Ionophore 4-BrA23187 Transports Zn^{2+} and Mn^{2+} with High Selectivity Over Ca^{2+} †

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ABSTRACT: The cation transport selectivities of the Ca^{2+} ionophores A23187, Ionomycin, and 4-BrA23187 have been determined using a model system comprised of phospholipid vesicles loaded with the chelator/indicator Quin-2. At pH 7.00 and a 100 μM concentration of the cations, A23187 displays the transport selectivity sequence $Zn^{2+} > Mn^{2+} > Ca^{2+} > Co^{2+} > Ni^{2+} > Sr^{2+}$, with the absolute rates of transport spanning ∼3 orders of magnitude. Similar data are obtained with Ionomycin, although the relative transport rates of Zn^{2+} and Mn^{2+} are equivalent, and the range of absolute rates is decreased by a factor of ∼3. When values are normalized to those of Ca^{2+}, transport selectivity is seen to be only weakly related to complexation or extraction selectivity. It is also seen that, when used to manipulate Ca^{2+} (or Mg^{2+}), both ionophores can be expected to alter the distribution of additional divalent cations which have known biological activities. 4-BrA23187 is a low-activity ionophore for Ca^{2+}, compared to A23187 and Ionomycin, while retaining comparable activities as an ionophore for the other cations. As a consequence, 4-BrA23187 is highly selective for the transport of Zn^{2+} and Mn^{2+}, compared to Ca^{2+}, with selectivity ratios approaching that of valinomycin for K^+ over Na^+ when conditions are optimal. Plots of the log of the rate of cation transport *vs* the log of the ionophore concentration indicate that Ca^{2+} is transported primarily as a 2:1 complex by A23187 and 4-BrA23187, but Zn^{2+} and Mn^{2+} are transported, in part, as 1:1 complexes. These findings, together with a postulated low stability of 2:1, compared to 1:1 complexes between 4-BrA23187 and divalent cations, partially explain the novel transport selectivity of this compound. Unlike A23187 or Ionomycin, 4-BrA23187 may be useful for investigating cell regulation by Zn^{2+} and Mn^{2+}, without interference by regulatory mechanisms which respond to Ca^{2+}.

The carboxylic acid ionophores A23187, 4-BrA23187, and Ionomycin have been invaluable tools for investigators interested in the role of Ca^{2+} as an intracellular mediator [see Woolley *et al.* (1995)]. In spite of the popularity of these compounds, their transport selectivities have not been widely studied. Much of the available data relate to solution chemical properties [see Erdahl *et al.* (1994)] or capacities to extract cations from a bulk aqueous to a bulk organic phase (Painter & Pressman, 1982; Taylor *et al.*, 1982). In the case of A23187 and Ionomycin, extraction data show that several divalent cations found in biological systems form charge neutral complexes, some of which are much more stable than the corresponding Ca^{2+} complex (Pfeiffer & Lardy, 1976; Young & Gomperts, 1977; Liu & Herman, 1978; Mimouni *et al.*, 1992). Solution chemical studies indicate that a wide variety of mono-, di-, and trivalent cations form complexes of 2:1 and/or 1:1 stoichiometry (ionophore to cation) (Tissier *et al.*, 1979, 1985; Bolte *et al.*, 1982; Taylor *et al.*, 1985; Chapman *et al.*, 1987; Divakar & Easwaran, 1987). In some cases, the complexes are protonated or contain hydroxide anion, such that species which bear a net charge of 0, +1, or +2 are known (Chapman *et al.*, 1987, 1990a; Stiles *et al.*, 1991). The complex stability constants span ∼10 orders of magnitude (Chapman *et al.*, 1990a; Tissier *et al.*, 1993). Among the alkaline earth and first transition series divalent cations, these values conform to the extended Irving−Williams sequence (Chapman *et al.*, 1987, 1990a; Stiles *et al.*, 1991; Tissier *et al.*, 1993; Taylor *et al.*, 1993) which is typically seen with flexible chelating ligands which can adopt the preferred donor atom geometry of the cation (Irving & Williams, 1953). These properties led us to propose that A23187 and Ionomycin should be expected to transport a variety of cations found in biological systems by mechanisms which are electroneutral, in some cases electrogenic, or mechanisms which involve the cotransport of anions (Chapman *et al.*, 1990a). There is relatively little extraction or solution chemical data available for 4-BrA23187 (Debono *et al.*, 1981; Deber *et al.*, 1985), and it is generally assumed that the properties of this compound are similar to those of A23187.

