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Aequorin chimeras as valuable tool in the measurement of Ca^{2+} concentration during cadmium injury

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Abstract

The ability of cadmium to disrupt calcium homeostasis has been known since a long time, but the precise cellular targets of its toxic action are still debated. A great problem in the interpretation of data has been associated with the ability of cadmium to strongly bind traditional calcium probes. Aequorin, the well-characterized calcium-sensitive photoprotein, was used as intracellular calcium indicator during cadmium injury in NIH 3T3 murine fibroblasts. NIH 3T3 cells were transfected with a cDNA construct containing aequorin fused to a truncated glutamate receptor, which directs the probe to the outer surface of intracellular membranes. At first, we tested if different cadmium concentrations were able to modify the rate of light emission by aequorin showing that cadmium concentrations <15 μM were ineffective on aequorin luminescence. Hence, aequorin chimeras revealed as a useful tool in the analyses of Cd^{2+}/Ca^{2+} interference. To directly investigate the role of Cd^{2+} in Ca^{2+} homeostasis, we have started to selectively measure the free Ca^{2+} concentration in different cell compartments. Here, we report that cadmium reduces the transient free calcium signal after stimulation of cells with bradykinin. Further studies are in progress to clarify the role of mitochondria and endoplasmic reticulum in cadmium-induced alterations of Ca^{2+} homeostasis in order to link signal transduction modifications with the onset of apoptosis induced by cadmium exposure.

Keywords: Cadmium; NIH 3T3 murine fibroblasts; Aequorin chimeras; Calcium homeostasis

Abbreviations: $[Ca^{2+}]_c$, free cytosolic calcium concentration; $[Ca^{2+}]_{ER}$, free calcium concentration into endoplasmic reticulum; A.A.S., atomic absorption spectroscopy; AEQ, aequorin; Bk, bradykinin; cytAEQ, aequorin chimera localized to cytosol; GSH, reduced glutathione; HA1, short epitope tag derived from haemagglutinin; mGluR1/AEQ, aequorin chimera obtained by the fusion of the COOH-terminus of a truncated metabotropic glutamate receptor (mGluR1) to aequorin molecule

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1. Introduction

Cadmium is a potent toxic metal, harmful to experimental animals and humans, inducing a vast array of remarkably adverse effects as teratogenicity, neurotoxicity and carcinogenicity (Wong and Klaassen, 1982; IARC, 1993; Rogers, 1996; Calevro et al., 1998; Waalkes, 2000). Because of its wide distribution and extensive use in industrial and other activities, human exposure to cadmium is virtually inevitable. Moreover, cadmium has a long lifetime and persists in the ecosystems. Defining the mechanisms of cadmium-induced toxicity has been difficult and not yet completely understood because of the intricate nature of its interactions with living systems. Critical biological events as gene expression, cell proliferation and cell death are affected by cadmium exposure (Waisberg et al., 2003). Recently, several reports indicate that apoptosis probably plays an important role in acute and chronic cadmium exposure. Among various pathways of cell death regulation, intracellular Ca^{2+} homeostasis is very important and its perturbation can lead to apoptosis (Berridge et al., 1998; Pinton et al., 2001). The Cd^{2+} ion has been shown to interfere at various levels with Ca^{2+} homeostasis and Ca^{2+}-mediated signal transduction. In human dermal fibroblasts, for example, cadmium evokes a hormone-like Ca^{2+} mobilization by acting at the signal reception level on the cell surface (Smith et al., 1989); on the other hand, this metal is able to inhibit Ca^{2+} influx across the plasma membrane by interacting with the receptor activated Ca^{2+} influx systems in hepatocytes (McNulty and Taylor, 1999) or with voltage-operated Ca^{2+} channels in neuronal cells (Usai et al., 1999). Even if the interference of cadmium with the regulation of Ca^{2+} homeostasis and Ca^{2+} signals has been extensively studied, this scenario still remains poorly understood. The major problem in the interpretation of the results is associated with the use of the fluorescent indicators, such as Fura-2, Fluo-3 or Quin-2. As reported by different authors, the majority of fluorescent probes bind Cd^{2+} and other divalent cations with very high affinity (Hinkle et al., 1992). Moreover, in the case of Fura-2, the binding of Cd^{2+} modifies the absorption spectrum similarly to that of Ca^{2+} and causes an increase in fluorescence even more intense than that of Ca^{2+}. Hence, it has been difficult to distinguish between calcium and cadmium signals and neither the changes in intracellular free Ca^{2+} induced by Cd^{2+}, nor intracellular concentration of the toxic ion could be assessed unambiguously.

