

Inhibitors of membrane transport system for organic anions block fura-2 excretion from PC12 and N2A cells

Francesco DI VIRGILIO,*† Cristina FASOLATO* and Thomas H. STEINBERG†

*C.N.R. Unit for the Study of the Physiology of Mitochondria and Institute of General Pathology, Via Loredan 16, 35131 Padova, Italy, and †Departments of Medicine and Physiology and Cellular Biophysics, Columbia University, 630 West 168 Street, New York, N.Y. 10032, U.S.A.

The neuroblastoma-like cell line N2A and the pheochromocytoma-like cell line PC12 excrete about 20–25 % of the intracellular fluorescent Ca^{2+} indicator fura-2 during 10 min of incubation at 37 °C. The drug probenecid, known to inhibit membrane systems for the transport of organic anions [Cunningham, Israili & Dayton (1981) *Clin. Pharmacol.* 6, 135–151], inhibited fura-2 excretion in both cell types. However, probenecid also had untoward effects on intracellular Ca^{2+} homeostasis in N2A and PC12 cells. We therefore tested the drug sulphipyrazone, another known inhibitor of organic-anion transport systems. Sulphipyrazone fully inhibited excretion of fura-2 at 250 μM , a concentration one order of magnitude lower than that of probenecid. At this concentration and for incubation times up to 20 min, sulphipyrazone had no untoward effects on cell viability and metabolic functions. Fura-2 was also loaded into the cytoplasm of N2A cells by permeabilization of the plasma membrane with extracellular ATP. In this case as well, the dye was rapidly released from the cells and the efflux was blocked by sulphipyrazone. These findings suggest that N2A and PC12 cells possess a membrane system for the transport of the free-acid form of fura-2. This transport system is probably responsible for the excretion of fura-2 from these cells. Incubation of N2A and PC12 cells with sulphipyrazone may help overcome problems arising in the investigation of $[\text{Ca}^{2+}]_i$ homeostasis in these cell types.

INTRODUCTION

The new generation of fluorescent indicators of cytoplasmic free calcium concentration ($[\text{Ca}^{2+}]_i$), epitomized by fura-2, has become an extremely powerful tool in the investigation of cellular metabolic responses. However, fura-2 has been plagued from the beginning by two problems, namely sequestration of dye into a yet undefined subcellular compartment and a high rate of dye leakage into the extracellular medium. Although it is frequently unmentioned in papers reporting experiments using this probe, there is practically no cell type which is free, to a lesser or greater extent, from such problems. Sequestration of fura-2 inside intracellular organelles or excretion into the extracellular medium or both have been described in a large variety of cell types: smooth muscle cells [2]; rat mast cells [3]; PtK_1 cells [4]; cytotoxic T lymphocytes [5]; fibroblasts [6]; and inflammatory and J774 mouse macrophages [7,8]. It is obvious that these findings raise a number of experimental problems and question measurements of $[\text{Ca}^{2+}]_i$ performed in the presence of a high rate of fura-2 excretion and sequestration. While some tentative explanations of fura-2 excretion and sequestration have been provided [3,6] a reliable means to block these processes is still lacking.

We have recently shown that a well-known inhibitor of organic anion transport, the drug probenecid [1], is a powerful inhibitor of fura-2 excretion and sequestration in the macrophage cell line J774 without any short-term

side-effects on macrophage viability [7,8]. However, when we used probenecid to prevent fura-2 excretion in two neuronal cell lines, PC12 and N2A, some side-effects of this drug became apparent. At a concentration sufficient to block fura-2 excretion, probenecid also reduced by 50 % the rise in $[\text{Ca}^{2+}]_i$ induced by depolarization of the plasma membrane and by the neuropeptide bradykinin. Furthermore, probenecid was unable to prevent fura-2 leakage in Hit cells, an insulin-secreting cell line (C. B. Wollheim, personal communication). Therefore, we tested the congener of probenecid, sulphipyrazone and discovered that sulphipyrazone was a powerful inhibitor of fura-2 excretion in N2A and PC12 cells at a concentration 10-fold lower than probenecid. At this concentration it had no untoward effects on cell viability as judged from cell morphology, plasma membrane potential, resting $[\text{Ca}^{2+}]_i$ level, responses to calcium-mobilizing receptor-linked agonists and to plasma membrane depolarization. Therefore, we wish to suggest sulphipyrazone as the drug of choice for the investigation of $[\text{Ca}^{2+}]_i$ homeostasis with fura-2.

