AN EVALUATION OF PARAMAGNETIC BROADENING AGENTS FOR SPIN PROBE STUDIES OF INTACT MAMMALIAN CELLS

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ABSTRACT Six transition metal ion complexes have been examined for their effects on the cell survival as well as their effectiveness in inducing the broadening of the electron spin resonance (ESR) spectra of nitroxide spin probes. These paramagnetic species are Ni(EDTA), Ni(DTPA), potassium tris(oxalato) chromate (chromium oxalate), $K_3Fe(CN)_6$, Cu(DTPA), and $NiCl_2$. At 100 mM concentration, the typical concentration used in cell studies to broaden the extracellular nitroxide ESR signal, only Ni(EDTA) and Ni(DTPA) are found to be non-toxic to Chinese hamster ovary cells. The relative cytotoxicities of the six metal ion complexes are Cu(DTPA) > $K_3Fe(CN)_6$ > $NiCl_2$ > chromium oxalate > Ni(DTPA) > Ni(EDTA). Thus, potassium ferricyanide and $NiCl_2$, two most commonly used paramagnetic broadening agents, are relatively toxic to the cell. In contrast, among the six paramagnetic species tested here, chromium oxalate appears to be the most effective agent at non-toxic concentrations in inducing the broadening of the ESR spectra of both cationic and neutral nitroxide spin probes. By considering both their cytotoxicity and their effectiveness in causing line broadening of the nitroxide ESR spectra, chromium oxalate is a good paramagnetic broadening agent for spin probe studies of intact mammalian cells.

INTRODUCTION

Nitroxide spin probe methods have been used to study a variety of intracellular properties including viscosity and molecular diffusion in the cytoplasm (1, 2). These techniques generally require the use of a membrane-impermeable paramagnetic broadening agent to quench the extracellular nitroxide signal, thus allowing the detection of the nitroxide signal arising mainly from the probe inside the cell. Exchange interaction is probably the major mechanism by which transition metal ion complexes broaden the nitroxide electron spin resonance (ESR)[1] spectra in aqueous solution (3–5). Metal ion salts such as $NiCl_2$ and potassium ferricyanide are commonly used as paramagnetic broadening agents for spin probe studies of intact mammalian cells (1, 2). In our point of view, an ideal paramagnetic broadening agent for cell research should be one that is membrane impermeable, chemically inert under experimental conditions, effective at low concentrations, and relatively non-toxic to the cells, and that has short enough electron spin relaxation times so that no ESR signal is produced at experimental temperatures. In our search for such a broadening agent, we have determined the effects of six transition metal ion salts or complexes on the survival of Chinese hamster ovary (CHO) cells and their effectiveness in causing the broadening of the ESR spectra of nitroxide spin probes. Four spin probes commonly used for cell studies are used in this study, e.g., 4-amino-2,2,6,6-tetramethylpiperidino-1-oxy (Tempamine) and 4-oxy-2,2,6,6-tetramethylpiperidino-1-oxy (Tempone) have been used to study molecular diffusion in the cells (1, 2), 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy (CTPO) for measuring oxygen consumption (6), and 3-carboxyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy (CTPC) for measuring cell volume (7). Among the six paramagnetic metal ion complexes used here, potassium tris(oxalato) chromate (chromium oxalate) (8), potassium ferricyanide (2), and $NiCl_2$ (1) are frequently used, and Ni(EDTA) is less frequently used (3, 9), whereas to our knowledge, Ni(DTPA) and Cu(DTPA) have not been used for cell studies. We report here that two commonly used broadening agents, namely, $NiCl_2$ and potassium ferricyanide, are relatively toxic to the cell. In contrast, chromium oxalate appears to meet the above criteria and is the best broadening agent among the six paramagnetic agents tested here for spin probe studies of intact mammalian cells.

[1]Abbreviations used in this paper: CHO, Chinese hamster ovary; chromium oxalate, potassium tris(oxalato)chromate; CTPC, 3-carboxyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy; CTPO, 3-carbamoyl-2,2,5,5-tetramethy-3-pyrroline-1-yloxy; DTPA, diethylenetriaminepentaacetic acid; ESR, electron spin resonance; Tempamine, 4-amino-2,2,6,6-tetramethylpiperidino-1-oxy; Tempone, 4-oxy-2,2,6,6-tetramethylpiperidino-1-oxy.