One approach was reported by Benters et al. (1997) by the use of ^{19}F-MNR spectroscopy technique in combination with the intracellular bivalent cation chelator 1,2-bis-(2-amino-5-fluorophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (5F-BAPTA). By this technique, they were able to detect simultaneously both intracellular free Ca^{2+} and other bivalent ions, such as Cd^{2+}. Unfortunately, this method is rather slow and does not permit to register fast changes in intracellular Ca^{2+}. Recently, other authors suggested that alterations of Fura-2 and Fluo-3 produced by heavy metals in free solution become negligible in the cellular environment; thus, the use of fluorescent probes and digital imaging could be considered a suitable technique for studying heavy metals interactions with cell calcium (Marchi et al., 2000). However, they reported that Cd^{2+} leads to a shift in the excitation and emission spectra of Fura-2, causing a signal drop at 380 nm. This suggests that the use of the ratiometric indicator Fura-2 to evaluate Cd^{2+} effect on cell calcium can lead to $[Ca^{2+}]_c$ overestimation.

In this study, we used a new methodology that strongly differs from traditional methods of cell Ca^{2+} measurements: a class of Ca^{2+} probes, which are intracellularly targeted chimeras of the Ca^{2+}-sensitive photoprotein aequorin (Rizzuto et al., 1992). By this approach, aequorin can be targeted to a variety of organelles and cell compartments thus obtaining information on the spatio-temporal changes in Ca^{2+} concentration during cadmium injury. In the present work, we demonstrate that the aequorin molecule can be useful to study cadmium–calcium interaction in living cells, because intracellular cadmium concentrations, lower than 15 μM, are ineffective on aequorin luminescence. Then, we report that cadmium treatment interferes with the transient Ca^{2+} spike evoked by bradykinin in NIH 3T3 murine fibroblasts. These data reinforce the idea for a major role of cadmium in the alteration of Ca^{2+} signalling, but further studies are in progress to investigate a possible link between cadmium-induced apoptosis and the disruption of calcium homeostasis.

2. Materials and methods

2.1. Cell culture and treatment

NIH 3T3 cells (American Tissue Culture Collection) were cultured using Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen) and 10% fetal bovine serum (FBS; Invitrogen) at 37 °C in humidified atmosphere of 5% CO_2. Cultures were passaged twice a week.

Sterilized aqueous solutions of $CdCl_2$ (Sigma–Aldrich) were used for cadmium administration.

2.2. Atomic absorption spectroscopy (AAS)

For the determination of total cellular cadmium content, NIH 3T3 were treated with different cadmium concentrations as indicated in the text. Cells were washed three times in phosphate buffer saline (PBS; Invitrogen) and detached in 1 ml of PBS. After centrifugation, cell pellets were weighted and 1 ml of 65% HNO_3 (Suprapure, Fluka) was added to each 50 mg of cell pellet. The mixture was heated at 70 °C for 10 min and total cadmium content in the supernatant, recollected after 15 min centrifugation at 10 000 rpm, was determined by graphite furnace atomic absorption, using a Perkin-Elmer 5100 atomic absorption spectrophotometer equipped with Zeeman HGA. The cadmium uptake by NIH 3T3 was calculated as:

$$(\%) = \left[\frac{\mathrm{Cd}\,(\mu\mathrm{g})_{\text{cell pellet}}}{\mathrm{Cd}\,(\mu\mathrm{g})_{\text{administered in culture medium}}} \times 100\right].$$

2.3. Constructs and transfection with aequorin

For aequorin measurements, NIH 3T3 cells were seeded onto 13-mm coverslips (BDH, Milan, Italy) previously coated with poly-D-lysine (1 μg/ml in PBS) and transfected with 0.8 μg of aequorin cDNA with Lipofectamine 2000 reagent (Invitrogen).