MATERIALS AND METHODS

Cells and chemicals

PC12 cells were obtained from Dr. P. Calissano (University of Rome, Rome, Italy) and N2A neuroblastoma cells from Dr. Laura Facci (FIDIA Research Labora-

Abbreviations used: fura-2-AM, fura-2 acetoxymethylester; fura-2, fura-2 free acid; $[\text{Ca}^{2+}]_i$, cytoplasmic free calcium concentration; DMEM, Dulbecco modified Eagle medium; DTPA, diethylenetriaminepenta-acetic acid; TPEN, NNN'N'-tetrakis(2-pyridylmethyl)ethylenediamine.

† To whom correspondence should be addressed at the following address: Institute of General Pathology, Via Loredan 16, 35131 Padova, Italy.

ories, Abano Terme, Italy). Both cell types were subcultured in our laboratory in Falcon plastic flasks (Falcon Labware, Division of Becton Dickinson & Co., Oxnard, CA, U.S.A.). PC12 cells were cultured in RPMI 1640 (Flow Laboratories, Milan, Italy) supplemented with 2 mM-glutamine, 10% (v/v) horse serum, 5% calf serum and 100 μ l of gentamicin/100 ml (40 mg/ml) as described previously [9]. N2A neuroblastoma cells were grown in Dulbecco modified Eagle medium (DMEM; Flow Laboratories, Milan, Italy) supplemented with 2 mM-glutamine, 10% horse serum, 5% calf serum, 100 i.u. of penicillin/ml and 100 μ g of streptomycin/ml. Fura-2-AM, fura-2 free acid and *NNN'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) were obtained from Molecular Probes Inc., Eugene, OR, U.S.A. Sulphinpyrazone, probenecid and diethylenetriaminepentaacetic acid (DTPA) were purchased from Sigma, St. Louis, MO, U.S.A.

For experiments cells were harvested, washed and resuspended at a concentration of 10^6 /ml in saline medium containing 125 mM-NaCl, 5 mM-KCl, 1 mM-MgSO₄, 1 mM-KH₂PO₄, 5.5 mM-glucose, 1 mM-CaCl₂, 20 mM-Hepes/NaOH buffer, pH 7.4, at 37 °C. This medium was used throughout this work and is referred to as standard saline.

Fura-2 loading

N2A and PC12 cells were centrifuged and resuspended in standard saline at a concentration of 10^7 /ml and loaded with fura-2-AM as previously described [9]. In some experiments (as indicated in the text) 2.5 mM-probenecid or 250 μ M-sulphinpyrazone were also present during the loading. In selected experiments fura-2 was loaded into N2A cells as the free acid by reversible permeabilization of the plasma membrane with ATP.

Measurement of [Ca²⁺]_i

Fura-2 fluorescence as a function of [Ca²⁺]_i was determined in a Perkin-Elmer LS5 spectrofluorimeter as previously described [9]. In some experiments, as indicated in the legends, 100 μ M-DTPA and 20 μ M-TPEN were also added to the incubation medium to relieve the quenching by heavy metals of intracellular fura-2 [8,10]. Fura-2 was calibrated as quin-2 using as excitation and emission wavelengths 340 and 500 nm respectively.

Measurement of plasma membrane potential

Plasma membrane potential was monitored with the lipophilic fluorescent dye bis-(1,3-diethylthio-barbiturate)trimethineoxonal (bis-oxonol), as previously described [11]. Excitation and emission wavelengths were 540 and 580 nm respectively.

Plasma membrane permeabilization

The plasma membrane of N2A neuroblastoma cells was permeabilized with ATP as already described [12,13]. Briefly, cells were suspended at a concentration of 10^7 /ml in standard saline, without added Ca²⁺, containing 250 μ M-fura-2 free acid and 2 mM-EDTA, pH 7.8, at 37 °C. ATP (5 mM) was then added and the incubation carried out for 10 min at 37 °C. The reaction was stopped by adding an equal volume of DMEM medium containing 5 mM-MgSO₄ at pH 6.8. Cells were then centrifuged at 600 *g* for 5 min and resuspended in standard saline. While almost 95% of N2A cells were permeable

after the treatment with ATP, only about 30% of PC12 cells could be permeabilized. Therefore, only experiments with N2A cells are reported.

