Ionomycin, a Carboxylic Acid Ionophore, Transports Pb$^{2+}$ with High Selectivity*

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Studies utilizing phospholipid vesicle loaded with chelator/indicators for polyvalent cations show that ionomycin transports divalent cations with the selectivity sequence Pb$^{2+}$ > Cd$^{2+}$ > Zn$^{2+}$ > Mn$^{2+}$ > Ca$^{2+}$ > Cu$^{2+}$ > Co$^{2+}$ > Ni$^{2+}$ > Sr$^{2+}$. The selectivity of this ionophore for Pb$^{2+}$ is in contrast to that observed for A23178 and 4-Br-A23187, which transport Pb$^{2+}$ at efficiencies that are intermediate between those of other cations. When the selectivity difference of ionomycin for Pb$^{2+}$ versus Ca$^{2+}$ was calculated from relative rates of transport, with either cation present individually and all other conditions held constant, a value of ∼450 was obtained. This rose to ∼3200 when both cations were present and transported simultaneously. 1 μM Pb$^{2+}$ inhibited the transport of 1 mM Ca$^{2+}$ by ∼50%, whereas the rate of Pb$^{2+}$ transport approached a maximum at a concentration of 10 μM Pb$^{2+}$ when 1 mM Ca$^{2+}$ was also present. Plots of log rate versus log ionomycin or log Pb$^{2+}$ concentration indicated that the transporting species is of 1:1 stoichiometry, ionomycin to Pb$^{2+}$, but that complexes containing an additional Pb$^{2+}$ may occur. The species transporting Pb$^{2+}$ may include H$_2$IPbOH, wherein ionomycin is ionized once and the presence of OH$^-$ maintains charge neutrality. Ionomycin retained a high efficiency for Pb$^{2+}$ transport in A20 B lymphoma cells loaded with Indo-1. Both Pb$^{2+}$ entry and efflux were observed. Ionomycin should be considered primarily as an ionophore for Pb$^{2+}$, rather than Ca$^{2+}$, of possible value for the investigation and treatment of Pb$^{2+}$ intoxication.

Lead remains as a pervasive toxin to which we are all exposed, albeit at levels that vary substantially with geographical location, occupation, and socioeconomic factors (1-3). To illustrate the magnitude of this problem, on the order of 1 in 10 American children currently possess blood lead levels above the toxic threshold of ∼0.5 μg/dL (reviewed in Ref. 4). The identification of mechanisms leading to lead toxicity is therefore of considerable interest and actively investigated. Work of this type conducted at the cellular level has been facilitated by the recent demonstration that entrapped Indo-1 can be used to monitor Pb$^{2+}$ transport into cells (5, 6), even though this fluorescent compound is responsive to changes in the intracellular free Ca$^{2+}$ concentration (7). Transport studies utilizing Indo-1-loaded cells of several types have shown that Pb$^{2+}$ enters via plasma membrane Ca$^{2+}$ channels that are activated upon depletion of internal Ca$^{2+}$ stores (5, 6), as is also true for other toxic cations (8, 9).

The investigation of lead toxicity at a cellular level would benefit from the availability of ionophores that transport Pb$^{2+}$ selectively in comparison with other cations; however, to date, no compound with this property has been identified. This situation may reflect the use of cells and subcellular preparations in early studies on the selectivity of ionophore-mediated transport (10). Biological systems are not stable to toxic cations such as Pb$^{2+}$, making it difficult to use them for such purposes. In addition, the difficulty inherent in distinguishing ionophore-derived transport from transport occurring via endogenous channels and carriers may also have discouraged attempts to identify ionophores that are selective for Pb$^{2+}$.

Unlike biological systems, phospholipid vesicles are stable to a wide range of cations and conditions and are free of endogenous transport activities. We have demonstrated that these structures, when prepared by freeze-thaw extrusion, are useful for investigating the transport mechanisms (11-13) and specificities (14, 15) of divalent cation ionophores such as A23187, 4-Br-A23187, and ionomycin. In this report, we extend this work to include Pb$^{2+}$ transport. The results indicate that ionomycin is substantially selective as an ionophore for Pb$^{2+}$ and that this property is displayed in both phospholipid vesicles and cultured A20 B lymphoma cells. Accordingly, ionomycin may be useful for manipulating levels of Pb$^{2+}$ during studies of its toxic properties in other cell types and at higher levels of biological organization.