The following aequorin constructs have been used in our experiments (Fig. 1):

2.3.1. cytAEQ

For the cytoplasm, no further modification is introduced in HA1-tagged aequorin. HA1, derived from haemagglutinin, is a short (9-amino-acid) epitope tag added to each chimeras described here (Brini et al., 1995). As aequorin is itself a fairly poor immunogen (and thus very good antibodies are not available), HA1 sequence was added and immunocytochemical labelling of transfected cells demonstrate the correct cell location of recombinant photoprotein.

2.3.2. mGluR1/AEQ

The mGluR1/AEQ was generated by the fusion of aequorin to the COOH-terminus of a truncated metabotropic glutamate receptor (mGluR1) (Rizzuto et al., 1998). This chimeric aequorin, aimed at measuring Ca^{2+} concentration under the plasma membrane, was instead retained in the endoplasmic reticulum (ER) and the Golgi apparatus. The mGluR1/AEQ is, thus, exposed to the Ca^{2+} concentration of the cytoplasm, while residing on the outer surface of internal membranes. Indeed, using this membrane-bound cytosolic probe (mGluR1/AEQ), we were able, in digitonin-permeabilized cells, to expose the aequorin molecule to different solutions.

All aequorin experiments were performed 36 h after transfection, as previously described (Rizzuto et al., 1995).

2.4. Ca^{2+} measurements with aequorin constructs

For cytosolic AEQ (cytAEQ), the coverslips with the control and Cd^{2+}-treated cells were incubated for

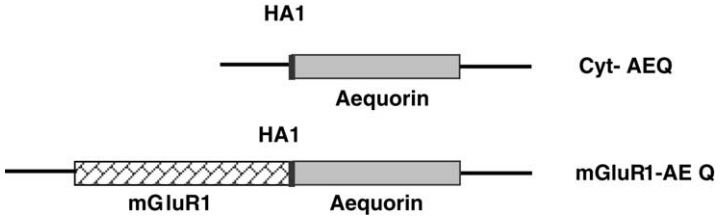


Fig. 1. Schematic representation of aequorin constructs used in our work: HA1, short epitope from haemagglutinin; mGluR1, COOH-terminus of a truncated metabotropic glutamate receptor.

2 h in KRB (Krebs–Ringer modified buffer: 125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 5,5 mM glucose, and 20 mM Hepes, pH 7.4) at 37 °C supplemented with wild-type (w) coelenterazine 5 μM and then transferred to the perfusion chamber.

Schematic experimental procedure for cyt-AEQ:

1. reconstitution in KRB + (Ca^{2+}) [1 mM] + 5 μM (w) coelenterazine (2 h at 37 °C);
2. perfusion in KRB + (Ca^{2+}) [1 mM];
3. agonist stimulation (Bk) [1 μM].

In experiments with permeabilized cells, using the membrane-bound cytosolic probe (mGluR1/AEQ), a buffer mimicking the cytosolic ionic composition (intracellular buffer (IB): 140 mM KCl, 10 mM NaCl, 1 mM K_3PO_4, 5.5 mM glucose, 2 mM $MgSO_4$, 1 mM ATP, 2 mM sodium succinate, 20 mM Hepes, pH 7.05 at 37 °C) was employed. The medium was switched from IB with 2mM EGTA to IB only. This buffer, without any further addition, still contains contaminant Ca^{2+} concentrations (~2 μM). In the experiments with $CdCl_2$, Cd^{2+} was added directly into IB as indicated in the following experimental procedure:

1. reconstitution in KRB + (Ca^{2+}) + 5 μM coelenterazine (w) (2 h at 37 °C);
2. perfusion in KRB + EGTA (100 μM);
3. perfusion in IB + EGTA (2 mM) + digitonine (100 μM);
4. perfusion in IB [$CdCl_2$ (5 or 15 μM) was added directly to IB where indicated].

All aequorin measurements were terminated by lysing the cells with 100 μM digitonin in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O) thus discharging the remaining AEQ pool. The light signal was collected and calibrated into [Ca^{2+}] values as previously described (Brini et al., 1995; Rizzuto et al., 1995). In brief, coverslip with the transfected cells was placed in a perfused, thermostated chamber located in the close proximity of a low-noise photomultiplier, with built-in amplifier discriminator. The output of the discriminator was captured by a Thorn–EMI photon counting board and stored in an IBM-compatible computer for further analyses. The AEQ luminescence data were calibrated off-line into [Ca^{2+}] values, using a computer algorithm based on the Ca^{2+} response curve of wild-type and mutant AEQs, as previously described (Brini et al., 1995; Barrero et al., 1997).