RESULTS

N2A neuroblastoma and PC12 cells loaded with fura-2-AM and incubated at 37 °C excrete the fluorescent dye into the extracellular medium. After 1 h of incubation of PC12 and N2A cells at 37 °C, about 60–65% of the fura-2 initially within the cells was recovered in the extracellular medium. In N2A cells dye excretion was observed after about 5 min of incubation at 37 °C; after 10 min almost 40% of total intracellular fura-2 was extracellular (Fig. 1). After this fast initial phase excretion of the dye continued at a fairly constant, but slower, rate. In the presence of 2.5 mM-probenecid or 250 μ M-sulphinpyrazone the rate of excretion was decreased and at the end of a 60 min incubation only about 15–20% of fura-2 was extracellular in the presence of either inhibitor. Both in the presence and absence of the inhibitors, 5–10% of fura-2 was already extracellular at the beginning of the incubation at 37 °C and after centrifugation of the cells. This extracellular dye was probably due to either carry-over or to cell damage during the manipulations which preceded the incubation and was subtracted in the calculation of fura-2 excreted. Excitation spectrum and 340/380 ratio at saturating Ca²⁺ (20 μ M) of fura-2 excreted during incubation at 37 °C were typical of the hydrolysed form of the dye.

The sulphinpyrazone concentration used in the previous experiment was near maximal for inhibition of

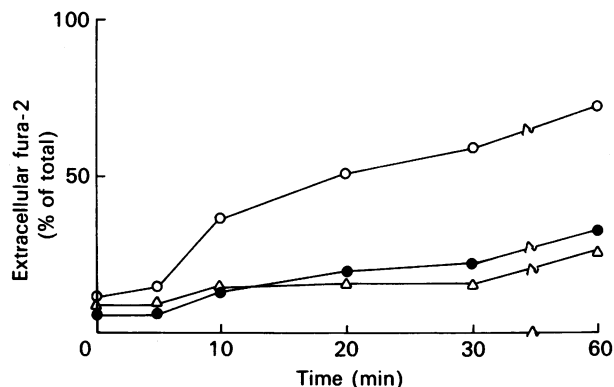


Fig. 1. Probenecid and sulphinpyrazone inhibit excretion of fura-2 from N2A neuroblastoma cells

N2A cells were loaded for 15 min with 3 μ M-fura-2-AM, washed, resuspended at a concentration of 5×10^6 /ml in a final volume of 100 μ l and incubated at 37 °C in standard medium for the time indicated. Probenecid (2.5 mM) (Δ) or 250 μ M-sulphinpyrazone (\bullet) were present throughout the loading with fura-2-AM and the following incubations. Control cells (\circ) were loaded with fura-2-AM and incubated in the absence of the inhibitors. At the end of the incubations, cells were centrifuged, supernatants were removed and the pellets lysed with 0.05% Triton X-100. Fluorescence in the pellets and in the supernatants was then measured. Intracellular fura-2 was 360 pmol/ 10^6 cells for control cells, 560 pmol/ 10^6 cells for cells incubated in the presence of sulphinpyrazone and 740 pmol/ 10^6 cells for cells incubated in the presence of probenecid.

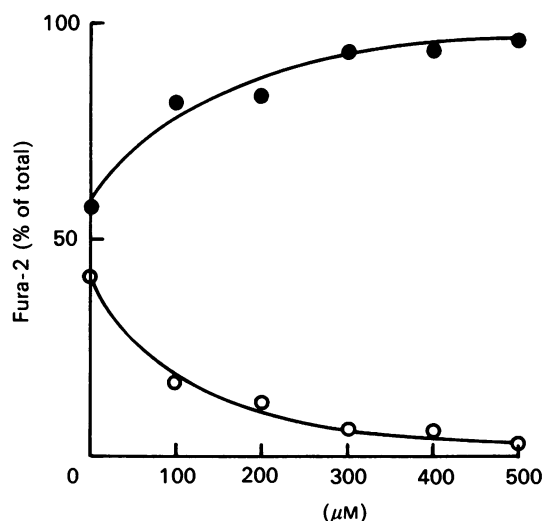


Fig. 2. Dependence of excretion of fura-2 on the sulphinyprazole concentration in N2A cells

