The Ionophore Nigericin Transports Pb^{2+} with High Activity and Selectivity: A Comparison to Monensin and Ionomycin†

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Received August 24, 2004; Revised Manuscript Received October 5, 2004

ABSTRACT: The K^+ ionophore nigericin is shown to be highly effective as an ionophore for Pb^{2+} but not other divalent cations, including Cu^{2+}, Zn^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Ni^{2+}, and Sr^{2+}. Among this group a minor activity for Cu^{2+} transport is seen, while for the others activity is near or below the limit of detection. The selectivity of nigericin for Pb^{2+} exceeds that of ionomycin or monensin and arises, at least in part, from a high stability of nigericin−Pb^{2+} complexes. Plots of log rate vs log Pb^{2+} or log ionophore concentration, together with the pH dependency, indicate that nigericin transports Pb^{2+} via the species NigPbOH and by a mechanism that is predominately electroneutral. As with monensin and ionomycin, a minor fraction of activity may be electrogenic, based upon a stimulation of rate that is produced by agents which prevent the formation of transmembrane electrical potentials. Nigericin-catalyzed Pb^{2+} transport is not inhibited by physiological concentrations of Ca^{2+} or Mg^{2+} and is only modestly affected by K^+ and Na^+ concentrations in the range of 0−100 mM. These characteristics, together with higher selectivity and efficiency, suggest that nigericin may be more useful than monensin in the treatment of Pb intoxication.

The antibiotic agent nigericin was first described in 1951 as a compound produced by a then unidentified *Streptomyces* species present in soil samples arising from Nigeria (*1*). The producing organism was later identified as *Streptomyces hydroscopicus*, and the compound was found to be identical to polyetherin A and X-464, which were independently described by other investigators (*2*, *3*). The actions of nigericin on mitochondria led to the discovery of its well-known activity as a K^+ ionophore, and it has long been used as a research tool in that regard (*4*).

Structurally, nigericin belongs to the polyether class of antibiotics, a group which also includes the well-known ionophores monensin and ionomycin (Figure 1). About 130 naturally occurring compounds belonging to this class have been described (*5*). Their ion transport properties are unknown in most cases, and when they have been investigated, a limited set of the classical methods were often employed. These include two- and three-phase bulk solvent extraction techniques, potentiometric methods based on stabilized membrane electrodes or Mueller−Rudin bilayers, and ion transport studies carried out with subcellular preparations. While data obtained by such methods are of interest, they are often difficult to apply when interpreting the action of ionophores on intact biological systems. For example, bulk solvent systems do not model the potential energy or structural characteristics of a phospholipid bilayer, which is the actual environment wherein the cation complexation and decomplexation reactions associated with transport occur. This problem is not manifest when subcellular preparations are employed; however, preparations of that type are generally unstable to extremes of pH, ionic strength, and other conditions that are of interest when investigating

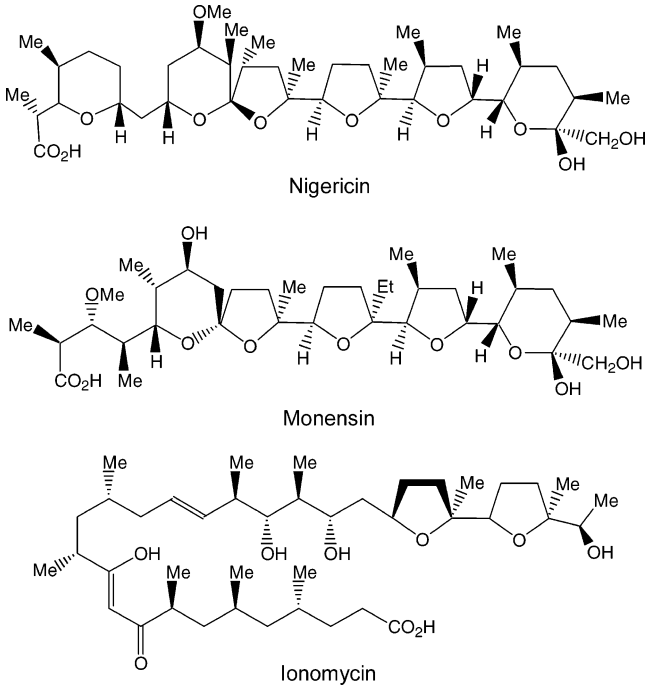

FIGURE 1: Structures of representative carboxylic acid ionophores.

† This research was supported by Grant GM 66206 from the National Institute of General Medical Sciences, National Institutes of Health, by Grant 0255017B from the American Heart Association, Ohio Valley Affiliate, and by Grant HR00-030 from the Oklahoma Center for the Advancement of Science and Technology.

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10.1021/bi048175z CCC: $27.50
Published on Web 11/24/2004

mechanisms of transport. In addition, they contain endogenous transporters which can obscure an activity parameter of interest and do not readily lend themselves to the investigation of toxic and trace cation transport, depending upon the cation in question and its concentration.

Several groups utilize model transport systems based upon phospholipid vesicles to circumvent the problems that are inherent with other systems. For example, these structures were employed in conjunction with temperature-jump techniques to investigate factors that limit rates of Na^+ and K^+ transport by monensin and nigericin (*6*, *7*). Riddell and coworkers used NMR-based methods for similar purposes (*8*−*13*), but that method of detection is limited to cations which are NMR active and requires their presence at high concentrations. We have been utilizing a highly defined system in which the vesicles are loaded with a chelator/indicator, allowing transport to be monitored by UV−vis or fluorescence spectroscopy. It is well suited to the determination of polyvalent cation transport and has so far been applied to investigate the properties of A23187, 4-BrA23187, and ionomycin (*14*−*19*), properties of monensin (*20*), and those of a synthetic ionophore for divalent cations called ETH 129 (*21*). Our interest, in part, is to identify ionophores that are efficient and selective for the transport of Pb^{2+} and then to apply these toward the development of improved treatments for Pb^{2+} intoxication. Among the three naturally occurring compounds that have been considered, all are ionophores for Pb^{2+}, but marked differences are seen in terms of selectivity. A23187 is relatively unselective, ionomycin is of intermediate selectivity, and monensin is highly selective for Pb^{2+} transport, compared to other divalent cations. This range of selectivity suggests that we may not yet have identified the most suitable compound for the intended application, and we are therefore investigating other members of the group.

The present report describes Pb^{2+} transport properties of nigericin and compares them to those of monensin and ionomycin. This compound was selected because it is similar to monensin in terms of structure but shows significant differences in cation complexation chemistry and transport selectivity. More specifically, nigericin contains a pyran ring between the spiroketal group and the terminal carboxylate that is not present in monensin (Figure 1). As a result, the carbon backbone chain length is increased from 26 to 30 atoms, which is associated with differing coordination modes for Na^+, K^+, and Ag^+, as seen in the solid state (*22*−*28*). It is also associated with a reversal in the Na^+/K^+ complexation selectivity where monensin favors Na^+, while nigericin forms stronger complexes with the larger K^+ ion (*29*, *30*). Accordingly, it seemed possible that nigericin might be particularly selective for a larger cation like Pb^{2+}, relative to smaller ones such as Ca^{2+}, Mg^{2+}, and Zn^{2+}. That is in fact the case as shown by the present communication.