2.5. Statistical analysis

Data in Figs. 3 and 5 were expressed as means ± S.E. (S.E. = S.D./$\sqrt{n}$; with n number of replicates) were analysed using Student's t-test and were considered statistically significant at $P < 0.05$.

3. Results

3.1. Interference between cadmium and aequorin

Aequorin protein from the luminescent jellyfish *Aequorea victoria* was used in our experiments. The ability of this molecule to emit light in presence of calcium ions has led to the use of aequorin as calcium indicator in biological samples. The validity of this use depends on the fact that only calcium (and to a lesser extent Sr^{2+}) can elicit light emission from aequorin. However, some authors have reported that cadmium is able to bind to aequorin and to elicit light emission in cell free assay (Izutsu et al., 1972).

In order to study if aequorin molecule could be employed to investigate Cd^{2+}/Ca^{2+} interactions, we used the chimeric probe mGluR1/AEQ, that was generated by the fusion of aequorin to the COOH-terminus of a truncated metabotropic glutamate receptor (mGluR1) (Rizzuto et al., 1998). This chimeric aequorin is retained on the outer surface of intracellular membranes, such as that of endoplasmic reticulum and Golgi apparatus. This probe (mGluR1/AEQ) was transfected into the cells. After digitonin permebilization, aequorin-expressing cells were exposed to different solutions in a perfusion apparatus. In control experiments, only intracellular buffer was perfused. IB composition mimics intracellular conditions (Rizzuto et al., 1998). No Ca^{2+} was added to IB, but contaminant concentrations of this cation were always found in this solution (nearly 2 μM). When IB alone was perfused, after an initial lag phase of about 150 s, the aequorin luminescence increases to a plateau at nearly 2.5 μM, as shown in Fig. 2 (dotted line). This increase is attributed to the contaminant calcium in IB. The retardation time, related to the time the solution needs to reach the cell preparation and to enter the cells, is linked to the rate

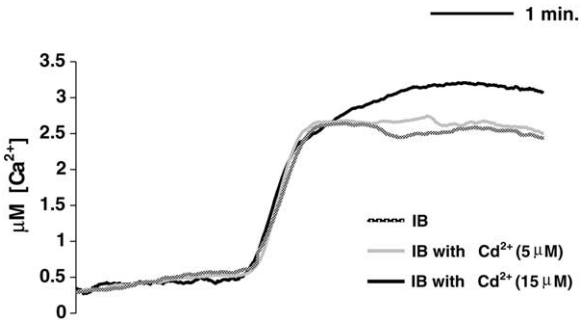


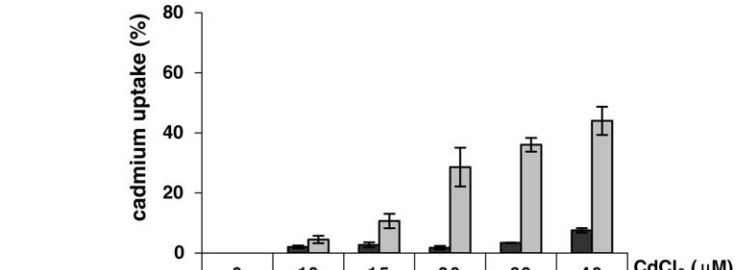
Fig. 2. mGluR1/AEQ probe was used, in digitonin-permeabilized cells, to expose aequorin molecule to different solutions. When IB alone was perfused (control), a significant increase in aequorin luminescence, reaching a value of about 2.56 ± 0.10 μM [Ca^{2+}] (dotted line), was observed. Cadmium concentrations of 5 μM, directly added to IB, were ineffective on aequorin luminescence, and the curve of light emission is rather the same in control and in cadmium-treated cells. The value of [Ca^{2+}] calibrated by the software is 2.65 ± 0.10 μM, not significantly different from control cells (grey line in comparison with dotted line). The use of higher cadmium concentration (15 μM) in IB caused a slight increase in aequorin luminescence in comparison with control experiments. The values determined by the software are around 3.3 ± 0.10 μM (line black in comparison with dotted line).

of IB perfusion (2.5–5 ml/min) and to the time for equilibration of the solution in the perfusion chamber.

