction of the response to SiO_2 NPs could be detected, neither in individual responses nor in averaged traces (SM, Figure S11C,D).

The second issue refers to the specific contribution of members of the TRPV family. To this purpose, specific blockers of TRPV1 (previously reported to be activated by environmental nano- and microparticles, Oortgiesen et al., 2000) and TRPV4 channels, the most strongly expressed member in the GT1-7 cell line, were used. As shown in SM, Figure S11E, F, theTRPV1 antagonist capsazepine (10 µM; Veronesi et al., 2003) did not affect the response to NPs, either on individual traces or on the average (271 cells from three experiments).

The picture was quite different with the selective TRPV4 antagonist GSK 2193874 (Thorneloe et al., 2012). GSK 2193874 (300 nM) induced a marked and irreversible reduction of the calcium signal activated by NPs in 51% of the cells (142 cells from three experiments). Subsequent addition of 5 µM RR did not induce a further decrease. In Figure 6E,F are shown two individual responses and the averaged trace from the same experiment (24 cells responsive to GSK 2193874 from a total of 35 cells). In the remaining cells, non responsive to GSK 2193874, addition of RR induced a small decrease in the response in only 12 cells (8% of the total population; not shown), suggesting that the contribution of other members of the TRPV family, if present, is quite marginal. A quantitative evaluation of the effect of GSK 2193874 on the oscillatory activity induced by the NPs by means of wavelet analysis was performed in two additional experiments (69 responsive cells from a total of 122), in which RR perfusion was omitted, in order to focus the analysis on the specific effect of the TRPV4 blocker. Fig. 6G shows two representative traces of responding cells from one experiment; in H the wavelet analysis of the time course of the oscillatory activity shows that the TRPV4 blocker sharply reduced the mean activity.

These findings provide evidence for an involvement of TRPV4 channels in the generation of the calcium signals elicited by the NPs, at least in a relevant subpopulation of cells.

Discussion

The data presented above provide evidence that the oscillatory changes in $[Ca^{2+}]_i$ observed following perfusion of neuronal cells with the silica NPs are elicited by their interaction at the cell

membrane, influx from the extracellular medium being apparently the exclusive pathway. The nanoparticles used in the present study, as many others, can be internalized (Accomasso et al., 2012; Miletto et al. 2010) and intracellular organelles are potential targets (Corazzari et al., 2013; Kettiger et al., 2013). However, the calcium imaging data are supported by the observation that the time course of internalization is slower than the onset of the calcium signals: even in the presence of the NPs, $[Ca^{2+}]_i$ returns to baseline in about 4 h, when internalization has reached levels comparable to observations at 24 and 72 h (Miletto et al., 2010). Therefore, the search for the target(s) responsible for the onset of these responses has to be focused on the plasma membrane, the primary gate for all interactions of cells with the external milieu. The reversibility of the NPs-induced signals and their differential activation at delimited subcompartments allow to rule out a non-specific perturbation of membrane permeability, such as a disruption of the plasmamembrane lipid bilayer, pointing to the activation of one or more physiological pathways. While these electrically excitable cells express several types of VDCCs, also other calcium permeable channels are potential candidates. We provide evidence of the involvement of both VDCCs and TRPVchannels. The latter possess peculiar polymodal activation properties and are regarded as sensors of irritants (Nilius et al., 2007) and of pollutants (Veronesi et al., 2006). The interaction between these two channel families, with the potential contribution of other calcium permeable channels, may contribute to the oscillatory behaviour observed in most cells. By comparing NPs of different sizes and physico-chemical properties, our data suggest that neither size, nor surface charge or agglomeration (Thomassen et al., 2011) taken separately are sufficient to define the biological response, but that it depends in a combined way from the whole set of parameters. The finding that the NPs-induced calcium oscillations at non cytotoxic doses, albeit long lasting, are transient on the time scale of a few hours may provide a useful criterion for studies aimed at large scale screening of the neurotoxicity and/or biocompatibility of environmental and engineered NPs.

The fact that the same NPs can elicit quite similar oscillations in $[Ca^{2+}]_i$ in another neuronal cell line, showing different functional properties and different expression of membrane channels, provides evidence that the mechanisms involved may be quite general.

Finally, the precise identification of the molecular targets responsible for the cellular response to the NPs is a crucial step in designing nanomaterials for biomedical use tailored to the specific cell type or tissue of interest.

The early findings that the inflammatory response to particulate matter (PM) in airway epithelial cells (Veronesi et al., 1999) and peripheral neurons (Oortgiesen et al., 2000; Veronesi et al., 2002a) was at least partly dependent on activation of calcium influx through the capsaicin-activated, acid sensing TRPV1 channel, were the first report of an involvement of a member of the TRPV family in

response to micro- and nanoparticles. In these and in a following paper (Veronesi et al, 2002b) the same group attributed their activation to the negative charge present on the surface of many PM components. However, the specimens employed were quite heterogeneous regarding size (from nanometers to micrometers) and other properties; these data were confirmed by using engineered polymeric particles, but these were in the micrometer range (Veronesi et al., 2003).