N2A cells were loaded with 3 μM -fura-2-AM, washed and resuspended at a concentration of $7.5 \times 10^6/\text{ml}$ for 15 min at 37 °C in the presence and absence of sulphinyprazole. Intracellular fura-2 concentration was 180 pmol/ 10^6 cells. At the end of the incubation cells were centrifuged, supernatants removed and pellets lysed with Triton X-100. (●), pellets; (○), supernatants.

fura-2 excretion, as shown in Fig. 2, where the dependence of fura-2 excretion on the sulphinyprazole concentration is reported. In this experiment, after a 15 min incubation at 37 °C in the absence of sulphinyprazole, we recovered nearly 40% of fura-2 in the extracellular medium. This amount was decreased to 10% and 3% in the presence of 200 and 500 μM -sulphinyprazole respectively.

We had previously used probenecid in macrophages and observed no side-effects of this drug on cell viability or functional responses [7,8,13]. However, incubation of N2A and PC12 cells in the presence of 2.5 mM-probenecid caused a significant attenuation of the rise in $[\text{Ca}^{2+}]_i$ induced by depolarization of the plasma membrane and by the neuropeptide bradykinin (results not shown). This observation raised an important objection to the use of probenecid in studies on $[\text{Ca}^{2+}]_i$ homeostasis in these cell types. Since sulphinyprazole can be used at much lower concentrations, we directed our attention to this inhibitor with the hope that it would be free of this disturbing side-effect. Fig. 3 and Tables 1 and 2 show that a short (10 min) incubation in the presence of 250 μM -sulphinyprazole did not affect the rise in $[\text{Ca}^{2+}]_i$ induced by either plasma membrane depolarization or by bradykinin and at the same time reduced greatly excretion of fura-2 (compare in Fig. 3 the baseline of trace *a*, no sulphinyprazole in the medium, with that of trace *b*, 250 μM -sulphinyprazole). In control cells depolarization of the plasma membrane with 60 mM-KCl caused a rise in $[\text{Ca}^{2+}]_i$ from 90 to 870 nM. As described previously [14], this spike of increase in $[\text{Ca}^{2+}]_i$ was followed by a fast decrease and by a plateau at a level of about 270 nM. Note that the continuous excretion of fura-2 into the extracellular medium made it impossible to record a flat baseline and would give a much higher $[\text{Ca}^{2+}]_i$ level for the plateau if not accounted for. The addition of 20 μM -verapamil lowered $[\text{Ca}^{2+}]_i$ to resting level, thus showing

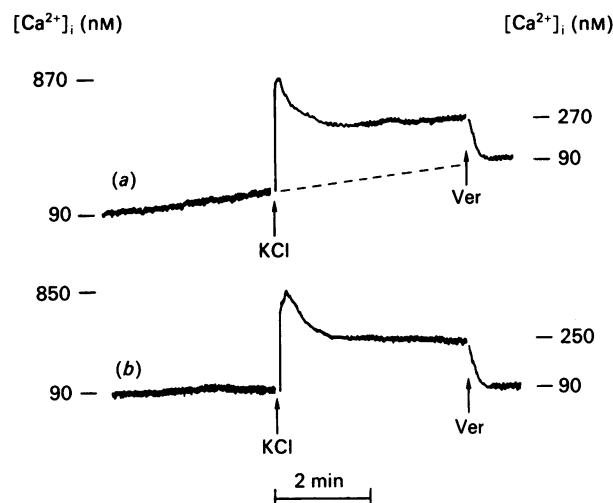


Fig. 3. Changes in $[\text{Ca}^{2+}]_i$ induced by depolarization of plasma membrane in PC12 cells incubated in the presence and absence of sulphinyprazole