EXPERIMENTAL PROCEDURES

Reagents—Synthetic 1-palmitoyl-2-oleoyl-sn-glyero-phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids, Inc. Purity was confirmed by thin-layer chromatography before use. Ionomycin, A23187, and 4-Br-A23187 were obtained from Sigma, Calbiochem, or Teflabs and were used without further purification. Stock solutions in dimethyl sulfoxide were standardized by titration with a primary standard.

Preparation of Phospholipid Vesicles—Freeze-thaw-extruded POPC vesicles loaded with Quin-2 were prepared as described previously (17, 18). Briefly, 300 mg of POPC in paraffin 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 5 mM purified Quin-2 (Ca⁺) and 10.0 mM Hepes adjusted to pH 7.00 with Chelex-treated CsOH (11). 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 (19) to remove extraneous solutes at the following concentrations; Quin-2, 10.5 ± 0.8 mM; Hepes, 34 ± 8 mM (pH 7.4); and Cs⁺, 60 ± 5 mM. Specific values for Quin-2 and Ca⁺ were determined for each preparation by the methods described previously (11, 12). Briefly, entrapped Quin-2 was determined by spectrophotometric titration with standard CaCl₂ following dispersion of the vesicles in deoxycholate. Entrapped Cs⁺ was determined by atomic absorption spectroscopy following replacement of the external medium with one not containing Cs⁺ and dispersion of the vesicles in 0.1 N HCl. When of interest, buffer entrainment was determined from the other values by calculation using the Henderson-Hasselbalch equation, the buffer pKa, and the internal pH. When buffer entrainment was to be determined, the vesicles also contained the fluorescent pH indicator 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) so that the internal pH could 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 (17, 18).

Vesicles loaded with BAPTA plus Indo-1 were prepared and characterized in an analogous way. For those preparations, the formation medium contained 5 mM BAPTA (in place of Quin-2) and 100 μM Indo-1. Accordingly, the resulting vesicles contained a relatively high level of BAPTA and a much lower level of Indo-1. The efficiency of BAPTA entrainment was similar to that of Quin-2 and was determined by titrating the intact vesicles with standard CaCl₂ in the presence of excess ionomycin. Fluorescence of the Indo-1-Ca⁺ complex was monitored at 400 nm (excitation at 360 nm) using an SLM-AMINCO 8100 spectrophotometer operated in the analog mode. The end point can be taken to represent the end point for the simultaneous reaction of Ca⁺² with BAPTA because the two compounds have essentially the same affinity for Ca⁺² (7, 21), as described further under “Results.” Knowledge of Indo-1 entrainment was not required during the present investigation, and that parameter was not determined.

Cell Culture and Loading of Cells with Indo-1—A20 B lymphoma cells (ATCC TIB-208) were grown to a density of 1.5–2.5 × 10⁶ cells/ml at 37 °C under an atmosphere of 95% air and 5% CO₂. RPMI 1640 medium was employed and was supplemented with 25 mM Na+-Hepes, pH 7.4, 100 units/ml penicillin, 100 μM streptomycin, 2 mM L-glutamine, and 5% fetal bovine serum albumin (22). For loading with Indo-1, the cells were incubated for 45 min at 37 °C in a medium containing 4 μM Indo-1/AM plus 140 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM Na⁺-Hepes, pH 7.4, and 10 mM L-glutamate (23). 1.0 mM probenecid was also present to inhibit the release of Indo-1 after loading (24). Following this, the loaded cells were maintained at 25 °C with gentle shaking and were used within 6 h.

Cation Buffers and Determination of Transport—Pb⁺² and Ca⁺² were usually present from a buffer system for transport into the vesicles or cells. 15 mM citrate was employed to buffer the concentration of cations, whereas 10 mM each Hepes and Mes were present to buffer H⁺. Seventeen equilibria involving citrate³⁻, H⁺, Pb⁺², and OH⁻ were accounted for when calculating the free Pb⁺² concentration when this cation was present alone. Additional equilibria involving Ca⁺² were also considered, but both Pb⁺²- and Ca⁺² were present and the free concentration of both cations was of interest. The respective equilibrium constants were taken from literature sources (25, 26), and when necessary, the Davies equation (27) was used to correct these to an ionic strength of 100 mM. The species distribution program COMICS (28) was used to solve the applicable sets of simultaneous equations at experimental conditions of interest and to allow the generation of standard curves. As examples of the latter, Fig. 1A shows how free Pb⁺² and PbOH⁺ vary in this system as a function of total Pb⁺² when the medium pH is 7.0. Fig. 1B shows the pH dependence of this system.