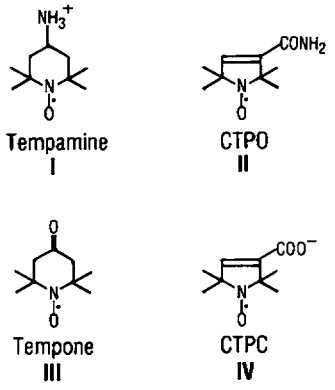

FIGURE 1 Spin probes used in this study.

MATERIALS AND METHODS

Tempamine (*I*), CTPO (*II*), Tempone (*III*), and CTPC (*IV*) (Fig. 1) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Phosphate-buffered saline (PBS, Dulbecco's solution A without Mg^{2+} and Ca^{2+}, pH 7.4) was purchased from Oxoid Ltd., England. $NiCl_2 \cdot 6\ H_2O$, EDTA, diethylenetriaminepentaacetic acid (DTPA), and $CuSO_4 \cdot 2\ H_2O$ were obtained from Aldrich Chemical Co. Chromium oxalate was obtained from Alpha Products, Inc. (Chicago, IL). Potassium ferricyanide is a product of Fisher Scientific Co. (Pittsburgh, PA).

Preparation of Transition Metal Ion Complexes

Transition metal ions chelated with EDTA or DTPA were prepared as follows. Metal ions added to PBS buffer were heated to 90°C until completely dissolved. EDTA or DTPA was added to the above metal ion solution at a stoichiometry of 1:1. The solution was allowed to cool to 22°C and the pH of the solution was adjusted to 7.2–7.4. Chromium oxalate and potassium ferricyanide were readily dissolved in PBS buffer. $NiCl_2$ solution was prepared in saline because it precipitates in the presence of PBS buffer. All stock solutions of spin probes were prepared in water. The samples containing spin probes and metal ion complexes were used directly for ESR measurement without removal of oxygen (10).

Cell Lines

CHO cells were maintained routinely in monolayer culture as previously described (11). Cells were subcultured by trypsin detachment 3 d per week before the cells reached confluence. The cells were grown in α-minimum essential medium (K. C. Biological, Inc., Lenexa, KS) with 10% fetal calf serum (K. C. Biological Inc.) and antibiotics at pH 7.2 in a humidified CO_2 incubator regulated at 37°C. Under these conditions the cells had a consistent doubling time of 14 h. New cultures were initiated every 3 mo from mycoplasma-free frozen stocks.

Cytotoxicity Experiments

On the day before the toxicity experiments, cells were detached from monolayer cultures and added to spinner flasks at a concentration of 1×10^5/ml. The flasks were stirred at 1.5–2.0 rps in a humidified CO_2 incubator. On the day of the experiment when the concentration had reached $4–5 \times 10^5$/ml (still exponential growth), 10×10^6 cells were removed for each toxicity assay, centrifuged, and washed in PBS buffer. The cell pellet was resuspended in 200 μl of the appropriate concentration of paramagnetic broadening agent, which had been diluted in PBS. The cell suspension was drawn up into the tip of a Pasteur pipette that was sealed with Miniseal (American Scientific Products, McGaw Park, IL) and placed in a 37°C water bath for 30 min. Then the end was snipped with wire cutters, and the cells were flushed out with 1 ml of culture medium, resuspended, and counted in an electronic particle counter (Electro Zone/Celloscope; Particle Data, Inc., Elmhurst, IL). Appropriate dilutions were made and two different cell numbers plated into replicate plates to accommodate different survival levels. The plates were incubated for 7–9 d in a CO_2 incubator and then rinsed with Hanks' balanced salt solution. The colonies were fixed with methanol and stained with crystal violet. Colonies with more than 50 cells were scored as viable. Cells treated in exactly the same manner except for the omission of broadening agents served as control. Survival was calculated as the number of colonies per number of cells plated divided by the plating efficiency (number of colonies per number of cells plated in the control).