We repeated the same experiments by using cadmium concentrations of 5 or 15 μM, directly added to IB from the beginning of the registration. As Fig. 2 clearly shows (grey line), cadmium concentrations of 5 μM are ineffective on aequorin luminescence and the curve of light emission in presence or absence of cadmium chloride is nearly the same. The value of free [Ca^{2+}] calculated is 2.65 ± 0.10 μM, which is not significantly different from control cells. Hence, as contaminant calcium is present in both the solutions used, the step-rise in aequorin luminescence is likely to be only caused by calcium either in control experiments and in cadmium treatment (5 μM).

However, in accordance with the observations presented by Izutsu et al. (1972), the use of higher cadmium concentration (15 μM) in IB causes a slight increase in aequorin luminescence in comparison with control experiments (black line). Here, the value determined is 3.3 ± 0.10 μM. The statistical analysis by Student's test demonstrated that the values of cadmium-treated (15 μM) and control cells are significantly different. Thus, for high intracellular cadmium concentrations, the use of the aequorin probe could lead to an overestimation of [Ca^{2+}].

In order to avoid misunderstandings in calcium measurements, it is important to have precise information on the extent of total cadmium uptake into the cells. For this purpose, we monitored cadmium concentration in NIH 3T3 cells by atomic absorption spectroscopy (A.A.S.). After exposition to different cadmium concentrations for 12 or 24 h, cadmium uptake into NIH 3T3 cells (expressed as percentage of cadmium present in cell pellet versus cadmium administered in culture medium) was dose- and time dependent (Fig. 3). More-



	0	10	15	20	30	40
Cadmium treatment for 12h	0	2.00	2.67	1.74	3.38	7.53
Cadmium treatment for 24 h		4.46	10.64	28.60	36.06	43.99

Fig. 3. Cellular cadmium uptake in NIH 3T3 cells after exposition to different cadmium concentrations for 12 or 24 h. Cellular cadmium uptake was calculated by A.A.S as: (%) = [$Cd(\mu g)_{\text{cell pellet}}/Cd(\mu g)_{\text{administered in culture medium}} \times 100$]. The values reported on the table under the graphic represent average of three different experiments, and S.D. is shown.

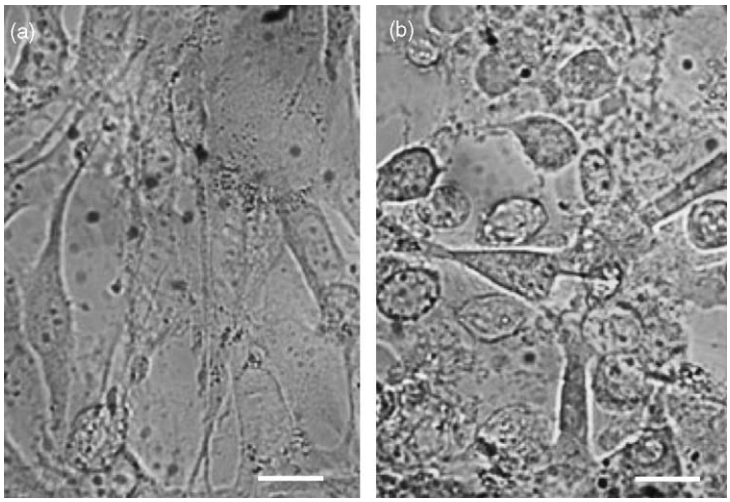


Fig. 4. Phase contrast micrographs of NIH 3T3 cells: (a) control cells; (b) cadmium (15 μM)-treated cells for 24 h. In cadmium-treated cells, the number of round, lucent dead cells is much higher in comparison with control fibroblasts. Scale bar: 10 μm.

over, we observed that after 12 h treatment with 40 μM $CdCl_2$, nearly 7% of Cd^{2+} administered in culture medium was found in the cell pellet. Longer periods of treatment increase the cellular uptake of cadmium and after treatment with 40 μM $CdCl_2$ for 24 h, the percentage of cadmium in the pellet reaches nearly 45% (Fig. 3).