PC12 cells were loaded with 6 μM -fura-2-AM, washed and resuspended in the fluorimeter cuvette at a concentration of $7.5 \times 10^5/\text{ml}$. Intracellular fura-2 concentration was about 70 pmol/ 10^6 cells. Cells were incubated for 10 min at 37 °C in the presence and absence of sulphinyprazole before adding KCl (60 mM). Verapamil (Ver) was 20 μM . DTPA (100 μM) and 20 μM -TPEN were also present in the incubation medium to prevent quenching of fura-2 fluorescence by medium heavy metals. In trace (*a*), due to the continuous excretion of fura-2 in the external medium, there was an apparent increase of resting $[\text{Ca}^{2+}]_i$ during the incubation at 37 °C. The contribution of external fura-2 to baseline fluorescence was taken into account in the calculation of true $[\text{Ca}^{2+}]_i$. The dotted line indicates the upward drift of baseline fluorescence. The contribution of external fura-2 to $[\text{Ca}^{2+}]_i$ in the presence of sulphinyprazole was negligible.

that the increase in $[\text{Ca}^{2+}]_i$ caused by KCl was due to the opening of voltage-gated Ca^{2+} channels. In Fig. 3, trace *b*, 250 μM -sulphinyprazole was added 10 min before the addition of KCl. Both the spike in $[\text{Ca}^{2+}]_i$ increase and the following plateau were qualitatively and quantitatively similar to those obtained in the absence of the inhibitor. Also in the presence of sulphinyprazole, verapamil lowered $[\text{Ca}^{2+}]_i$ to pre-depolarization level. We found that N2A and PC12 cells could be incubated for as long as 15–20 min at 37 °C in the presence of sulphinyprazole without any untoward effect on $[\text{Ca}^{2+}]_i$ mobilization. A 60 min incubation caused a 40% reduction of the increases in $[\text{Ca}^{2+}]_i$ induced by both plasma membrane depolarization and by bradykinin (Tables 1 and 2). Both the initial spike and the plateau phase of the depolarization-induced rise in $[\text{Ca}^{2+}]_i$ were reduced by sulphinyprazole roughly to the same extent (Tables 1 and 2). However, even after 5 h of incubation in the presence of sulphinyprazole plasma membrane depolarization and bradykinin induced an increase in $[\text{Ca}^{2+}]_i$, which was still about 50% of that of freshly harvested cells.

Sulphinyprazole did not perturb either resting $[\text{Ca}^{2+}]_i$ (Tables 1 and 2) or depolarization of plasma membrane potential induced by KCl, as measured with bis-oxonol

Table 1. Increases in $[Ca^{2+}]_i$ induced by plasma membrane depolarization in PC12 cells incubated in the presence and absence of sulphinyprazone

Experimental conditions as in Fig. 2. Data are means \pm s.d. of the number of experiments indicated in parentheses. Cells were kept at 37 °C under continuous stirring throughout the experiment. Peak $[Ca^{2+}]_i$ indicates maximal $[Ca^{2+}]_i$ reached immediately after the addition of KCl; plateau $[Ca^{2+}]_i$ indicates $[Ca^{2+}]_i$ 2 min after the addition of KCl (cf. Fig. 3).

Conditions	$[Ca^{2+}]_i$ (nM)		
	Basal	Peak	Plateau
Control cells	100 \pm 24 (6)	740 \pm 220 (6)	240 \pm 40 (6)
Cells incubated in sulphinyprazone for 10 min	90 \pm 10 (9)	750 \pm 140 (9)	220 \pm 30 (9)
Cells incubated in sulphinyprazone for 60 min	90 \pm 20 (4)	450 \pm 50 (4)	130 \pm 20 (4)

Table 2. Increases in $[Ca^{2+}]_i$ induced by bradykinin in N2A cells incubated in the presence and absence of sulphinyprazone

N2A cells were loaded with 3 μ M-fura-2-AM (final intracellular fura-2 content 400 pmol/ 10^6 cells), washed and resuspended at a concentration of 5×10^5 /ml. Cells were kept at 37 °C under continuous stirring throughout. Sulphinyprazone was 250 μ M. Peak $[Ca^{2+}]_i$ indicates maximal increase in $[Ca^{2+}]_i$ after the addition of 400 nM-bradykinin. Data are means \pm s.d. of the number of experiments indicated in parentheses.