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 spectrophotically. Vesicles containing Quin-2 were present at a nominal POPC concentration of 1.0 mM in a medium that also contained 50 mM CaCl₂ and 10 mM each Hepes and Mes. The medium pH ranged from 6.0 to 8.0 and was adjusted with CsOH that had been passed over Chelex 100 columns to remove contaminating divalent cations (11). To maintain internal pH at the external value, valinomycin (0.5 μM) and carbonyl cyanide m-chlorophenylhydrazone (5 μM) were also present (12). Specific concentrations of ionophores and divalent cation chlorides and pH values are shown in the figure legends. Reactions were started by the addition of the divalent cation ionophore following an initial 2–5-min period that was allowed for the equilibration of transmembrane pH.

The formation of Quin-2-cation complexes was followed continuously by difference absorbance spectroscopy using an SLM-AMINCO DW2a spectrophotometer operated in the dual wavelength mode. An Oril 59800 band-pass filter was used between the cuvette and the beam scanner-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 described previously (14). Data were collected on disc using Unkel Scope software.

The vesicles loaded with BAPTA plus Indo-1 were used to monitor the simultaneous transport of Pb\(^{2+}\) and Ca\(^{2+}\), as further described under “Results.” Pb\(^{2+}\) transport into Indo-1-loaded A20 B lymphoma cells was monitored as described by Kerper and Hinkle (5, 6), with modifications that are also described under “Results.”

Other Methods—To determine initial transport rates, an early portion of the progress curves was fit to Equation 1 using standard nonlinear least-squares methods.

\[
A_p = A_o + Bt + Ct^2
\]

(Eq. 1)

In this expression, \(A_p\) and \(A_o\) are the observed and the initial absorbance values, respectively; \(B\) is the initial rate in units of absorbance/s; \(C\) is a correction factor for nonlinearity; and \(t\) is time. The values presented are in units of micromolar external cation transported into the vesicles/s. \(B\) values obtained from Equation 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) Cs deoxycholate (11, 14). This was with the exception of Pb\(^{2+}\), where apparent multiple equilibria between the cation and Quin-2 required a slightly modified approach (see “Results”). Transport selectivities are expressed as \(S\) values defined by Equation 2.

\[
S_{M} = \frac{\text{initial rate of M\(^{2+}\) transport}}{\text{initial rate of Ca\(^{2+}\) transport}}
\]

(Eq. 2)

When determining \(S\), an equal concentration of the cation in question is substituted for Ca\(^{2+}\) with all other conditions held constant (14). All data were obtained at 25.0 °C.

RESULTS

Use of Quin-2-loaded Vesicles to Investigate Ionophore-catalyzed Pb\(^{2+}\) Transport—The vesicle-based transport system employed here is useful for determining selectivity and mechanisms of ionophore-mediated cation transport (11–15), but there are features of importance that must be accounted for when applying the system to citrate-buffered solutions of Pb\(^{2+}\) (PbOH\(^{+}\)). The first is an apparent slow permeation of Pb\(^{2+}\) in the absence of an ionophore, as illustrated in Fig. 2A. This is seen at much lower free Pb\(^{2+}\) concentrations than with Ca\(^{2+}\) permeation, with the uncatalyzed movements of both cations showing a complex dependence on concentration and pH (Fig. 2B). Under the conditions we utilized, the rate of uncatalyzed transport was usually negligible, but was determined, and data were corrected accordingly when it exceeded 2–3% of the ionophore-dependent rate.

The second feature relates to an apparent formation of multiple complexes between Pb\(^{2+}\) and entrapped Quin-2 and to how this impacts on the calibration procedure used to relate the Quin-2 difference absorbance measurements to Pb\(^{2+}\) transport. When the vesicles were suspended in an unbuffered Pb\(^{2+}\) solution and the total Pb\(^{2+}\) available for transport exceeded the total Quin-2 trapped within the vesicles, a biphasic progress curve was obtained upon the addition of a Pb\(^{2+}\) ionophore (Fig. 3A). During the first phase, entrapped Quin-2 approached an apparent saturation; however, as time proceeded, a second phase was observed that would indicate a partial release of previously accumulated Pb\(^{2+}\) if it were taken at face value (Fig. 3A). However, titrating vesicle-entrapped Quin-2 with Pb\(^{2+}\) in the presence of excess ionophore also produced a biphasic pattern, wherein the apparent degree of saturation decreased as Pb\(^{2+}\) was added beyond the 1:1 equivalence point (Fig. 3B). In addition, biphasic transport curves were not seen when Quin-2 was present in excess of total Pb\(^{2+}\) (data not shown), and titration data were less multiphasic when the vesicles had been lysed to dilute the internal concentration of Quin-2 that originally existed (Fig. 3B).