ESR Measurement

ESR spectra were obtained on a Varian Century line spectrometer (Varian Associates, Inc., Palo Alto, CA) operating at 9.5 GHz and equipped with a Varian temperature regulator. All spectra were recorded at 22°C. For the measurement of the peak-to-peak linewidths, the modulation amplitude used was about one-tenth of the linewidth to avoid spectral distortion and to ensure accuracy in linewidth determination.

RESULTS AND DISCUSSION

Broadening of the Nitroxide ESR Spectra by Paramagnetic Metal Ion Complexes

Collisional interactions between nitroxide spin probes and transition metal ion complexes cause the broadening of the ESR spectra of nitroxides. The broadening has been shown to be linearly proportional to the concentration of metal ions (3–5, 10). We have determined the broadening effect of the ESR spectra of the four nitroxide spin probes by the six metal ion complexes. The results are summarized in Table I. To quantify the effectiveness of line broadening, we have used the term, the broadening factor, which is defined as the value of the line broadening (in gauss) divided by the concentration of the paramagnetic ion (in millimolar). As shown in Table I, anionic chromium oxalate is clearly the most effective broadening agent among the six paramagnetic ions tested when it is used with cationic nitroxides (Tempamine and CTPO) and neutral nitroxide (Tempone). As expected, for anionic nitroxide CTPC, cationic $NiCl_2$ is an effective broadening agent. In contrast, Cu(DTPA) is found to be more effective than either Ni(DTPA) or Ni(EDTA) in inducing the broadening of the nitroxide ESR spectra. This is consistent with our previous observation that the interaction of copper ions with the nitroxide radical is "strong-exchange" type, whereas the interaction of nickel ions with the nitroxide is "weak-exchange" type (12). Our finding that potassium ferricyanide is a good broadening agent for Tempamine is in agreement with observations made by other investigators (2). The reason why potassium ferricyanide is less effective for CTPO than for Tempamine is not clear. For charge effect (see Table I), all metal complexes show a value between 2.4 and 5.2 except for potassium ferricyanide where a charge effect of 15 is noted. The effect of the charge of metal ions and nitroxides upon the broadening of the nitroxide spectra is a complicated subject and has been extensively discussed by Dalal et al. (10) and by Molin et al. (13).

TABLE 1
COMPARISON OF THE EFFECTIVENESS OF VARIOUS METAL ION COMPLEXES IN INDUCING LINE BROADENING OF THE NITROXIDE COMPOUNDS

Metal complexes	Broadening factors‡				Charge effect	
	Tempamine I	CTPO II	Tempone III	CTPC IV	I/IV	IV/I
	gauss/mM					
Chromium oxalate	0.198	0.120	0.175	0.038	5.2	
Ni(DTPA)	0.043	0.033	0.043	0.018	2.4	
Ni(EDTA)	0.032	0.022	0.037	0.012	2.7	
Cu(DTPA)	0.118	0.080	0.092	0.040	3.0	
$K_3Fe(CN)_6$	0.150	0.025	0.073	0.010	15.0	
$NiCl_2$	0.035	0.085	0.081	0.130		3.7

*The experiments were carried out by keeping the nitroxide concentration constant at 0.1 mM and varying the metal ion concentration between 0 and 40 mM. The ESR measurement was performed as described in Materials and Methods. The standard errors of the mean for the data presented here are ~ ±10%.

‡Broadening factor is defined in the text.

Cytotoxicity of Paramagnetic Metal Ion Complexes

The effects of the six paramagnetic metal complexes on the survival of CHO cells are depicted in Fig. 2. In this study, the cells were prepared in the same manner as those prepared for usual ESR experiments (see Materials and Methods). At 100 mM concentration, Ni(EDTA) and Ni(DTPA) have no effect on cell survival. Whereas chromium oxalate has a moderate effect on the cell viability, potassium ferricyanide, Cu(DTPA), and $NiCl_2$ are relatively toxic to the cell (Fig. 2, *solid bars*). We then used the amount of each of the six paramagnetic agents that produces the same broadening effect on the ESR spectra of CTPO (not shown) to determine their toxicities. The results are shown as open bars in Fig. 2. It is worth noting that chromium oxalate at 25 mM broadens the nitroxide spectra to the same extent as potassium ferricyanide at 88 mM, whereas the former is not toxic to the cell and the latter is. Even though $NiCl_2$ at 35 mM produces the same broadening as chromium oxalate at 25 mM, the former is relatively toxic to the cell. Because the actual concentration of metal ions is affected by their potential binding to the culture medium and/or to the cell whose exact volume is uncertain under these conditions, the concentrations of the metal ions reported in this work therefore are apparent concentrations rather than actual concentrations.