Our results, obtained by means of NPs of controlled size and surface properties, partially diverge from these previous observations by pointing to a complex relationship between size, agglomeration and surface charge, and providing evidence for a contribution, at least partial, of TRPV4 channels. The mechanism may be either a direct or an indirect one: TRPV4 can be activated by arachidonic acid (AA) and some of its metabolites (Everaerts et al., 2010); some isoforms of the enzyme phospholipase A2, that catalyzes AA synthesis, may be activated following mechanical stress (Lehtonen and Kinnunen, 1995; Munaron, 2011; Oike et al., 1994). The indirect hypothesis could explain the relatively long delay (up to a few minutes) observed between NPs perfusion and the onset of the calcium response.

In conclusion, the data reported above can be a useful tool to set the basis for testing the specificity of the effects of different types of nano-objects on selected cellular models.

Conflict of interest

The Authors declare no conflict of interest.

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Figure legends

Figure 1. 50 nm SiO_2 NPs (20 µg mL^{-1}) elicit long lasting and oscillatory calcium signals in GT1-7 cells, totally dependent from influx from the extracellular medium. (A) Three individual responses. (B) Averaged data from 64 cells from the same experiment as in (A). Here and in following figures the response to 40 mM KCl is shown at left. (C) The calcium signals elicited by 50 nm SiO2 NPs have in some cases a sharp spatial specificity. In the example shown above, three regions of interest (ROIs) were selected at three subcellular compartments: the increase in $[Ca^{2+}]_i$ was observed first at the terminal of a neurite (*), subsequently at the cell soma (▲) and finally at the opposite neuritic compartment (★), thus producing a characteristic spatiotemporal pattern at the single cell level. Scale bar: 20 µm. (D) Recordings at the three ROIs shown in C.

Fig. 2. Perfusion with an extracellular calcium-free solution completely and reversibly abolished the NPs-induced calcium signals. (A) Single traces. (B) Averaged response. (C) In a calcium free solution, no response to NPs could be observed. Reintroduction of the solution containing 2 mM $CaCl_2$ elicited the oscillatory responses in individual cells (not shown) and a strong increase in the averaged signal.The response to NPs could be observed also following activation of store-operated calcium entry (SOCE) induced by 10 nM thapsigargin. D. Two sample cells; E: averaged response from 35 cells from the same experiment as in D.

Figure 3. Reversibility of the NPs-induced increases in $[Ca^{2+}]_i$. (A) The NPs were perfused for about 6 minutes and then washed out. After about 50 min, perfusion was stopped and the recording was continued at a slower acquisition frequency. The averaged trace from 44 cells returned to basal levels in about 4 hours. (B) Same as in A, but the flux was stopped after 40 min and the NPs were kept in the bath for more than 4 hours; again, the averaged trace (20 cells) returned to baseline. The star denotes the response to 40 mM KCl.

Figure 4. Time course of incorporation of fluorescent SiO_2 NPs into GT1-7 cells. 3-D reconstructions of XYZ confocal stacks. Insets represent the virtual section along the white/yellow lines. Scale bars: 10 µm in the images, 2 µm in the insets. (A) After 7 min of incubation in DMEM, no NPs could be observed inside the cells. (B,C) At 30 min and 4 h incubation, internalization could be observed.

Figure 5. Effects of the blockers of L- and T-type voltage dependent calcium channels (respectively nifedipine, 10 µM and nickel, 100 µM) on NPs-induced calcium signals. (A) The two blockers abolished calcium oscillations in three cells showing strong responses to high KCl. (B) A reduction in average response amplitude was observed (13 cells, same experiment as in A). (C) Wavelet analysis from the same experiment as in A,B shows that the two VDCC blockers exerted a significant reduction in the oscillatory activity. Time 0 corresponds to the start of perfusion in E (arrow). Inset: reduction of mean value of the activity index <J> following perfusion with the blockers. * $p < 0.05$.

Figure 6. TRPV channels are involved in the calcium signals elicited in GT1-7 cells by SiO_2 NPs. (A) Expression of transcripts of the members of the TRPC and TRPV families. The TRPV channel blocker Ruthenium Red (RR; 5 µM) abolished oscillations in single cells (B; two sample traces representative of 56 cells from one experiment) and reduced the averaged response (C). (D) Wavelet analysis from the same experiment as in B,C shows that RR exerted a significant reduction in the oscillatory activity. Time 0 corresponds to the start of perfusion in E (arrow). Inset: reduction of mean value of the activity index <J> following perfusion with RR. * $p < 0.05$. The TRPV4 antagonist GSK 2193874 (300 nM) markedly reduced the calcium responses to NPs. (E) Two individual traces and (F) averaged trace from 24 cells responsive to GSK 2193874 from one experiment (total of 37 cells). When 5 µM RR was further added to the bath, no additional decrease could be detected. (G) Two individual traces (representative of 44 responsive cells) from another experiment in which RR was omitted showing the decrease in oscillatory activity induced by GSK 2193874. (H). Wavelet analysis from the same experiment as in G. Time 0 corresponds to the start of perfusion in E (arrow). Inset: reduction of mean value of the activity index <J> following perfusion with the blocker. * $p < 0.05$.