We interpret these data to indicate that more than one complex is formed between Pb\(^{2+}\) and the indicator under conditions that pertain in the lumen of intact vesicles. There was no indication of multiple complexes when this system was employed to investigate ionophore-catalyzed transport of Ca\(^{2+}\) and other divalent cations, such that this factor was not considered in the previous studies that established procedures for utilizing the vesicle system to investigate ionophore-mediated transport (11–15, 17, 18). Here we have avoided possible errors in the reported rate and selectivity values by deriving these parameters from the initial portion of the progress curves, where internal Quin-2 was present in excess of internal Pb\(^{2+}\).

There was no disagreement between the internal and external calibration methods within this region (Fig. 3B), indicating that the same complex is considered in both cases.
Selectivity and Mechanism of Transport—Ionomycin transported $\text{Pb}^{2+}$ more efficiently than other divalent cations as shown in Fig. 4A. This is in contrast to A23187 (Fig. 4B) and 4-BrA23187 (data not shown), where rates of $\text{Pb}^{2+}$ transport were intermediate in comparison with the others. Selectivity for $\text{Pb}^{2+}$ compared with $\text{Ca}^{2+}$ is of particular interest because $\text{Pb}^{2+}$ enters cells via channels that normally transport $\text{Ca}^{2+}$ (5, 6, 29) and because competition between these cations at intracellular sites is important in mechanisms of $\text{Pb}^{2+}$ toxicity (30, 31). Using Equation 2 and the data in Fig. 4, it can be shown that $S_{\text{Pb}}$ is 98 for ionomycin and only 2.3 for A23187 under the conditions of this figure.

The rate of ionomycin-catalyzed $\text{Pb}^{2+}$ transport is a function of the ionophore (Fig. 5) and the $\text{Pb}^{2+}$ (Fig. 6) concentrations, as expected for a mechanism where a discrete complex between the cation and the ionophore underlies activity. When the ionophore concentration was varied, a plot of log initial rate versus log of the ionophore concentration was well represented by a straight line of slope 1.0, as was also true for $\text{Ca}^{2+}$ transport (Fig. 5) (11). Varying the free $\text{Pb}^{2+}$ concentration produced a log versus log plot that also had a slope near 1 within the lower portion of the range examined, but a progressively smaller slope as the free $\text{Pb}^{2+}$ concentration rose (Fig. 6). This nonlinear behavior was less apparent with $\text{Ca}^{2+}$ transport (Fig. 6), which resulted in lower values of $S_{\text{Pb}}$, being obtained from Equation 2 as higher concentrations of the two cations were compared. To avoid this circumstance and to obtain a more representative value of $S_{\text{Pb}}$, we calculated a value based upon the $x$ axis separation of the plots within regions where (near) linear behaviors were observed. Within these regions, both sets of data were separated by 2.65 log units, which translates into an $S_{\text{Pb}}$ value of $-450$.

Competitive Transport of $\text{Pb}^{2+}$ Versus $\text{Ca}^{2+}$ in Vesicles—To determine if ionomycin retains high transport selectivity for $\text{Pb}^{2+}$ over $\text{Ca}^{2+}$ when both cations are present, we utilized vesicles loaded with BAPTA plus a small amount of Indo-1, rather than Quin-2. Quin-2-loaded vesicles are not suitable for investigating simultaneous transport because there is no spectral distinction between the primary $\text{Pb}^{2+}$ and $\text{Ca}^{2+}$ complexes that are formed with this indicator. The same is true for the corresponding BAPTA complexes; however, the $\text{Pb}^{2+}$ and $\text{Ca}^{2+}$ complexes of Indo-1 can be distinguished by their fluorescence properties as reported by Kerper and Hinkle (5, 6).

Fig. 7 illustrates, in part, how the differences they described can be exploited to allow initial rates to be obtained for the two cations individually when both are present. Titration of vesicles containing BAPTA plus Indo-1 with $\text{Pb}^{2+}$ in the presence of high ionomycin allowed the external cation to equilibrate with
the internal chelators, as shown earlier for Quin-2-loaded vesicles titrated with Ca$^{2+}$ (11). As such a titration proceeded, Pb$^{2+}$ association with Indo-1 quenched its fluorescence emission at 452 nm (Em$_{452}$), while having a much smaller effect on Em$_{400}$ (Fig. 7A). The former wavelength is an isosbestic point between the emission spectra of free Indo-1 and its Ca$^{2+}$ complex (5, 6), whereas the latter is a maximum in the spectrum of the Ca$^{2+}$ complex (7) and is little affected upon the binding of Pb$^{2+}$ (6). Accordingly, the entry of Pb$^{2+}$ during a continuous transport experiment should progressively decrease Em$_{452}$, without affecting Em$_{400}$, and this was observed (Fig. 7B). Likewise, titrating the vesicles with Ca$^{2+}$ increased Em$_{400}$ and had a much smaller effect on Em$_{452}$ (Fig. 7C), whereas the same features were again apparent during entry of Ca$^{2+}$ by continuous transport (Fig. 7D).

