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Neuroscience Letters 248 (1998) 9–12

Neuroscience Letters

High sensitivity quantitative analysis of cobalt uptake in rat cerebellar granule cells with and without excitatory amino acids

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Received 26 January 1998; received in revised form 1 April 1998; accepted 1 April 1998

Abstract

We quantified the effect of the excitatory amino acids kainate and glutamate on the uptake of cobalt in primary rat cerebellar granule neurons, by using inductively coupled plasma-atomic emission spectroscopy (ICP-AES). We quantitatively demonstrated that Co^{2+} uptake, although enhanced by glutamate and kainate also takes place in the absence of excitatory amino acids. We also found that cobalt uptake is not significantly altered by the presence of glutamate receptor competitive or non-competitive antagonists, indicating that cobalt uptake in granule neurons does not require glutamate receptor stimulation. Our results suggest, therefore, that Co^{2+} may enter the cell by passive diffusion through the plasma membrane. © 1998 Elsevier Science Ireland Ltd.

Keywords: Inductively coupled plasma-atomic emission spectroscopy; Trace-Element; Quantitative analysis; Cobalt; Kainate; Glutamate; Calcium-channels

In recent years, cobalt uptake has been increasingly used to label neurons that express excitatory amino acid and Ca^{2+} permeable (±)-a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate receptors. Such studies were performed in hippocampal [19] and cerebellar tissue sections [15], as well as in primary cultures of dorsal root ganglia [13], hippocampal [4], cortical [11,20], retinal [1] and cerebellar neurons [3,8,10].

In these experiments, cobalt staining was performed following the protocols described by Pruss et al. [2]. With this histochemical technique, the authors provide evidence that intracellular cobalt staining is exclusively due to the activation by kainate of a subset of ligand-gated glutamate receptors (i.e. AMPA/kainate). No uptake was observed in control neurons, therefore, it is believed that in kainate-stimulated uptake, cobalt might replace calcium and enter the cell through distinct types of AMPA/kainate receptors. Such a conclusion is strengthened by specific studies with other techniques including calcium fluorescence quenching electrophysiology and the use of specific antagonists.

To our knowledge, no attempts were made to quantify the uptake of cobalt ions by neuron cells.

Using another technique, synchrotron spectromicroscopy [7,9], we observed a basal uptake of cobalt by neurons. We found that Co was detectable in cultured granule cells also in the absence of added excitatory amino acid [8,10].

In another previous work performed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) we had already found evidence of cobalt uptake in the absence of excitatory amino acids, but such work was to

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PII S0304-3940(98)00296-1

be considered only a feasibility test [10]. We decided therefore to address this issue with the present quantitative analysis of the Co^{2+} internalized by cultured cerebellar granule neurons using ICP-AES, [14,18] a technique for chemical analysis of liquid samples with a sensitivity of a few parts per billion.

Cell culture: primary cultures >95% enriched in granule cells were obtained by enzymatic and mechanical dissociation of 8-day-old rat cerebellum, essentially as described in [5,12].

Suspended cells were plated at a density of 2.5×10^5 cells/cm^2 either on 12-well culture plates (Costar) or in 35 mm Petri dishes (NUNC), previously coated with poly-D-lysine. Cells were grown in a humidified atmosphere of 5% CO_2 at 36°C in BME containing 10% fetal bovine serum (GIBCO), 2 mM glutamine and 100 μg/ml gentamycine. AraC (10 μM) was added at 18 h after plating in order to prevent proliferation of non-neuronal cells.

Cobalt labeling and detection: Co^{2+} labeling was carried out in uptake buffer, mainly as described in [5,8]. After 8 days in vitro (DIV) the cultures were washed and exposed to Co^{2+} (1–5 mM in uptake buffer), alone or in the presence of 100 μM kainate or glutamate.

Control cultures were treated in parallel, incubating them in uptake buffer with no cobalt.

In experiments with glutamate receptor antagonists, the culture medium was replaced with uptake buffer supplemented with 10 μM 6-cyano-7-nitro-quinoxaline-2,3-dione (RBI), 100 μM L(+)-2-amino-3-phosphonopropionic acid (RBI), or 10 μM MK801 ((+)-MK-801 hydrogen maleate; RBI). Part of the cultures were incubated with these antagonists for 5 min prior to the exposure to Co^{2+} with or without glutamate or kainate.

After incubating the cells for 20 min at room temperature, the cultures were washed with uptake buffer and solubilized in 1 N HNO_3 for 30 min for ICP-AES. All the experiments were run in duplicate and on a total of seven cell preparations.