EXPERIMENTAL PROCEDURES

Reagents and Solvents. High-purity nitric acid (Fisher, trace metal) and perchloric acid (GFS Chemicals, doubly distilled) were obtained from commercial sources. Synthetic 1-palmitoyl-2-oleoyl-*sn*-glycerophosphatidylcholine (POPC)[1] was obtained from Avanti Polar Lipids, Inc. Purity was confirmed by thin-layer chromatography before use. Nigericin was obtained from Calbiochem and used without further purification. For the transport studies, ionophore stock solutions were prepared in ethanol and were standardized gravimetrically or by titration with Me_4NOH. Quin-2 (K^+ salt) from Sigma was purified by passage over Chelex 100 resin (100−200 mesh) in the Cs^+ form as described previously (*14*) or in the Na^+ form when Na^+-loaded vesicles were employed. The nitrate and chloride salts of divalent cations were the ultrapure grade from Alfa Products. Stock solutions were standardized by titration with a primary standard EDTA solution (*31*) or by atomic absorption spectroscopy using certified solutions (Fisher) for calibration. Solvents containing Et_4NClO_4 and H^+ buffering compounds were further deionized by passage over Chelex 100. For this purpose the resin was in the Et_4N^+ form, which was prepared as previously described (*32*).

Preparation of Phospholipid Vesicles. The preparation of freeze−thaw extruded POPC vesicles loaded with Quin-2 has also been described previously (*33*, *34*). Briefly, 300 mg of POPC in chloroform was dried by rotation under a nitrogen stream to produce a film on the wall of a 25 × 150 mm culture tube. Residual solvent was removed under high vacuum (4 h), and the film was subsequently hydrated in 6 mL of a solution containing 6.6 mM purified Quin-2 and 10.0 mM Hepes buffer adjusted to pH 7.00 with Chelex-treated CsOH or NaOH (*14*), depending on the internal composition required. The mixture was vortexed, and the resulting multilamellar vesicles were frozen in a dry ice−acetone bath, thawed in lukewarm water, and vortexed again. The freeze−thaw and vortexing procedures were repeated two additional times, after which the vesicles were extruded three times through two stacked 100 nm polycarbonate membrane filters. This step was followed by six additional freeze−thaw cycles coupled with additional extrusions. The resulting preparations were applied to Sephadex G-50 minicolumns (*35*) to remove extravesicular Quin-2. These columns were eluted by low-speed centrifugation and had previously been equilibrated with a solution containing 10 mM Hepes buffer, pH 7.00. A single pass over such columns effectively removes the external Quin-2 (*14*, *33*, *34*).

The nominal concentration of POPC in the final preparations was determined by measurement of lipid phosphorus (*36*) and was near 80 mM. The average vesicle diameter is 71 nm (range = 35−110 nm) as determined by freeze−fracture electron microscopy (*33*), and they contain entrapped solutes at the following concentrations: Quin-2, 10.5 ± 0.8 mM; Hepes, 34 ± 8 mM (pH ≈7.4); and Cs^+/Na^+, 60 ± 5 mM. Specific values for Quin-2 and Cs^+/Na^+ were determined for each preparation by the methods described before (*14*, *15*). Briefly, entrapped Quin-2 is determined by spectrophotometric titration with $CaCl_2$ following dispersion of the vesicles in deoxycholate. The entrapped monovalent cation is determined by atomic absorption spectroscopy,

[1] Abbreviations: BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphatidylcholine; TEA^+, tetraethylammonium cation; TEAP, tetraethylammonium perchlorate; VAL, valinomycin.

following replacement of the external medium with one containing a different cation, and dispersion of the vesicles in 0.1 N HCl. When of interest, buffer entrapment is determined from the other values by calculation, using the Henderson−Hasselbach equation, the buffer pK_a, and the internal pH. When buffer entrapment is to be determined, the vesicles also contain the fluorescent pH indicator BCECF, so that the internal pH can be ascertained. The internal pH and solute concentrations differ from those of the vesicle formation medium because of a freeze−thaw-driven solute concentrating effect that operates during preparation of the vesicles (*33*, *34*).

Pb^{2+} Buffers and Determination of Transport. A buffer system was used to control the concentration of Pb^{2+} available for transport into the vesicles. Citrate (15 or 5 mM) was employed to buffer the concentration of this cation, whereas 10 mM each of Hepes, Mes, and Ches were present to buffer H^+. Seventeen equilibria involving citrate^{3-}, H^+, Pb^{2+}, and OH^- were accounted for when calculating the free Pb^{2+} concentration. When Ca^{2+} or Mg^{2+} was to be buffered simultaneously, three additional equilibria involving those cations were also considered. The respective equilibrium constants were taken from literature sources (*37*, *38*), and when necessary, the Davies equation (*39*) was used to correct these to an ionic strength of 100 mM. The species distribution program COMICS (*40*) was used to solve the applicable sets of simultaneous equations at experimental conditions of interest and to allow the generation of standard curves. Examples of the latter were shown previously (*19*).

The transport of Pb^{2+} and other divalent cations into Quin-2-loaded vesicles was determined by monitoring formation of the Quin-2−cation complexes spectroscopically. Unless otherwise indicated, vesicles containing Quin-2 were present at a nominal POPC concentration of 1.0 mM in a medium that also contained 50 mM $CsNO_3$ and 10 mM each of Hepes, Mes, and Ches. The medium pH ranged from 6.00 to 9.50 and was adjusted with CsOH that had been passed over Chelex 100 columns to remove contaminating divalent cations (*14*). In some cases, valinomycin (VAL) (0.5 μM) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (5 μM) were also present to maintain the internal pH at the external value and to dissipate any transmembrane electrical potential that might otherwise arise (*15*). Specific concentrations of ionophores, divalent cations, and pH values are given in the figure legends. Reactions were started by addition of the ionophore, following an initial 2 min preincubation to allow equilibration of transmembrane pH.

The formation of Quin-2−cation complexes was followed continuously by difference absorbance spectroscopy using an Aminco DW2a spectrophotometer operated in the dual wavelength mode. An Oriel no. 59800 band-pass filter was used between the cuvette and the beam scrambler-photomultiplier assembly to prevent detection of the fluorescent light emitted by Quin-2. The sample wavelength used for all cations was 264 nm. The reference wavelengths were at an isosbestic point in the Quin-2/Quin-2−cation complex difference spectrum of interest. These wavelengths vary slightly from cation to cation, as previously described (*16*). Data were collected on a disk using Unkel Scope software (Unkel Software, Inc., Lexington, MA).

To determine initial transport rates, an early portion of the progress curves was fit to eq 1 using standard nonlinear least-squares methods. In this expression, A_T and A_0 are the observed and the initial absorbance values, respectively, B is the initial rate in units of absorbance per second, C is a correction factor for nonlinearity, and t is time in seconds. The values presented are in units of micromolar external cation transported into the vesicles per second. B values obtained from eq 1 were converted to the latter unit by referring to a standard curve for the cation of interest that was generated by titrating the vesicles in the presence of excess ionophore or after they had been lysed with 0.33% (w/v) of Cs^+ deoxycholate (*14*, *16*). Transport selectivities are expressed as S values defined by eq 2. When determining

$$A_T = A_0 + Bt + Ct^2 \quad (1)$$

$$S = (\text{initial rate of } Pb^{2+} \text{ transport})/(\text{initial rate of } Ca^{2+} \text{ transport}) \quad (2)$$

S, the total concentration of the Pb^{2+} or Ca^{2+} was 20 μM and all other conditions were held constant. All transport data were obtained at 25.0 °C.