Thus, the accumulation of Pb$^{2+}$ or Ca$^{2+}$ gives rise to distinct fluorescence signals from the entrapped Indo-1. However, as the accumulation of either cation proceeds, most of the internal quantity is associated with BAPTA, whereas Indo-1 equilibrates with the fraction that is free. To relate the wavelength-specific fluorescence changes to rates of transport, the relationships between fractional Indo-1 and BAPTA saturation must be taken into account. The stability constants of the BAPTA and Indo-1 complexes with Ca$^{2+}$ are similar at $6.03 \times 10^6$ and $4.36 \times 10^6$, respectively (7, 21). In the case of Pb$^{2+}$, the corresponding values are $1.99 \times 10^{11}$ (21) and $2.88 \times 10^{10}$ (5). Given the inherent uncertainties, the Ca$^{2+}$ values could probably be considered equivalent; however, this is not true in the case of Pb$^{2+}$, which is bound 6.9-fold more tightly by BAPTA than by Indo-1. Ignoring this discrepancy would cause the Pb$^{2+}$ transport rate to be underestimated significantly.

To correct for the discrepancy, we used the maximum and minimum Em$_{452}$ values observed when no Pb$^{2+}$ and saturating Pb$^{2+}$ were present, respectively, to calculate the Indo-1/Pb$^{2+}$ complex ratio at each point in the progress curves. These values were converted to free Pb$^{2+}$ concentrations using the stability constant of the Indo-1-Pb$^{2+}$ complex, and thereafter, the BAPTA/BAPTA-Pb$^{2+}$ ratio could be calculated using the stability constant for the complex formed between that ligand and Pb$^{2+}$. From the resulting values and the amount of BAPTA entrapped, Pb$^{2+}$ accumulated as a function of time could be calculated (free Pb$^{2+}$ and Pb$^{2+}$ associated with Indo-1 can be ignored because they are small compared with the

![Fig. 5. Effect of ionophore concentration on the initial rate of Pb$^{2+}$ transport. A, experiments were conducted as described under “Experimental Procedures” and in the legend to Fig. 4, except that free Pb$^{2+}$ was buffered at 1.0 $\mu$M using citrate (see legend to Fig. 2), and the ionomycin concentration was varied as shown to the right of the individual traces. B, shown is the log of the initial Pb$^{2+}$ (●) or Ca$^{2+}$ (○) transport rate as a function of log ionomycin concentration. The data for Ca$^{2+}$ transport were determined from experiments like those shown in A, wherein 1.0 $\mu$M free Ca$^{2+}$ replaced Pb$^{2+}$.](image)

![Fig. 6. Effect of Pb$^{2+}$ concentration on the initial rate of Pb$^{2+}$ transport. A, experiments are analogous to those shown in Fig. 5, except that the ionomycin concentration was held constant at 0.10 $\mu$M, and the free Pb$^{2+}$ concentration was varied as shown to the right of the individual traces. B, shown is the log of the initial Pb$^{2+}$ (●) or Ca$^{2+}$ (○) transport rate as a function of log concentration for that cation. The data for Ca$^{2+}$ transport were obtained from experiments like those shown in A, wherein the free Ca$^{2+}$ concentration was varied instead of free Pb$^{2+}$.](image)
Fig. 7. Indo-1 fluorescence distinguishes between Pb\(^{2+}\) and Ca\(^{2+}\) transport. A, POPC vesicles loaded with BAPTA plus Indo-1 were incubated in a medium containing 50 mM Ca\(_{2+}\) plus 10 mM each HEPES and MES. The nominal concentrations of POPC and BAPTA were 1.0 mM and 20 \(\mu\)M, respectively, and Indo-1 was present at <1 \(\mu\)M. The external pH was adjusted to 7.00 using purified Ca\(_{2+}\). Valinomycin (0.5 \(\mu\)M) plus carbonyl cyanide m-chlorophenylhydrazine (5 \(\mu\)M) were present to maintain the internal pH at the external value, and 1.0 \(\mu\)M ionomycin was present to rapidly equilibrate external Pb\(^{2+}\) with the internal chelators. C, change in Indo-1 fluorescence at 400 and 452 nm, respectively (excitation at 336 nm), during titration of the system with Pb(NO\(_3\))\(_2\). B, same as A, except that the vesicles were initially titrated with Ca\(^{2+}\) rather than Pb\(^{2+}\), until the total Ca\(^{2+}\) added reached 41 \(\mu\)M. Thereafter, the titration was continued by adding Pb\(^{2+}\). Above 41 \(\mu\)M, the x axis units refer to the sum of Ca\(^{2+}\) and Pb\(^{2+}\) that had been added. D, same as B, except that 3.00 mM Ca(NO\(_3\))\(_2\) replaced Pb(NO\(_3\))\(_2\) to establish a free Ca\(^{2+}\) concentration of 89 \(\mu\)M.