Data analysis. The data of Table 1 were first normalized to 1×10^6 cells in 1 ml HNO_3 solution, then to the volume of cells (approximately 1 μl). Afterwards, statistical analysis was performed by one-way analysis of variance (ANOVA) for replicated measures and Duncan's Multiple Comparison Test or *t*-test for difference of means for paired or unpaired samples, to assess the significance of the observed numerical differences (statistical analysis package for Windows, Stat-100 from Biosoft). For the ANOVA *P*-value test, we took a $P \leq 0.05$ as significant. Hereafter we will refer to the one-way ANOVA *P*-value as '*P*', and to the Duncan's test as '*D*'. *D* = 's' or 'n.s.' identify a test as significant or not significant.

A summary of our ICP-AES results is reported in Table 1 and Fig. 1.

Specific kainate-stimulated Co^{2+} uptake in neurons, which express AMPA/kainate receptor-gated Ca^{2+} permeable channels, has been previously demonstrated by observing that neither NMDA, glutamate or high potassium stimulation of granule cells were able to induce cobalt uptake [15]. As shown in Table 1, we now report that in primary cultures of postnatal rat cerebellar granule cells, the internalization of Co^{2+} does occur and it is concentration dependent: exposure to 1 mM $CoCl_2$ gave an uptake that was significantly lower than that obtained when exposing the cells to 5 mM cobalt (140 and 340 p.p.m. respectively, $P < 10^{-2}$ and *D* = s).

In control samples, not exposed to cobalt, we found only trace amounts of Co^{2+}, at the detection limit of ICP-AES.

In order to verify if the uptake observed in the absence of kainate was due to excitatory compounds (e.g. glutamate) possibly released by the neurons during the 20 min of incubation with Co^{2+}, we treated the cultures with specific glutamate receptor antagonists, before adding 5 mM Co^{2+}. As reported in Fig. 1, the uptake of cobalt was not significantly affected by the presence of: (1) MK801 (in the histogram of Fig. 1, compare the AP3 + MK bin with Ctrl, and MK + Glu with Glu), which is an NMDA open calcium channel blocker; (2) AP3 (compare AP3 and AP3 + MK with Ctrl, and AP3 + MK + Glu with Glu), which acts at the level of the metabotropic glutamate receptor; (3) CNQX (compare CNQX with Ctrl), an antagonist of non-NMDA glutamate receptors. These results suggest that basal Co^{2+} uptake by granule cells is not mediated by glutamate-receptors.

Table 1

ICP-AES results on Co uptake by granule cells with or without excitatory amino acids (exposure time: 20 min)

Number of analyzed specimens	$CoCl_2$ exposure concentration (mM)	Excitatory amino acid	Co concentration in cells (p.p.m.)
10	0	NO	8 ± 3
7	1	NO	140 ± 30
7	5	NO	340 ± 70
6	1	100 μM glutamate	270 ± 50
6	5	100 μM glutamate	450 ± 200
7	1	100 μM kainate	600 ± 50
10	5	100 μM kainate	950 ± 100

The results for Co concentration are the arithmetic mean ± SEM of the concentrations measured on the several samples from six different cell preparations. The concentrations are given in parts per million (p.p.m.) and are normalized to the volume of cells.

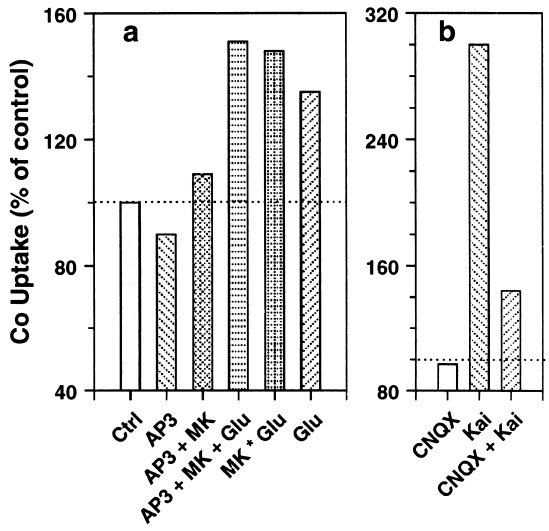

Fig. 1. Histogram of the ICP-AES results on 5 mM Co uptake by granule cells with or without excitatory amino acids (exposure time: 20 min) and with or without antagonists. The results reported here are originating from separate cell preparations and are expressed as percentage of the control specimen. The deviation from the single bin value in the histogram is less than 10%. (a) Ctrl, cells exposed to 5 mM Co, average over $n = 7$ samples; AP3, cells exposed to 5 mM Co + AP3, $n = 2$; AP3 + MK, cells + Co + AP3 + MK801, $n = 2$; AP3 + MK + Glu, cells + Co + AP3 + MK801 + glutamate, $n = 2$; MK + Glu, cells + Co + MK801 + glutamate, $n = 2$; Glu, cells + Co + glutamate, $n = 8$. (b) CNQX, cells + Co + CNQX, $n = 4$; Kai, cells + cobalt + kainate, $n = 15$; CNQX + Kai, cells + Co + CNQX + kainate, $n = 4$.