Taking into consideration these results, for all the following aequorin measurements, we used a 24 h treatment with 15 μM $CdCl_2$ directly administered to the culture medium. In these conditions, according to our A.A.S. experiments, only around 10% of cadmium present in culture medium is found in the cell pellet. These conditions, able to induce morphological alterations characteristic of programmed cell death (Fig. 4) in accordance with previous observations (Biagioli et al., 2001), are ineffective on aequorin measurements as the intracellular Cd^{2+} concentration remains well below the critical point of 15 μM. In particular, Fig. 4 shows contrast phase micrographs of control cells (a) and fibroblasts treated for 24 h with 15 μM cadmium (b). In cadmium-treated cells (Fig. 4b), the number of round, lucent dead cells is much higher in comparison with control fibroblasts. Moreover, our previous studies have shown, that cadmium treatment (10–20 μM for 24 h) is able to induce internucleosomal DNA fragmentation and apoptotic bodies formation which represent characteristics of apoptosis (Biagioli et al., 2001).

3.2. Cadmium administration and bradykinin stimulation

A most pertinant question is whether cadmium administration was able to modify the free cytosolic Ca^{2+} concentration either by acting on membrane receptor or directly by interference with intracellular Ca^{2+} homeostasis. We were not able to observe any modification in cytosolic calcium concentration in response to 15 or 50 μM $CdCl_2$ for different times (from 30 s to 3 min). Also, treatment of cells for longer times (12 and 24 h) using 15 μM of $CdCl_2$ resulted in no significant changes in intracellular calcium concentrations. Aequorin molecule, in fact, is able to monitor increases in Ca^{2+} concentration ranging from 0.5 μM to approximately 5/10 μM, and cytoplasmic resting free calcium concentrations in our cells are assumed to be around 70–80 nM, well below the minimum detectable by the probe. Thus, we decided to examine the influence of cadmium on changes in the intracellular Ca^{2+} concentration elicited during bradykinin stimulation. This hormone mediates Ca^{2+} mobilization by binding to surface receptors. Murine fibroblasts NIH 3T3 have been show to possess this kind of receptors and to be sensitive to bradykinin stimulation (Okano et al., 1991).

Bradykinin evokes a Ca^{2+} signal mainly due to the generation of inositol-1,4,5-triphosphate, which in turn, causes a release of Ca^{2+} from internal stores. In

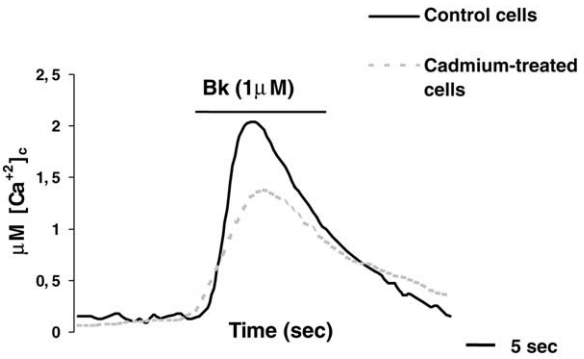


Fig. 5. Kinetics of cytoplasmic Ca^{2+} homeostasis in control and cadmium-treated cells. Cyt-AEQ was transfected into NIH 3T3 cells and 36 h later, the measurement of AEQ luminescence was carried out and calibrated into $[Ca^{2+}]$ values. Where indicated, the cells perfused with KRB were challenged with 1 μM Bk added to the same buffer. The $[Ca^{2+}]_c$ in NIH 3T3 cells raises from 70–80 nM to 2.39 ± 0.10 μM during Bk stimulation.

NIH, 3T3 cells transfected with Cyt/AEQ, stimulation with 1 μM bradykinin evokes a rapid calcium transient, reaching a peak value of about 2.39 ± 0.10 μM (Fig. 5), followed by a gradually declining signal. With cells treated for 24h with 15 μM cadmium, a reduction of the bradykinin-stimulated Ca^{2+} transient is clearly evident in the same figure. The reduction in the bradykinin-evoked Ca^{2+} transient by cadmium treatment is nearly 25% of the control value and the calcium peak amounts to 1.83 ± 0.05 μM (Fig. 6). Moreover, nearly the same reduction in bradykinin-stimulated Ca^{2+} response was found in NIH 3T3 cells treated with 15 μM of $CdCl_2$ for 12 h (1.78 ± 0.34 μM).