BAPTA-associated Pb\(^{2+}\)\). An analogous procedure was applied to the Ca\(^{2+}\)-accumulation data obtained as Em\(_{400}\).

Fig. 8 shows the results of these procedures when applied to determine \(S_{\text{Pb}^{2+}}\). When the medium free Ca\(^{2+}\)/free Pb\(^{2+}\) ratio was <900, the initial rate of Pb\(^{2+}\) transport was 3.5-fold greater than the rate of Ca\(^{2+}\) transport. Thus, under conditions of simultaneous transport, \(S_{\text{Pb}^{2+}}\) is ~3000 in comparison with Ca\(^{2+}\). The release of Ca\(^{2+}\) that was seen as Pb\(^{2+}\) accumulation proceeded represents the displacement of Ca\(^{2+}\) from Indo-1 (and BAPTA) by the more tightly bound Pb\(^{2+}\) (Fig. 8). This was also seen when vesicles were first titrated with Ca\(^{2+}\) and then with Pb\(^{2+}\) (Fig. 7C).

Fig. 8 (inset) shows the dependence of Pb\(^{2+}\) and Ca\(^{2+}\) transport on Pb\(^{2+}\) concentration when the external free Ca\(^{2+}\) concentration is 1 mM. Approximately 1 \(\mu\)M free Pb\(^{2+}\) decreased the Ca\(^{2+}\) transport rate by 50\%, whereas the rate for Pb\(^{2+}\) transport approached a maximum as the free Pb\(^{2+}\) concentration approached 10 \(\mu\)M. The methods we employed to correct the data for differing stabilities of the Indo-1 and BAPTA complexes with Pb\(^{2+}\) and Ca\(^{2+}\) do not account for all factors of possible interest; however, it is clear that a very high selectivity for Pb\(^{2+}\) over Ca\(^{2+}\) is manifested by ionomycin when both cations are present.

Pb\(^{2+}\) Transport in Cells—Ionomycin-catalyzed Pb\(^{2+}\) transport into A20 B lymphoma cells is demonstrated in Fig. 9. Fig. 9A was obtained using cells that were maintained in growth medium during loading with Indo-1. \(N,N,N',N'-\text{Tetrakis}(2\text{-pyridylmethyl})\text{ethylenediamine (TPEN)}, the membrane-permeant chelator of heavy metal cations (32), increased Em\(_{452}\) when added to these cells, indicating the presence of contaminants (Fig. 9A). This was not seen when the cells were in the defined medium during loading (Fig. 9B and data not shown) so that condition was adopted. The addition of the impermeant chelator DTPA (Fig. 9B) or 1 \(\mu\)M free Pb\(^{2+}\) (Fig. 9C) was also without an immediate effect on Em\(_{452}\), demonstrating the absence of Indo-1 and its Ca\(^{2+}\) complex in the external volume. Thus, these cells, when loaded with Indo-1, are an appropriate system for the investigation of Pb\(^{2+}\) transport.

As with other cell types, Pb\(^{2+}\) entered A20 lymphoma cells slowly when it was present at a free concentration of 1 \(\mu\)M (Fig. 9C). The rate was markedly accelerated by 0.5 \(\mu\)M ionomycin (Fig. 9D), demonstrating that the compound is active as a Pb\(^{2+}\) ionophore in this biological system. As with vesicles, the rate of transport is a function of the Pb\(^{2+}\) and ionomycin concentrations over a broad range of either parameter. This is shown by Fig. 9E, which summarizes concentration dependence data covering 2 orders of magnitude in both cases.