Conditions	$[Ca^{2+}]_i$ (nM)	
	Basal	Peak
Control cells	80 \pm 10 (5)	380 \pm 80 (5)
Cells incubated in sulphinyprazone for 10 min	80 \pm 10 (4)	430 \pm 90 (4)
Cell incubated in sulphinyprazone for 60 min	80 \pm 20 (3)	250 \pm 40 (3)

(Fig. 4). However, it did cause consistently a small and transient hyperpolarization of resting plasma membrane potential (Fig. 4).

Fura-2 is usually loaded into the cytoplasm as the acetoxymethyl ester conjugate (fura-2-AM). Fura-2-AM is hydrolysed and fura-2 free acid is trapped intracellularly. We have shown in macrophages that it is the hydrolysed species that is transported across intracellular membranes and, therefore, excreted and/or sequestered [7,8]. We suggest that the same also holds true in the case of fura-2 excretion in the neuronal cell lines investigated here. N2A neuroblastoma cells can be reversibly permeabilized to membrane-impermeant water-soluble solutes by a brief extracellular application of ATP (F. Di Virgilio, unpublished observations). Thanks to this tech-

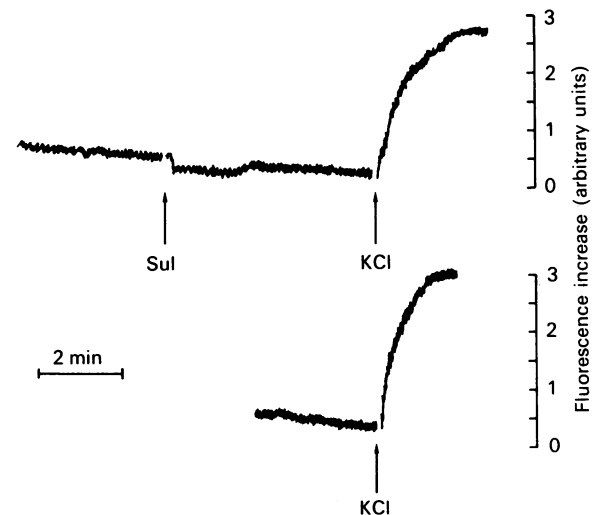


Fig. 4. Effect of sulphinyprazone on plasma membrane potential in PC12 cells

PC12 cells were resuspended at a concentration of 2.5×10^5 /ml. Bis-oxonol was 100 nM. Sulphinyprazone (Sul) and KCl were added at a concentration of 250 μ M and 60 mM respectively.

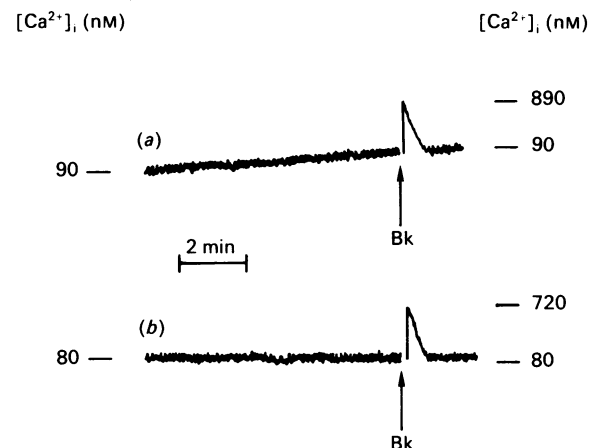


Fig. 5. Effect of sulphinyprazone on excretion of fura-2 in N2A cells loaded by ATP permeabilization

N2A cells were loaded with fura-2 by ATP⁴⁻ permeabilization, washed and resuspended in the fluorimeter cuvette at a concentration of 2.5×10^5 /ml. Intracellular fura-2 content was 24 pmol/ 10^6 cells. Trace (a), control cells; trace (b), cells treated with 250 μ M-sulphinyprazone. Bradykinin (Bk) was 400 nM.