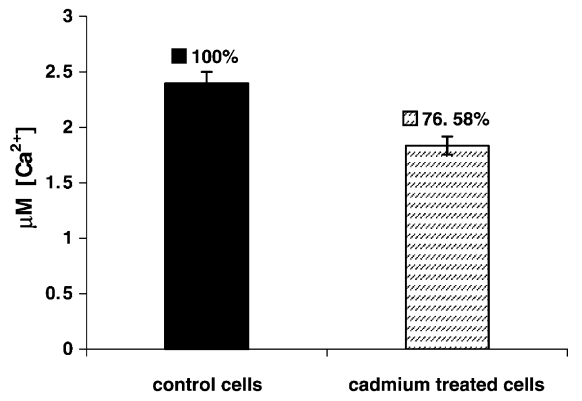


Fig. 6. Alteration in cytoplasmic Ca^{2+} homeostasis after cadmium treatment: percent values. It is evident that $CdCl_2$ treatment for 12/24 h significantly (~25%) reduces the ability of the cells to react to Bk stimulation.

4. Discussion

Calcium is a ubiquitous intracellular signal responsible for the regulation of different processes including proliferation, differentiation, development and cell death (Berridge et al., 2000). It has been reported that cadmium is able to interfere with calcium homeostasis by stimulating both calcium influx and intracellular calcium mobilization (Yamagami et al., 1998). However, even if the interference of this heavy metal with Ca^{2+} regulation has been studied previously, the pathways by which cadmium is able to disrupt calcium signalling still remain debated. We decided to test if aequorin, the well-characterized calcium-sensitive photoprotein, could be used as intracellular calcium indicator during cadmium injury. Due to the intricate nature of cellular effects of cadmium, the use of aequorin chimeras, able to measure calcium concentration at the level of different intracellular organelles, could be important in order to elucidate possible links between different cellular targets of this toxic metal and to correlate possible initial effects, such as disruption of calcium homeostasis and induction of oxidative stress to the onset of apoptosis.

In this report, we first tested if different cadmium concentrations were able to modify the rate of light emission of aequorin. Previous works, in fact, demonstrated that aequorin light emission could be elicited not only by Ca^{2+}, but also by other divalent ions, such as Sr^{2+} and Cd^{2+}. Hence, we exposed aequorin to solutions containing different amount of cadmium, showing that intracellular cadmium concentrations <15 μM were ineffective on aequorin luminescence. The exposition to a higher cadmium concentration (15 μM), instead, induced a slight increase in light emission. We performed these experiments in digitonin-permeabilized cells, thus exposing the aequorin molecule directly to the concentration of cadmium present in the perfusion medium. However, for all the other experiments, NIH 3T3 cells were treated with a fixed cadmium concentration for extended times (12–24 h). In these conditions, it was important to assess the total quantity of cadmium able to enter the cells after a specific treatment and for a defined time. We

monitored intracellular cadmium content by atomic absorption spectroscopy in order to avoid misunderstandings in the interpretation of aequorin results. In our model, A.A.S. experiments revealed that cadmium uptake is dose- and time dependent, and cadmium content found in the cell pellet after 24 h of treatment with 10 and 40 μM, ranges from 5.34 to 40.68% of the amount of cadmium administered in culture medium, respectively. However, the time and the extent of cadmium uptake is expected to be different in different cell lines. We also have to consider that by A.A.S. monitors total cellular cadmium and cannot distinguish between intracellular free Cd^{2+} ions and cadmium-bound to membranes and proteins. As only free cadmium ions are able to binds aequorin and to elicit light emission, intracellular free cadmium content in living system is expected to be several orders of magnitude lower than the values assessed by A.A.S. In fact, our observations demonstrated that after only 6–12 h of cadmium treatment, a significant increase in metallothioneins, a class of small metal binding proteins, was observed (data not shown). Moreover, cadmium could also bind to glutathione inside the cell, thus being trapped far away from the aequorin molecule. In accordance with this considerations, are the data by Marchi et al. (2000). These authors observed an increase in the fluorescence of the Ca^{2+} probe Fura-2 evoked by cadmium, whereas the addition of glutathione prior to or after metal addition, showed a dose-dependent restoration of the Fura-2 signal, indicating the ability of the thiol groups of glutathione to bind Cd^{2+} ions and to reverse the alteration of the Fura-2 signal caused by cadmium. These conclusions made us even more confident that the use of aequorin molecule in our experimental model could be a valid way to study intracellular cadmium–calcium interactions, as the intracellular free cadmium concentration remains always lower than the critical level, even at the high extracellular concentration of 15 μM $CdCl_2$.