Fig. 10 demonstrates that ionomycin-mediated Pb\(^{2+}\) transport in cells is reversible. To obtain these data, the Pb\(^{2+}\)/citrate buffer system was replaced by 20 \(\mu\)M Pb(NO\(_3\))\(_2\) to yield a free
Pb²⁺ concentration in that range. As with other cells, Pb²⁺ rapidly entered A20 B lymphoma cells through endogenous activities when present at this higher concentration (Fig. 10, data obtained as Em₄₀₀). The entry was accelerated by ionomycin addition and reversed by the subsequent addition of excess EDTA. Like DTPA, EDTA is membrane-impermeant and binds Pb²⁺ and Ca²⁺ with high affinity. Thus, the recovery of Em₄₀₀ following the addition of EDTA indicates that internal Pb²⁺ was released from Indo-1, transported out of the cells, and chelated in the external volume. Also shown are the parallel changes in Em₄₀₀, reflecting, in part, the accompanying changes in free Ca²⁺. Em₄₀₀ decreased as Pb²⁺ entered the cells initially, consistent with a displacement of endogenous Ca²⁺ from Indo-1 by the more tightly bound Pb²⁺. Conversely, Em₄₀₀ rose to a high level upon EDTA addition and Pb²⁺ depletion, indicating that the cytoplasmic Ca²⁺ concentration is increased progressively as Pb²⁺ is removed. This rising Ca²⁺ level presumably reflects an ionomycin-mediated entry of Ca²⁺ from the external volume and possibly a release of Ca²⁺ from internal stores into the cytoplasm.

**DISCUSSION**

Early studies utilizing solvent extraction systems, sarcoplasmic reticulum vesicles, and mitochondrial preparations established that ionomycin is a Ca²⁺ ionophore (33–35), and it has long been utilized in that regard. More recently, it has become clear that this compound binds and transports a wide range of cations, including alkaline earth and first transition series divalent cations and lanthanide series trivalent cations (14, 15, 36). Among all the cations considered to date, ionomycin is the most active by far as an ionophore for Pb²⁺. Accordingly, it seems appropriate to describe this compound as a Pb²⁺ ionophore that has a limited activity for the transport of other polyvalent cations such as Ca²⁺. Pb²⁺ transport by ionomycin is a first-order function of ionophore concentration, as indicated by the linear character and slope value of 1.0 that are seen in plots of log initial rate versus log ionomycin concentration (Fig. 5). The same is true with respect to Pb²⁺ concentration within the lower portion of the range examined (Fig. 6). Where these relationships hold, the rate of Pb²⁺ transport can be described by Equation 3, in which $k_{trans}$ is a second-order rate constant that is valid at the pH and temperature employed.

\[
Rate = k_{trans}[\text{ionomycin}][\text{Pb}^{2+}]
\]  
(Eq. 3)

The versatility of the vesicle transport system with respect to
cations that can be considered and the range of conditions that are accessible makes it possible to express the efficiency and selectivity of ionophore-catalyzed transport in terms of rate constants. These values are usually not accessible when traditional methods are employed to characterize ionophores. From the present data, $k_{\text{trans}}$ values for Pb$^{2+}$ and Ca$^{2+}$ transport are $1.2 \times 10^6$ and $2.7 \times 10^5$ M$^{-1}$ s$^{-1}$, respectively.

Within the concentration regions where Equation 3 is applicable, the stoichiometry of the transporting species must be 1:1, ionophore to cation, based upon the first-order kinetic characteristics (slope values of 1.0 in the log versus log plots). Ionomycin is a monocarboxylic acid that contains an enolized $\beta$-diketone moiety (37) and can therefore ionize twice to form 1:1 charge neutral complexes with divalent cations (36). Thus, it is possible that Pb$^{2+}$ is transported as the neutral species IPb, formed according to Equation 4, wherein $\text{H}_2\text{I}$ is the fully protonated form.

$$\text{H}_2\text{I} + \text{Pb}^{2+} \rightleftharpoons \text{IPb} + 2\text{H}^+ \quad \text{(Eq. 4)}$$

However, monoprotonated complexes between ionomycin and several divalent cations have been identified (36), and charge neutral complexes of 1:1 stoichiometry are formed between ionomycin and trivalent lanthanide cations, apparently by the inclusion of an OH$^-$ (15). Since Pb$^{2+}$ and Pb$^{2+}$ complexes are prone to hydrolyze (38–40), it is then possible that the weakly acidic enolized $\beta$-diketone moiety remains protonated in the transporting species and that this species contains OH$^-$. Thus, a mixed complex of stoichiometry H$^+$IPb-OH might also be responsible for transporting Pb$^{2+}$, wholly or in part. An unambiguous identification of the transporting species must await determination of the mode of transport (electroneutral, electrogenic, or mixed) and investigation of the complexation equilibria between this ionophore and Pb$^{2+}$.