Potentiometric Titrations and Determination of pH in Aqueous Methanol. For solution chemical studies a mixed solvent of 80% (w/w) methanol in water was prepared gravimetrically, using distilled deionized water and reagent grade methanol (Fisher) that had been freshly distilled. The Et_4NClO_4 that was used to maintain ionic strength in this solvent was prepared by reaction of Et_4NOH (Aldrich) with 70% perchloric acid (GSH Chemicals, distilled). The salt obtained was recrystallized four times from water. The protonation constants and complex formation constants of nigericin were measured by potentiometric methods in the mixed solvent 80% (w/w) methanol−water. For these studies, Na^+ nigericin (Fluka, Fermentek) was purified by column chromatography on silica gel using ethyl acetate as the eluent. The Na^+ salt was converted to the acid form by repeated back-extraction of a $CHCl_3$ solution with 1.0 M HCl. The $CHCl_3$ solution was then washed three times with distilled water, the solvent removed, and the product dried under vacuum. Analysis by flame atomic emission showed that less than 0.05% Na^+ (w/w) remained.

$Pb(ClO_4)_2$ and $Zn(ClO_4)_2$ were prepared by reaction of the metal oxide [Aesar, (Pb) 99.9995%, (Zn) 99.99%] with 70% $HClO_4$ (GFS Chemicals, distilled). $CaCl_2 \cdot xH_2O$ (99.999%) and $MgCl_2 \cdot \times b06H_2O$ (Aldrich, 99.995%) were used as received. Stock solutions of Pb^{2+}, Zn^{2+}, Ca^{2+}, and Mg^{2+} salts were standardized by titration using EDTA (*31*). NaCl (Aldrich, 99.999%) and KCl (Aesar, 99.997%) were dried at 110 °C, and stock solutions were prepared gravimetrically.

Test solutions for potentiometric titration typically contained 0.5−1.0 mM nigericin as the free acid (HL) and, where appropriate, the metal ion (M) at concentration ratios ([HL]:[M]) in the range 1.0−3.0. In the case of Mg^{2+} and K^+, [HL]:[M] ratios of 0.2−0.25 were also used. Ionic strength was maintained at 0.050 using Et_4NClO_4, except in the case of K^+ where Et_4NCl (Fluka, >99%) was used. The titrations were carried out using a digital buret (Metrohm, model 665) and pH meter (Fisher 825MP) that was interfaced to a computer (*41*). pH* measurements were made using double junction combination electrodes (Sensorex S1021CD, Orion Ross 8175BN, Thomas 4080-B49) where the external

filling solution was replaced with 0.1 M Et_4NClO_4 or 0.1 M Me_4NCl (for K^+) in 20% methanol−water.

The pH meter−electrode system was calibrated in aqueous solution using standard buffers (Fisher Gram-Pac); then the electrodes were equilibrated in 80% methanol−water for at least 2 h prior to measurement. The operational pH* scales developed by de Ligny et al. (*42*, *43*) and Gelsema et al. (*44*, *45*) were utilized to determine the value of pH*. The term pH* is defined as $-\log a_H^*$, where a_H^* is the activity of H^+ in the mixed solvent. Accordingly, the term pH* when used in reference to a specific methanol/water mixture has the same meaning as the term pH when used in reference to an aqueous solution (see ref *46* and references cited therein).

All titrations were carried out at 25.0 °C using a thermostated cell and 20 mM Me_4NOH (Fluka) as titrant. A nitrogen or argon atmosphere was maintained to minimize contamination by CO_2. The Me_4NOH was standardized using KH_2PO_4 and checked for carbonate content (*47*). Typical titrations consisted of 50−100 pairs of pH*−mL of Me_4NOH readings. The titration data were analyzed using the computer programs PKAS for the protonation constants (*48*) and BEST for the metal ion complexation constants (*49*).

RESULTS

The activity of nigericin as an ionophore for divalent cations is compared to that of monensin (*20*) and ionomycin (*19*) in Figure 2. Under the conditions employed all three compounds are effective as Pb^{2+} ionophores, with monensin and nigericin being particularly selective in that regard. In the past we have used *S* values, as defined by eq 2, to express the selectivity of ionophores for the transport of Pb^{2+} compared to Ca^{2+} transport (*19*, *20*). When considered this way, the selectivity sequence for the three compounds is monensin (3340) > nigericin (2890) > ionomycin (100), with the numerical values in parentheses being the calculated value for that compound. However, a close comparison of panels A and B of Figure 2 shows that the overall selectivity of nigericin exceeds that of monensin when the total set of cations investigated is considered. That is particularly apparent when the efficiencies of Cu^{2+}, Zn^{2+}, and Cd^{2+} transport by the two compounds are compared.

As with monensin and ionomycin, the predominant complex by which nigericin transports Pb^{2+} has a 1:1 stoichiometry, ionophore:cation, as indicated by plots of log rate vs log of the free Pb^{2+} concentration (Figure 3B) or log rate vs log of the nigericin concentration (Figure 4B). In both cases the slopes of these plots are near to 1.0, as expected for a transporting species of that stoichiometry.[2] Figures 3B and 4B also contain log rate vs log Pb^{2+} or log ionophore concentration data obtained with ionomycin and monensin.