Furthermore, we found that the uptake of Co^{2+} by granule neurons was independent of extracellular calcium (not shown).

The addition of 100 μM glutamate increased Co^{2+} uptake by 92 and 32%, for the 1 and 5 mM Co^{2+} concentrations (see Table 1). Such glutamate-induced increase is significant at 1 mM cobalt ($P = 0.04$, D = s), while at 5 mM it is not ($P = 0.46$, D = n.s).

The group of experiments with kainate gave the following results: an increase of about 2- to 3-fold in the amount of Co^{2+} uptake, as compared to control samples. At 1 mM Co concentration, adding kainate gave a 330% increase ($P = < 10^{-4}$ and D = s); at 5 mM Co it gave a +180% variation ($P = < 10^{-3}$ and D = s).

As shown in Fig. 1b, the AMPA/kainate receptor antagonist CNQX almost totally blocked the cobalt uptake induced by kainate (compare CNQX + Kai with Kai).

We have extensively studied cobalt uptake with spectromicroscopy, and found no evidence of extracellular cobalt [8].

The high sensitivity ICP-AES technique has been previously employed to measure the concentration of trace elements present in biological samples, such as human brain [2], blood, serum and urine [6,17], or in samples of urban wastes or to estimate dietary elements in foods. In such studies, elements of either toxic or clinical and occupational significance were measured. In primary cultures of cerebellar neurons ICP-AES was also employed for a comparative study of metal ions uptake [10].

In the present work, we use the ICP-AES high sensitivity quantitative approach to demonstrate that Co^{2+} is internalized in significant amounts, even in the absence of glutamate receptor stimulation. This neuronal property was never described by other authors, since the techniques used to detect Co^{2+} in cell preparations were not quantitative and rather 'visual' methods, that revealed only neuronal cells expressing Ca^{2+} permeable receptor-channels, activated by the neurotoxin kainate; furthermore no detection limit was provided for the histochemical techniques employed.

We demonstrate that cobalt uptake does indeed take place in the absence of excitatory amino acids, that it depends on the extracellular concentration of Co^{2+} ions and that is not inhibited by glutamate receptor competitive and non competitive antagonists.

These results rule out the possibility that excitatory compounds are released in the medium by the neurons themselves and demonstrate that Co^{2+} enters the cell by passive diffusion through the plasma membrane.

This conclusion is supported by the recent finding of Reichling et al. [16]. They in fact report that, although at 45°C, pharmacological agents that block divalent cations or depolarizing concentrations of potassium were not able to reduce heat evoked cobalt staining.

Histochemistry did not reveal any basal cobalt staining in granule neurons [1,4,11,13,15,20]. The apparent discrepancy with our results is solved if one considers that cobalt staining techniques are not as sensitive as ICP-AES. From our studies the detection limit of the histochemical technique appears to be around 500 p.p.m.

We also found that glutamate stimulated the uptake of Co^{2+}. This could be explained by a partial activation, in granule cells, of glutamate receptors other than NMDA and metabotropic, as suggested by the experiments performed in the presence of MK801 and AP3. On the other hand, it could also be partially attributed to 'a minority of cells' present in the neuronal cultures, which were found to be Co^{2+} stained after glutamate stimulation, and identified as type-2-astrocytes and as O-2A progenitors by Pruss et al. [15].

Our results on kainate and CNQX confirm instead what was previously found by other investigators: cobalt is taken up by neurons expressing AMPA/kainate calcium permeable-receptor channels, when cells are stimulated by this excitatory compound. This therefore proves the reliability of the ICP-AES approach we used to quantify Co^{2+} uptake in cultured cells.

We demonstrated the superiority of the ICP-AES approach over the histochemical staining techniques for the detection of cobalt in trace concentrations. While further studies in other types of primary neuronal cultures are needed to demonstrate that non-receptor-mediated uptake of Co^{2+} is a general neuronal behavior, our observations on granule cells may already play a crucial role for physio-

logical and toxicological studies, and add a warning for the indirect measurement of calcium fluctuations in cells exposed to divalent cations.

This work was supported by the Italian CNR Progetto Bilaterale Italia USA (to D.M.), the Fonds National Suisse and the EPF-Lausanne. We are indebted to Dr. C. Volonté for a critical review of the manuscript.

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