nique we were able to load into the cytoplasm of N2A cells fura-2 as the water-soluble free acid. Upon removal of ATP the lesions in the plasma membrane resealed and fura-2 was trapped inside the cytoplasm (Fig. 5). In the absence of sulphinyprazone there was a continuous excretion of fura-2 from N2A cells into the extracellular medium (indicated by the upward drift of the baseline, trace a) which was blocked by the addition of this inhibitor (trace b). These permeabilized-resealed cells responded normally to bradykinin whether in the presence or absence of sulphinyprazone (Fig. 5). In addition,

resting $[Ca^{2+}]_i$ was normal. We also loaded into the cytoplasm of N2A cells by ATP permeabilization another organic anion not related to fura-2, the highly hydrophilic fluorescent dye Lucifer Yellow. Sulphinpyrazone also prevented excretion of this dye (F. Di Virgilio, unpublished observations). This finding strengthens our suggestion that these cells are equipped with a membrane system for the extrusion of organic anions, such as fura-2.

DISCUSSION

The accurate measurement of Ca^{2+} concentration in the cytoplasm requires that the probe used is localized in the cytoplasm. This statement can sound all too obvious, but with our increasing experience of the fluorescent indicators of $[Ca^{2+}]_i$ developed by Tsien [15], it is clear that very often these probes, even after short incubations at physiological temperatures (37 °C), are trapped within subcellular organelles and/or excreted from the cytoplasm. Efflux of fura-2 into the extracellular medium of PC12 and N2A cells is particularly disturbing because it is most rapid during the initial 10 min of incubation at 37 °C, i.e. the standard duration of a fura-2 experiment.

Two main interpretations have been provided so far for the sequestration and the excretion of fura-2: (i) it is fura-2-AM which diffuses into intracellular compartments where it is or it is not hydrolysed, depending on the cell type [3,6]; and (ii) fura-2-AM is endocytosed from the extracellular milieu during the loading and is then transported into an acidic compartment where it is finally hydrolysed [6]. Although different pathways for fura-2 excretion and sequestration may well exist, even in the same cell type, our results show that in PC12 and N2A cells it is the free acid form of fura-2 which is excreted. Crucial to this point is the demonstration that the dye loaded in its free-acid form via reversible plasma-membrane permeabilization was also rapidly excreted. However, it is inconceivable to think that a molecule with five negative charges is rapidly diffusible across cellular membranes, rather we suggest that fura-2 (which is an organic anion) is excreted via a transport system for organic anions. In agreement with this suggestion fura-2 excretion was blocked by the two known inhibitors of organic anion transport, probenecid and sulphinpyrazone. However, probenecid, initially characterized by us in mouse macrophages, turned out to be of difficult application in other cell types, such as N2A and PC12 cells where it significantly reduced the rise in $[Ca^{2+}]_i$ induced by depolarization of the plasma membrane or by a receptor-directed agonist such as bradykinin. Quite luckily sulphinpyrazone proved to be free of undesired side-effects for incubation times up to 15 min and preliminary results indicate that it is also a powerful inhibitor of fura-2 excretion in systems where probenecid is ineffective (C. B. Wollheim, personal communication).

However, sulphinpyrazone, by inhibiting the organic-anion transport system could, in principle, affect $[Ca^{2+}]_i$ homeostasis in cell types or with agonists other than

those tested in the present investigation. It is therefore important to perform careful control experiments whenever this compound is used to prevent leakage of fura-2.

In this report sequestration of fura-2 within subcellular organelles was not investigated. We found that in PC12 and N2A cells, in contrast to macrophages, sequestration of fura-2 was poor and difficult to quantify. In most experiments with either cell suspensions or monolayers we observed a diffuse pattern of fluorescence, even after prolonged incubations at 37 °C, whether sulphinpyrazone was present or not.

As a final remark, we wish to point out that, besides the methodological applications in the study of $[Ca^{2+}]_i$ homeostasis, the observations reported here also raise the question of the role of this hitherto undescribed system for the transport of organic anions in neuronal cells.

We wish to thank Dr. Samuel C. Silverstein and Dr. Tullio Pozzan for advice; Dr. Roger Y. Tsien for pointing out to us the similarities between probenecid and sulphinpyrazone; Dr. Laura Facci for the gift of N2A cells and Dr. Daria Milani for her kindness. This work was supported by grants from the Italian Ministry of Public Education (40% and 60%), from the Italian National Research Council (Special Project Oncology, grant no. 85 0074544) and from AIRC to F. Di V.

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