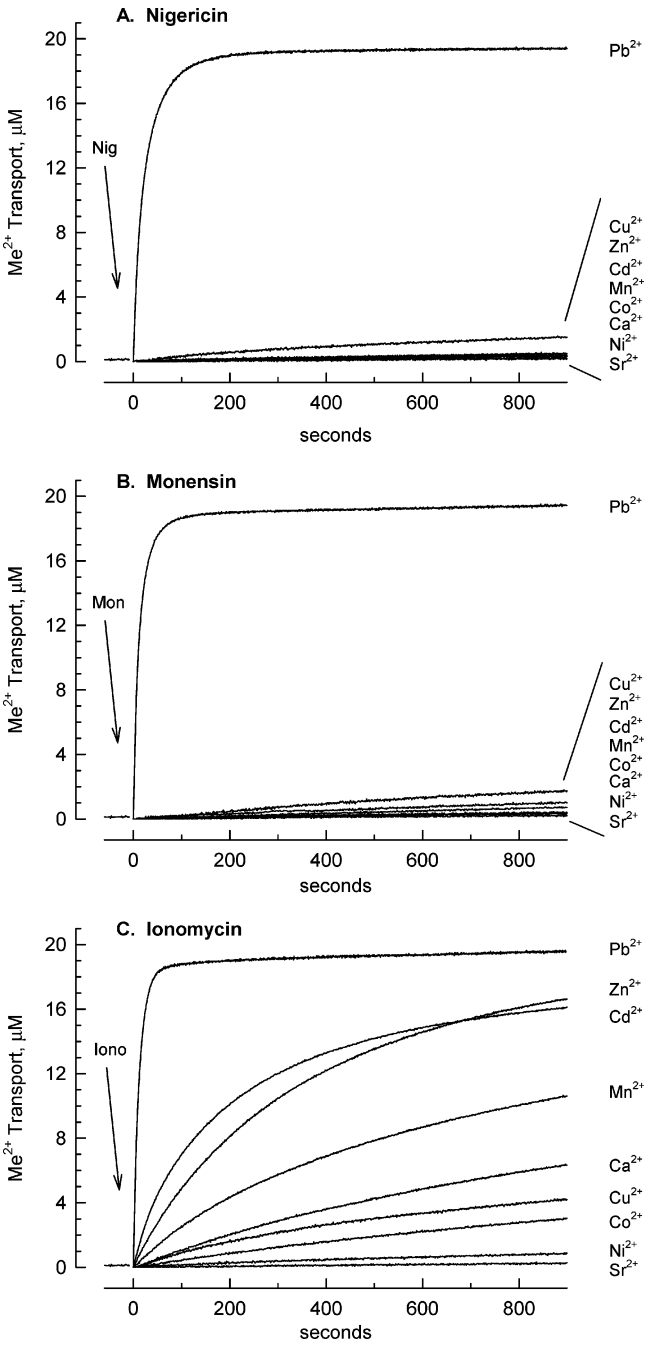

FIGURE 2: Transport of Pb^{2+} and other divalent cations by nigericin (panel A), monensin (panel B), and ionomycin (panel C). POPC vesicles loaded with Quin-2 (Cs^+) were prepared as described in Experimental Procedures. They were incubated at 25 °C and a nominal phospholipid concentration of 1.0 mM. The external medium contained 50 mM $CsNO_3$, 10 mM each of Mes and Hepes (both Cs^+), pH 7.00, 0.5 μM valinomycin, 5.0 μM carbonyl cyanide *m*-chlorophenylhydrazone, and 20 μM indicated divalent cation (nitrate in the case of Pb^{2+}, chlorides for all others). After a 2 min preincubation 0.10 μM ionophore was added to initiate transport (designated as 0 s in the figure). The sequence of cations shown on the right corresponds to their relative rate of transport as observed directly for panel C or on an expanded scale for panels A and B. The *y*-axis unit refers to cation concentration in the external medium. When this parameter reaches ~20 μM, all of the cation that was originally present in the external medium has been transported into the vesicles and sequestered by Quin-2.

A comparison of these plots shows the relative efficiency sequence of the three compounds as ionophores for Pb^{2+}, which is ionomycin > nigericin > monensin.

[2] In Figure 3B, at log Pb^{2+} values greater than approximately −6, the plots show a negative deviation from linearity and slope values that decrease progressively from 1.0. We attribute this to approaching saturation of the ionophores with Pb^{2+}, based upon the complex stability constants that are discussed later, in reference to Table 1. To obtain the slopes for these lines, we considered only the data at log [Pb^{2+}] values <−6.5, thus avoiding the range where saturation is approached. This yielded slope values of 1.07, 1.01, and 0.98 for ionomycin, nigericin, and monensin, respectively. For Figure 4B the full range of data was considered. The slope values were 1.00, 1.06, and 0.99 for ionomycin, nigericin, and monensin, respectively.

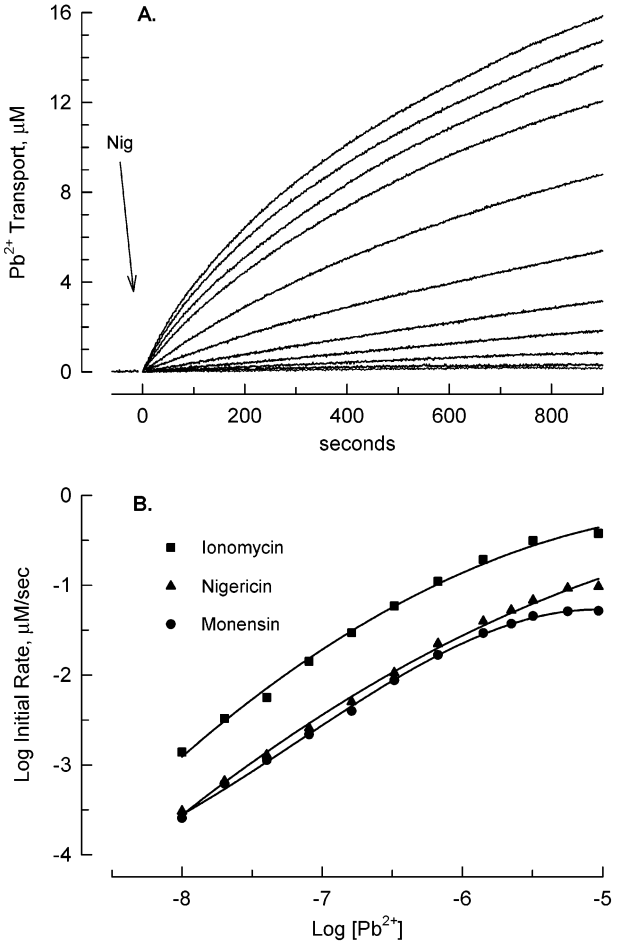

FIGURE 3: Dependence of Pb^{2+} transport on the concentration of free Pb^{2+}. Panel A: Data were obtained as described in the legend to Figure 2, except that the free Pb^{2+} concentration was buffered and varied as follows (beginning with the bottom most curve): 10 nM, 20.1 nM, 40.2 nM, 80.6 nM, 162 nM, 327 nM, 669 nM, 1.41 μM, 2.23 μM, 3.19 μM, 5.63 μM, and 10.0 μM (topmost curve). $CsNO_3$ was omitted from the medium, and 15 mM citrate was present to form the Pb^{2+} buffer, as further described in Experimental Procedures. Panel B: Initial rate values were obtained from the progress curves shown in panel A, as described in Experimental Procedures. The log of these values is shown vs log of the free Pb^{2+} concentration. Panel B also shows analogous data obtained with ionomycin and monensin (*19*, *20*).