Explanations for the progressive curvature in plots of log rate versus log Pb$^{2+}$ concentration that is evident at higher values of free Pb$^{2+}$ (Fig. 6) and the increase in $S_{\text{ip}}$ compared with Ca$^{2+}$ that is seen when both cations are present (Fig. 8) also await investigation of the complexation equilibria between Pb$^{2+}$ and ionomycin. Regarding the former, we suspect that curvature reflects an approach to saturation of the ionophore with Pb$^{2+}$ as the free concentration of the cation rises. However, it is also possible that it reflects the reaction of a 1:1 transporting species with a second Pb$^{2+}$ ion to form species such as IPb$_2$ (Equation 5, charge and hydroxylation status not specified) that are unable to cross membranes.

$$\text{IPb} + \text{Pb}^{2+} \rightleftharpoons \text{IPb}_2 \quad \text{(Eq. 5)}$$

If the higher order species were less stable than the transporting species, the result would be less ionophore contributing to transport as the free Pb$^{2+}$ concentration increased, producing the type of curvature that is observed (Fig. 6).

Regarding the high value of $S_{\text{ip}}$ that is seen when Pb$^{2+}$ and Ca$^{2+}$ are present, it seems possible that both complexation equilibria and differing transmembrane diffusion constants contribute to the explanation. For example, if Pb$^{2+}$ were bound more tightly than Ca$^{2+}$, but formed a transporting complex that crossed the membrane relatively slowly compared with the complex transporting Ca$^{2+}$, the result could be an increase in the apparent selectivity when both cations were present, which is what we observed (Fig. 8).

Legare et al. (29) reported that ionomycin transports Pb$^{2+}$ into primary cultures of rat astroglia cells, and our results with A20 B lymphoma cells extend their findings. We cannot compare the activity values in vesicles and cells quantitatively because the relationship between free and total Pb$^{2+}$ in the cells is unknown. In addition, the total aqueous/lipid phase volume ratios are not similar at the vesicle and cell concentrations we employed. Accordingly, and for other reasons, the fraction of ionophore that is partitioned to the lipid phase and engaged in transport is probably different in the two systems (much larger in the case of vesicles). Nevertheless, it is clear that ionomycin retains a high activity as a Pb$^{2+}$ ionophore in cells. This is seen qualitatively in Fig. 9D, where 0.5 $\mu$m ionomycin efficiently loaded the cells with Pb$^{2+}$ when the external free Pb$^{2+}$ concentration was 1 $\mu$M. The former value is typical of ionomycin concentrations used to load cells with Ca$^{2+}$, whereas the cation concentration employed in that case is normally much higher (millimolar). The Pb$^{2+}$ transport activity of ionomycin will, presumably, be a useful addition to the approaches used for investigating the toxic activities of Pb$^{2+}$ at a cellular level.

A final point relates to the reversibility of ionomycin-mediated Pb$^{2+}$ transport that is demonstrated in Fig. 10. It is interesting to note that the ionophore-mediated release of Pb$^{2+}$ appears to be more rapid than the initial accumulation. This same difference was seen earlier for Ca$^{2+}$ transport using the vesicle system, in which Ca$^{2+}$ release occurs 20 times faster than uptake (11). The differing rates are thought to reflect the large difference in volume of the aqueous phase compartments accessible from alternate sides of the membrane and the effect of this on ionophore levels at the two interfaces where transporting species are formed (11). It seems possible that efficient release of Pb$^{2+}$ from cells when ionomycin is present will extend to higher levels of biological organization and reach a practical application as follows. Lead intoxication is treated by the administration of chelating agents that complex Pb$^{2+}$, but formed a transporting complex that crossed the membrane relatively slowly compared with the complex transporting Ca$^{2+}$, the result could be an increase in the apparent selectivity when both cations were present, which is what we observed (Fig. 8).

There are reports that multiple hydrophilic chelators administered simultaneously remove accumulated lead more effectively than single compounds (43) and that a degree of hydrophobicity improves single compound effectiveness (44–46). Low levels of a lead ionophore administered together with a hydrophilic chelator might capture the multiple chelator advantage and the hydrophobicity advantage simultaneously. The result could be an improved treatment for lead intoxication, both in terms of the completeness of removal and a shortening of the treatment period required.

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