To directly investigate the role of Cd^{2+} in Ca^{2+} homeostasis, we have started to selectively measure the free Ca^{2+} concentration in the cytoplasm of cadmium treated and not-treated cells. The first observations, aimed to assess if cadmium administration during calcium registration was able to directly modify the cytosolic Ca^{2+} concentration either by acting on membrane receptor or directly by entering the cells, demonstrated no significant change between control and cadmium-exposed cells. This is probably due to the comparatively slow uptake of Cd^{2+} by NIH 3T3 cells (Morton et al., 1992) Moreover, cadmium seems not able to stimulate a membrane receptor and IP_3 production in NIH 3T3 cells, such as reported Smith et al. (1989) for human skin fibroblasts. We are now planning to use different cell lines, such as rat pheohromocytoma PC12 or human neuroblastoma (SH-SY5Y) cells to study cadmium–calcium interactions at the level of cell surface receptors. The range $[Ca^{2+}]$ endowed with aequorin, is between 0.5 and 10 μM whereas the estimated concentration of free Ca^{2+} in most of the cell lines considered is, about 60–80 nM, well below the minimum detectable by the probe. The combined use of other methods of Ca^{2+} measuring (such as Quin-2 or Fluo-3 and AEQ) might help to solve these problems and to elucidate cadmium/calcium interactions in more detail.

We further analysed the effect of Cd^{2+} exposure on the cytosolic Ca^{2+} signal elicited by an agonist, bradykinin, was investigated. In these experiments, NIH 3T3 cells were transfected with cytAEQ. Cells treated with 15 μM $CdCl_2$ for extended times 12–24 h, were challenged with bradykinin. This hormone evokes a Ca^{2+} signal due to release of Ca^{2+} from internal stores. Control cells were challenged the same way. Both in control and Cd^{2+}-treated cells, bradykinin stimulation causes a rapid rise in free cytoplasmic Ca^{2+} concentration, but the Ca^{2+} increases evoked by this hormone in Cd^{2+}-treated cells are significantly smaller than in controls. A possible explanation for the reduction in the agonist-dependent $[Ca^{2+}]_c$ increases is the decrease in the amount of Ca^{2+} released by the agonist-sensitive Ca^{2+} stores (i.e. ER). Cd^{2+} could reduce $[Ca^{2+}]_{ER}$ either by directly inhibiting Ca^{2+} uptake by the sarcoendoplasmic reticulum calcium ATPases (SERCAs) as reported by Hechtenberg and Beyersmann (1991) in rabbit muscle and by Zhang et al. (1990) in rat liver endoplasmic reticulum or by reducing the resting cytosolic $[Ca^{2+}]$. On the other hand, it could increase the passive diffusion of Ca^{2+} from the ER. Cadmium is known to strongly affect cytosolic $[Ca^{2+}]$ by acting either on receptor operated (ROCCs) and voltage operated (VOCCs) Ca^{2+} channels (McNulty and Taylor, 1999; Usai et al., 1999) thus reducing the resting cytosolic $[Ca^{2+}]$. The tight binding of cadmium to proteins, its ability to induce oxidative stress and tumor cell proliferation strongly complicate the interpreta-

tion of data of cadmium–calcium interactions. Other studies are in progress to gain insights about the specific intracellular targets of cadmium-toxicity by the use of specific aequorin chimeras directed to mitochondria and endoplasmic reticulum. These observations would be correlated with the activation of specific stress responses (such as caspases) and possibly also with alterations in the morphology of intracellular organelles during cadmium exposure in order to fully clarify the role of Ca^{2+} homeostasis disruption in cadmium-induced apoptosis.

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