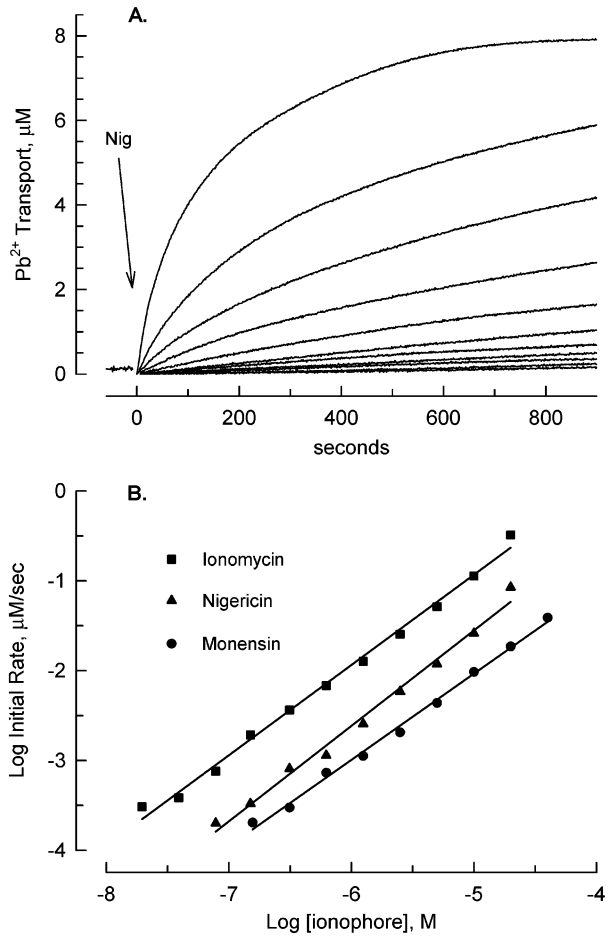

FIGURE 4: Dependence of Pb^{2+} transport on the concentration of nigericin. Panel A: Data were obtained as described in the legend to Figure 3 except that the nominal concentration of POPC was 0.50 mM and the free Pb^{2+} concentration was held constant at 10 nM. The concentration of nigericin was varied as follows (beginning with the bottom most curve): 0, 39.1 nM, 78.1 nM, 156 nM, 313 nM, 625 nM, 1.25 μM, 2.50 μM, 5.00 μM, 10.0 μM, and 20.0 μM (topmost curve). Panel B: Initial rate values were obtained from the progress curves shown in panel A, as described in Experimental Procedures. The log of these values is shown vs log of the nigericin concentration. Panel B also shows analogous data obtained with ionomycin and monensin (*19*, *20*).

Medium pH is an important factor determining the efficiency of Pb^{2+} transport catalyzed by monensin, with the rate increasing as pH rises over the range of 6.5−9 and with a half-maximal value obtained at pH 7.80 (*20*). Ionomycin and nigericin display similar dependencies, with half-maximal values obtained at pH 7.88 and 7.94, respectively (Figure 5). In the case of monensin, the pH dependence was thought to reflect the protonation equilibrium of the carboxylic acid function, together with hydrolysis of the 1:1 species ($MonPb^+$) to form MonPbOH, which is one of the transporting species (*20*). Ionomycin may form the analogous transporting species HIonoPbOH or may be ionized twice to form the neutral species IonoPb (*19*). Given that nigericin has a single ionizable function, like monensin (Figure 1), it is also unable to form an uncharged complex of 1:1 stoichiometry without participation of a second anion. Uncharged complexes are responsible for the nigericin-catalyzed Pb^{2+} transport seen in Figure 5 because no provision was made to collapse the membrane potential that would arise if electrogenic transport were occurring (i.e., valinomycin and CCCP were not present). On the basis of these considerations it seemed possible that the NigPbOH complex is a major species by which nigericin transports Pb^{2+} and that formation of this species accounts for the pH dependency seen in Figure 5.

To determine if the above view is consistent with the complexation chemistry of Pb^{2+} with nigericin, protonation constants and complex formation constants for several cations were determined by potentiometric methods using 80% methanol/water as the solvent. This medium provides a solvent polarity which is similar to that experienced by ionophores located at a POPC membrane interface, and so the values obtained can be used to analyze the mechanism of transport (*50*−*53*). These values are shown in Table 1, together with analogous values for monensin (*20*), and were used to construct the species distribution diagram shown in Figure 6. In Table 1 it is seen that nigericin is a slightly weaker acid than monensin but forms a 1:1 complex (PbL^+) with Pb^{2+} that is slightly more stable. As with monensin,

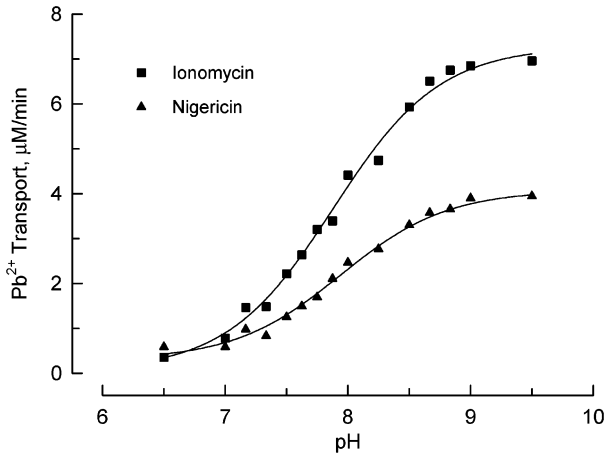

FIGURE 5: Dependence of Pb^{2+} transport on external pH. Data were obtained as described in the legend to Figure 3 except that the external medium contained 10 mM each of Mes, Ches, and Hepes to provide for buffering of H^+ across the full range of pHs examined. Cs^+ was used as the counterion to all buffer anions and to citrate or Quin-2 in the external and internal volumes, respectively. In addition, valinomycin and CCCP were not present, the free Pb^{2+} concentration was held constant at 100 nM, and the ionophores were used at this same concentration. Points shown are experimental and were fit to the Henderson−Hasselbalch equation to obtain the solid lines.

Table 1: Equilibrium Constants for Selected Reactions[a]

reaction	equilibrium constant	nigericin	monensin
$H^+ + L^- \rightleftharpoons HL$	$\log K_H =$	7.02	6.83
$Pb^{2+} + L^- \rightleftharpoons PbL^+$	$\log K_{ML} =$	7.57	7.25
$PbL^+ + OH^- \rightleftharpoons PbLOH$	$\log K_{MLOH} =$	6.69	7.10
$PbL^+ + L^- \rightleftharpoons PbL_2$	$\log K_{ML2} =$	3.80	3.35
$Pb^{2+} + L^- + OH^- \rightleftharpoons PbLOH$	$\log \beta_{PbLOH} =$	14.26	14.35
$Zn^{2+} + L^- + OH^- \rightleftharpoons ZnLOH$	$\log \beta_{ZnLOH} =$	9.46	10.41
$Ca^{2+} + L^- \rightleftharpoons CaL^+$	$\log K_{ML} =$	2.59	3.10
$Mg^{2+} + L^- \rightleftharpoons MgL^+$	$\log K_{ML} =$	2.46	3.16
$Zn^{2+} + L^- \rightleftharpoons ZnL^+$	$\log K_{ML} =$	2.99	3.74
$ZnL^+ + OH^- \rightleftharpoons ZnLOH$	$\log K_{MLOH} =$	6.47	6.67
$Na^+ + L^- \rightleftharpoons NaL$	$\log K_{ML} =$	2.89	5.00
$K^+ + L^- \rightleftharpoons KL$	$\log K_{ML} =$	3.77	3.76

[a] Equilibrium constants for the reactions shown were obtained by potentiometric titrations in an 80% methanol/water mixed solvent as described in Experimental Procedures (nigericin) or were taken from data reported previously (monensin) (*20*). The temperature was 25 °C, and the ionic strength was maintained constant at 0.05 by the presence of tetraethylammonium perchlorate. L^- refers to the ionized form of nigericin or monensin, as indicated. β refers to the overall equilibrium constant for formation of ternary species. It is obtained as the product of equilibrium constants for the stepwise reactions leading to the indicated product.

this complex can hydrolyze (react with OH^-) to form the charge neutral mixed complex NigPbOH, although the equilibrium constant is smaller with the latter compound. These differences tend to cancel each other, such that the overall formation constants (β_{PbLOH}) for MonPbOH and NigPbOH are effectively equal (Table 1).