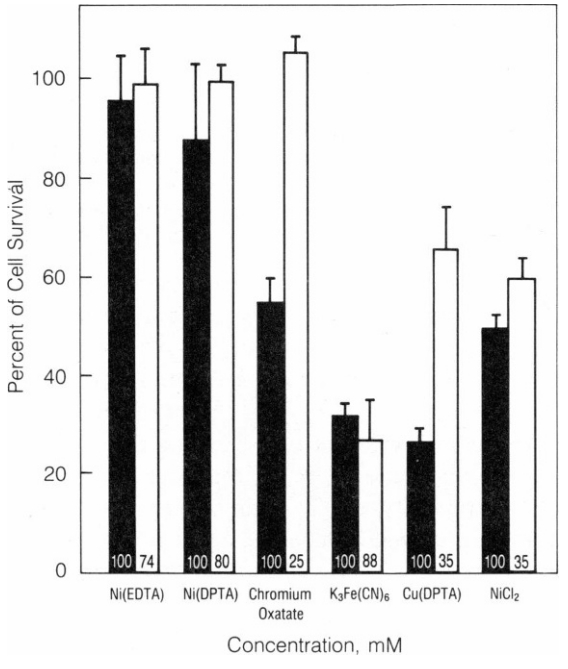

FIGURE 2 Effects of transition metal ions and their chelating complexes on the survival of CHO cells. The cell survival as affected by the metal complexes was determined as described in Materials and Methods. The six transition metal ion complexes used in this study are shown in abscissa. *Solid bars*, the concentration of the complexes was 100 mM. *Open bars*, the concentrations were varied as indicated. The data presented are means plus standard errors of the mean from three experiments.

The results reported here suggest that the two most commonly used paramagnetic metal ion complexes for spin probe studies of mammalian cells are relatively toxic to the cells. Lepock et al. (14) showed previously that potassium ferricyanide affects the survival of Chinese hamster lung (V79) cells. Our $NiCl_2$ toxicity data disagree with Mastro and Keith's report (15) in which 100 mM $NiCl_2$ was shown to have no effect on the growth of 3T3 fibroblastic cells. The discrepancies could be due to the differences in assay conditions: Colony formation is a more sensitive assay for killing than is growth delay. In our study, the cells in single cell suspension were treated with $NiCl_2$ for 30 min at 37°C, identical to the procedure used for usual ESR experiments. In their study, the cells on monolayer culture in flasks were exposed to $NiCl_2$ for 10 min. (The procedure used for their cytotoxicity assay is different from that used for their ESR experiments where the cells were scraped from the monolayer culture and then exposed to $NiCl_2$ before ESR experiments.) However, the possibility that the discrepancy may be due to the differences in sensitivity of

different cell lines to $NiCl_2$ cannot be ruled out. Another drawback of using $NiCl_2$ as a broadening agent involves its tendency of forming insoluble precipitates with buffers such as phosphate at pH 7 (16). Hence, isotonic $NiCl_2$ solution without any buffer generally was used to quench the extracellular nitroxide signal (15). The extracellular pH may be rather acidic under these conditions. In addition, in light of their low toxicity, Ni(EDTA) and Ni(DTPA) are potentially useful paramagnetic metal ion complexes for cell research. In particular, such complexes would certainly be membrane-impermeable and would not have any observable ESR signal at room temperature due to fast electron spin relaxation.

The results reported here have led to two important conclusions: (*a*) Two commonly used paramagnetic broadening agents, namely, $NiCl_2$ and potassium ferricyanide, at typical concentrations used in cell studies, are shown to be toxic to the cells. Caution should be taken in the use of these compounds for cell studies. (*b*) Because chromium oxalate shows little toxicity at concentrations that produce high broadening effects, its use is recommended for spin probe studies of intact mammalian cells.

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