The distribution diagram (Figure 6) shows that the species $NigPb^+$ is most prevalent near pH 7 and then declines as the pH rises, which is the opposite of what would be expected if it were the major species transporting Pb^{2+}, based on the behavior shown in Figure 5. The species $(Nig)_2Pb$, which a priori might transport Pb^{2+} in an electroneutral fashion, is not prominent at any of pH values examined, and under the present concentration conditions, whereas the prevalence of

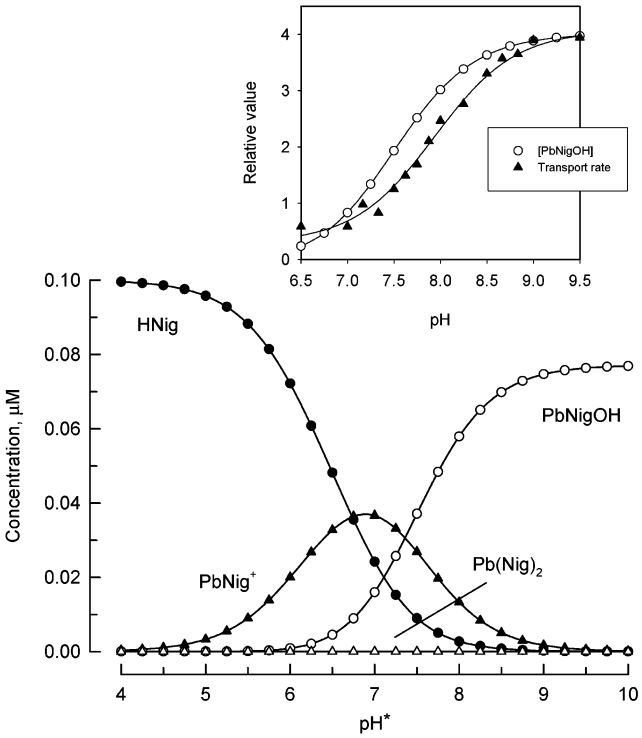

FIGURE 6: Equilibrium behavior of nigericin and Pb^{2+}. Main panel: Individual values were calculated using the program COMICS (*40*) and represent the distribution expected at total Pb^{2+} and nigericin concentrations of 100 nM. Equilibrium constants of interest were from Table 1, and the pK_a for hydrolysis of Pb^{2+} in 80% methanol/water was taken to be 7.96 (*60*). Inserted panel: The PbNigOH values from the main panel are plotted together with the nigericin transport data from Figure 5.

the NigPbOH complex closely parallels the effect of pH on the rate of transport. The similarity is emphasized by the inserted panel, wherein the two parameters are compared side by side. Thus we conclude that NigPbOH is primarily responsible for Pb^{2+} transport catalyzed by that compound, at least under conditions where an electroneutral mechanism is required.

The equilibrium constants shown in Table 1 also provide a rationale for the high selectivity of nigericin for Pb^{2+} transport, compared to other divalent cations (Figure 2). That is to say, the $NigCa^+$, $NigMg^+$, and $NigZn^+$ complexes are less stable than $NigPb^+$ by approximately 5 orders of magnitude, with this differential being greater than that seen with monensin by about 1 order of magnitude (Table 1). The same relative relationships are seen when comparing the overall stability constants (β) for the NigPbOH and the MonPbOH complexes while the analogous complexes formed with Ca^{2+} and Mg^{2+} could not be detected. These differences in complex stability constants appear adequate to explain the selectivity of nigericin for Pb^{2+} transport, among divalent cations, as was the case with monensin described previously (*20*).

With regard to monovalent cations, the equilibrium constant for formation of NigNa is similar to those for the analogous complexes formed with Ca^{2+}, Mg^{2+}, and Zn^{2+} and notably smaller than for the MonNa complex. This is in line with data showing that monensin is selective for Na^+ transport, among monovalent cations (*54*−*56*), and is the same pattern seen by others using different solvent conditions (*29*, *30*, *57*−*59*). The NigK complex is more stable than the

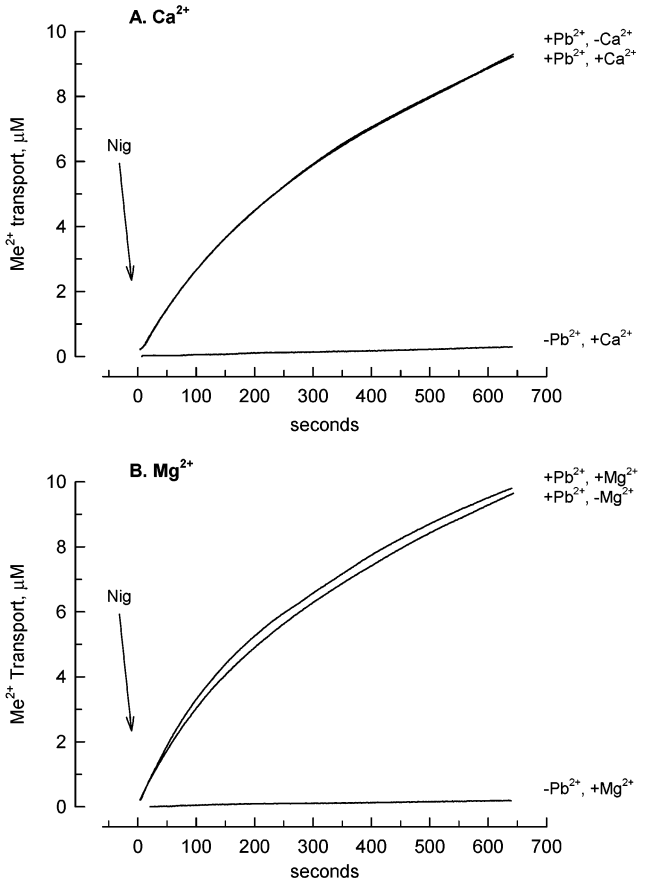

FIGURE 7: Actions of Ca^{2+} and Mg^{2+} as inhibitors of Pb^{2+} transport. Data were obtained as described in the legend to Figure 3 except, when present, the free Pb^{2+} concentration was held constant at 1.00 μM. Panel A: $CaCl_2$ was present with or without Pb^{2+}, as indicated in the figure, sufficient to give a free Ca^{2+} concentration of 1.00 mM. Panel B: Same as panel A except $MgCl_2$ was used instead of $CaCl_2$ to give a free Mg^{2+} concentration of 1.00 mM.

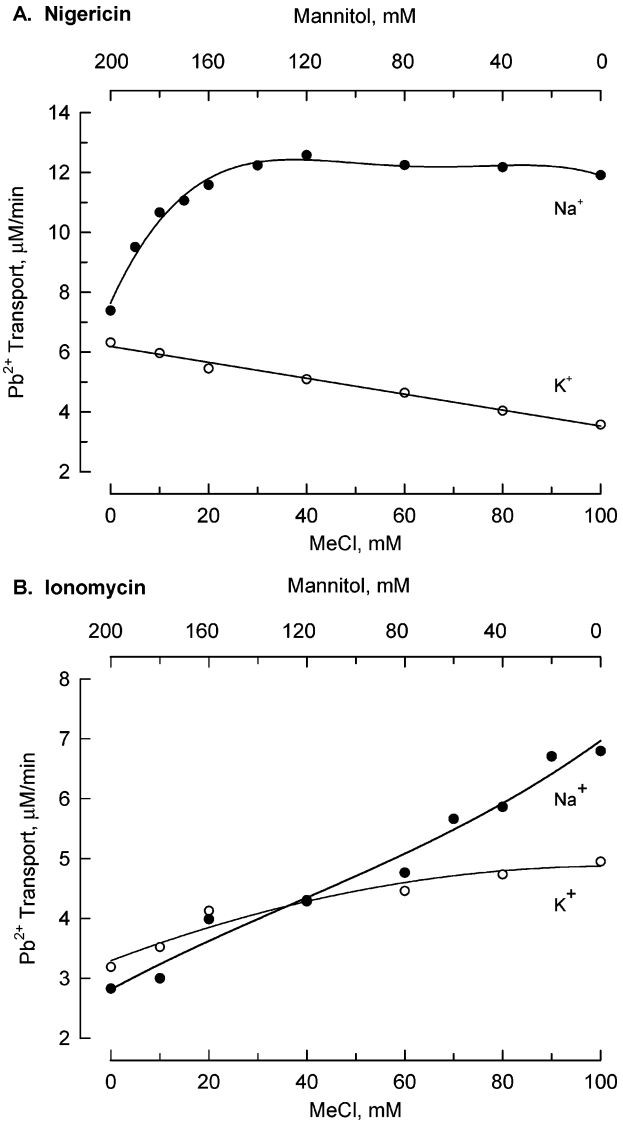

FIGURE 8: Actions of K^+ and Na^+ as inhibitors of Pb^{2+} transport. Vesicles were prepared as described in Experimental Procedures using Cs^+ as the counterion to Quin-2. The external medium contained 5 mM citrate plus $Pb(NO_3)_2$ sufficient to produce a free Pb^{2+} concentration of 100 nM. The external pH was buffered at 7.00 by 10 mM Hepes. NaOH or KOH was used to adjust the external pH, so as to match the monovalent cation that was under examination as a potential inhibitor. NaCl or KCl was also present in the external medium at the indicated concentrations. When these concentrations were 0, the medium also contained 200 mM mannitol, and this solute was decreased by twice the concentration of NaCl or KCl that was subsequently added so as to maintain a constant osmotic pressure. Nigericin (panel A) and ionomycin (panel B) were used at 100 and 50 nM, respectively.

analogous complexes with all divalent cations except Pb^{2+} and has the same stability as the MonK complex. All of these data are consistent with the possibility of nigericin acting as a Pb^{2+} ionophore in biological systems, although several physiological cations such as Ca^{2+}, Mg^{2+}, Na^+, and K^+ would be present at much higher concentrations and might reduce the rate of transport by competition.

To examine competitive relationships more directly, we determined if (near) physiological concentrations of Ca^{2+} and Mg^{2+} (1 mM) are inhibitory to Pb^{2+} transport at a free Pb^{2+} concentration of 1.0 μM. No inhibition was seen (Figure 7). There were effects of K^+ and Na^+, however, as shown in Figure 8. Na^+ produced a modest and concentration-dependent stimulation of Pb^{2+} transport, whereas K^+ had the opposite effect (Figure 8A). These monovalent cations also have small effects on the rate of Pb^{2+} transport catalyzed by ionomycin, and these are shown here (Figure 8B) to complete the asset of parallel data which are now available on Pb^{2+} transport catalyzed by both compounds and by monensin (refs *19* and *20* and the present communication).

DISCUSSION

On the basis of the present data, we can now add nigericin to the set of naturally occurring ionophores that transport Pb^{2+}. In comparison to the others (A23187, ionomycin, and monensin) nigericin has the greatest overall selectivity for Pb^{2+} as shown in part by Figure 2 and by work reported previously in the case of A23187 (*19*). The stability constants for ionophore−Pb^{2+} complexes which are available for monensin and nigericin (Table 1) confirm that the relative stability of complexes seen in solution is an important factor establishing transport selectivity. This can be seen in Table 1, in part by comparing the constants for 1:1 complexes formed with other divalent cations to that of the analogous complex formed with Pb^{2+}. With either compound the Pb^{2+} complex is the most stable, by 4−5 orders of magnitude.

The tendency of 1:1 complexes to hydrolyze, forming a ternary complex containing OH^-, appears to be another factor

that relates complex stability constants to the efficiency of Pb^{2+} transport and the transport of other divalent cations. Such complexes could not be detected for either compound interacting with Ca^{2+} or Mg^{2+}, and both of these cations are transported very poorly (Figure 2 and data not shown). A weak activity for Zn^{2+} transport was detected, as was the ternary complex ionophore−ZnOH formed with either compound. Assuming that Zn^{2+} is transported via these ternary complexes, as is Pb^{2+}, the overall equilibrium constants for their formation (log β values) can be compared to each other and to the relative transport efficiencies. log β for the ionophore−PbOH complexes is 4−5 orders of magnitude greater than those for the analogous Zn^{2+} complexes, in line with the much greater activity of both compounds for Pb^{2+} transport (Figure 2). Comparing the two compounds to each other shows that nigericin is more selective than monensin for Pb^{2+}, compared to Zn^{2+} ($\beta_{PbLOH}/\beta_{ZnLOH}$), and that this arises because β_{ZnLOH} for the monensin complex is greater than for the nigericin complex by ~10-fold. These differences are in line with the noticeably higher activity of monensin for Zn^{2+} transport and with the apparent higher selectivity of nigericin for Pb^{2+} transport compared to Zn^{2+}. Thus, one might suggest that relative complex stabilities seen in solution will predict the selectivities of other ionophores for Pb^{2+} transport. However, such parallel relationships were generally not found with A23187, 4BrA23187, and ionomycin transporting a different group of divalent cations (*16*) or transporting the lanthanide series trivalent cations (*18*). Accordingly, the patterns of complex formation constants and transport selectivities, as well as details of the transport mechanisms, must be determined for a larger group of ionophores and cations to establish the relationships among these characteristics.

The very high transport selectivity of nigericin and monensin for Pb^{2+} is one of the obstacles to obtaining fully quantitative expressions of a sequence which includes a more diverse set of divalent cations. This is because the range of initial rates that can be accurately determined is limited to about 5×10^3 when all conditions are held constant except for the divalent cation identity, whereas the selectivity values for Pb^{2+} will approach or exceed this limit in several cases. It should be possible to overcome these limitations with the compounds that have been investigated to date, however, given that they all transport Pb^{2+} predominantly through formation of a 1:1 complex, as indicated by broad regions in plots of log rate vs log ionophore and log Pb^{2+} concentrations, which are linear and have slopes near 1.0 (Figures 3 and 4). Within these regions the efficiency of transport can be expressed as a second-order rate constant, as defined by eq 3. Presumably, analogous constants for various cations

$$k_{Pb} = \text{rate}/[\text{ionophore}][Pb^{2+}] \quad (3)$$

can also be obtained, and these would be directly comparable even when different conditions of ionophore and cation concentration were used to obtain them. This approach may overcome the limitations associated with the range of rates that can be observed using a single set of conditions and allow a broader range of transport efficiencies to be determined and compared using the vesicle system. To initiate the use of rate constants when evaluating the transport selectivity in our system, we calculated k_{Pb} for monensin, nigericin, and ionomycin using the data shown in Figure 4B. The values obtained at pH 7.00 were 1.0×10^5, 2.4×10^5, and 11.9×10^5 M^{-1} s^{-1} for the three compounds, respectively. To the best of our knowledge these are the first examples of such constants to be reported for divalent cation transport by polyether ionophores.

The above type of quantitation in analyzing ionophore transport properties requires an understanding of the overall reaction and the stoichiometry of the transporting species, as well as the role that secondary medium conditions may play in establishing a rate (i.e., the role of medium components not involved in the transport process per se). Regarding the overall reaction, rapid transport in the absence of agents which would dissipate a membrane potential shows that an efficient electroneutral mechanism is operating (Figure 5), whereas ionophore−PbOH complexes are the apparent species responsible, as pointed out in the Results section, referable to Figures 5 and 6 and Table 1. It remains to be seen if other species contribute, depending upon conditions, and if significant electrogenic transport can occur. A small electrogenic component does seem possible because Pb^{2+} is transported slightly faster when VAL plus CCCP are present, compared to when they are absent (see preliminary data in Figure 9). This could indicate that a mixed mode arises when the membrane potential is dissipated and that this is shifted to strictly neutral when an opposing potential arises. Alternatively, CCCP may substitute for OH^- to form a neutral species of the type ionophore Pb−CCCP, similar to the mixed complex that was previously found to arise between ETH 129, Ca^{2+}, and the uncoupler (*21*). Other possible explanations for what is seen in Figure 9 relate to small changes in intravesicular pH that are brought about by VAL plus CCCP and the effects these have on rate by altering the distribution of ionophore between the two sides of the vesicle membrane (*15*).

Regarding secondary medium conditions, it is clear that these can be significant as shown here by the stimulation of Pb^{2+} transport that arises upon replacing mannitol with NaCl in an isoosmotic fashion (Figure 8). Another example is the most rapid rate of Pb^{2+} transport shown in Figure 3A, compared to the rate that is shown in Figure 2A. The latter rate is severalfold higher than the former despite the fact that the initial free Pb^{2+} concentration is higher by only a factor of 2 (20 μM for Figure 2A vs 10 μM for Figure 3A, uppermost curve). Apparently the substitution of citrate for nitrate as the major medium anion reduces the rate to a significant degree. The potential cause of effects such as these includes changing ionic strength and actions exerted at the membrane interface. Membrane interface effects could include alterations in membrane surface charge/surface polarity or changes in conformation of phospholipid headgroups. These in turn could alter the stability of interactions between the ionophore and the membrane, or the stability of membrane-associated complexes with the transported cations, and could therefore alter the rate of transport. Thus, medium conditions will remain important when comparing the properties of ionophores, even when the use of rate constants has been adopted.

Monensin has already been shown to promote the excretion of previously accumulated Pb^{2+} from rats (*20*) and to increase the effectiveness of dimercaptosuccinate, which is a hydrophilic chelating agent that is used clinically to treat Pb

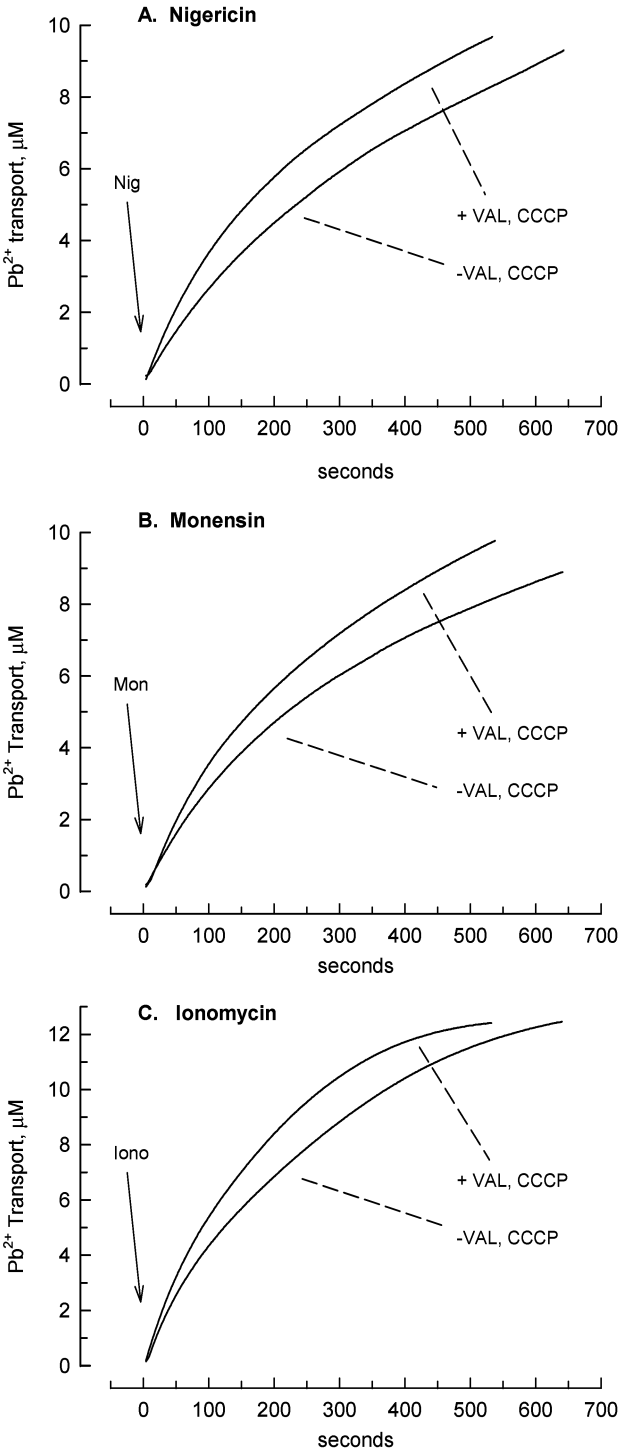

FIGURE 9: Possible involvement of electrogenic processes in ionophore-mediated Pb^{2+} transport. Experiments were conducted as described in Experimental Procedures at a free Pb^{2+} concentration of 1.00 μM. Valinomycin plus CCCP was present, or not present, as indicated in the figure. Nigericin (panel A), monensin (panel B), and ionomycin (panel C) were utilized at 100 nM.

intoxication (to be presented elsewhere). These actions are thought to reflect Pb^{2+} transport by that compound, facilitating the mobilization of Pb from membrane-bounded compartments. Given that nigericin is of higher activity as a Pb^{2+} ionophore (Figures 3 and 4), is apparently more selective for Pb^{2+} compared to other divalent cations (Figure 2), and is little affected by the major physiological cations in terms of its Pb^{2+} transport activity (Figures 7 and 8), it seems possible that nigericin may be the preferable compound for those types of applications. That possibility is under further investigation, as is the potential suitability of other polyether ionophores.

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BI048175Z