The transport selectivity of an ionophore reflects factors in addition to cation complex stabilities, including the kinetic properties of complexation/decomplexation reactions, the influence of ionophore−membrane interactions on these reactions, the transmembrane diffusion constants of the transporting species, and others. Thus, it is not clear to what extent the existing solution chemical and solvent extraction data can be applied to interpret the actions of Ca^{2+} iono-

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phores in biological systems. Model membrane systems which allow the quantitation of transport rates across a phospholipid bilayer have the potential to clarify questions in this area; however, the data available are limited. In a pioneering study, Pohl *et al.* (1980) used phosphatidylcholine vesicles prepared by sonication to show that A23187 transports cations into vesicles at rates which show the following rank order: $Zn^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+} \approx Li^{+} > Na^{+}$. A somewhat different order was observed for transport in the opposite direction. Limitations arising from the vesicle technology available at the time that study was conducted precluded detailed analysis of the origins of these properties. Yaguzhinsky and co-workers have described the application of planar bilayer methods coupled with electrical techniques to the investigation of the transport selectivities of carboxylic acid ionophores (Antonenko & Yaguzhinsky, 1983, 1990; Kovbasnjuk *et al.*, 1991) but have, in the case of Ca^{2+} ionophores, applied these methods only to A23187 and Lasalocid A transporting Ca^{2+} or Mg^{2+} (Antonenko & Yaguzhinsky, 1983, 1988; Pohl *et al.*, 1990).

The prospects for using model membrane systems to interrelate the transport and solution chemical properties of Ca^{2+} ionophores improved upon the introduction of extrusion methods for vesicle preparation (Hope *et al.*, 1985; Mayer *et al.*, 1986) and methods for accurately determining the concentration of solutes trapped within the preparations (Chapman *et al.*, 1990b, 1991). We have recently utilized these methods in detailed studies of the mechanisms by which A23187, 4-BrA23187, and Ionomycin transport Ca^{2+} (Erdahl *et al.*, 1994, 1995). Here we describe the use of the same system in investigating the transport selectivity of these ionophores. The most surprising aspects of the results are indications that A23187 and 4-BrA23187 transport Zn^{2+} and Mn^{2+} as both 1:1 and 2:1 complexes across a range of conditions. As a consequence of this, in part, and because Ca^{2+} is transported primarily as the 2:1 complex (ionophore−cation), 4-BrA23187 is highly selective for the transport of Zn^{2+} and Mn^{2+} over Ca^{2+}. When conditions are optimal, the selectivities approach that of valinomycin (VAL)[1] for K^{+} over Na^{+}. Thus, 4-BrA23187 may be useful for perturbing the levels of Zn^{2+} and Mn^{2+} in cells without significantly perturbing the levels of Ca^{2+}. Aspects of these findings have been reported in abstract form (Erdahl *et al.*, 1996).

EXPERIMENTAL PROCEDURES

Reagents. Synthetic 1-palmitoyl-2-oleoyl-*sn*-glycerophosphatidylcholine (POPC) was obtained from Avanti Polar Lipids, Inc. Purity was confirmed by thin-layer chromatography before use. A23187, 4-BrA23187, and Ionomycin were obtained from Sigma or Calbiochem and were used without further purification. Stock solutions in ethanol were standardized spectrophotometrically using the following extinction coefficients: 4-BrA23187 ($\epsilon_{290} = 15{,}600$), A23187 ($\epsilon_{278} = 21\,040$), and Ionomycin ($\epsilon_{278} = 13\,560$). Quin-2 ($K^{+}$ salt) from Sigma was purified by passage over Chelex 100 resin (100−200 mesh) in the Cs^{+} form as described previously (Erdahl *et al.*, 1994). The chloride salts of divalent cations were the ultrapure grade from Alfa Products. Stock solutions of these were standardized by titration with a primary standard EDTA solution (Vogel, 1961).

Preparation of Phospholipid Vesicles. Freeze−thaw extruded POPC vesicles were prepared as described previously (Chapman *et al.*, 1990b, 1991). Briefly, 250 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 5 mM purified Quin-2 (Cs^{+}) and 10.0 mM Hepes buffer adjusted to pH 7.00 with Chelex-treated CsOH (Erdahl *et al.*, 1994). 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 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 (Fry *et al.*, 1978) 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 at pH 7.00. A single pass over such columns effectively removes the external Quin-2 (Chapman *et al.*, 1990b, 1991; Erdahl *et al.*, 1994).

The nominal concentration of POPC in the final preparations was determined by measurement of lipid phosphorus (Bartlett, 1959) and was near 80 mM. The average diameter of these vesicles is 71 nm as determined by freeze−fracture electron microscopy (Chapman *et al.*, 1990b), and they contain entrapped solutes at the following concentrations: Quin-2, 10.5 ± 0.8 mM; Hepes, 33.7 ± 7.6 mM (pH ≈7.4); and Cs^{+}, 60 ± 5 mM. Specific values for Quin-2 and Cs^{+} were determined for each preparation by the methods described previously (Erdahl *et al.*, 1994, 1995). Briefly, entrapped Quin-2 is determined by spectrophotometric titration with standard $CaCl_2$ following dispersion of the vesicles in deoxycholate. Entrapped Cs^{+} is 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. 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 which operates during preparation of the vesicles (Chapman *et al.*, 1990b, 1991).

Determination of Cation Transport. The transport of divalent cations into Quin-2-loaded vesicles was determined by monitoring formation of the Quin-2−cation complexes spectroscopically. Vesicles containing Quin-2 were present at a nominal POPC concentration of 1.0 mM in a medium which also contained 50 mM CsCl, 10 mM Hepes, and 10 mM Mes. The medium pH ranged from 6.0 to 8.0 and was adjusted with CsOH which had been passed over Chelex 100

[1] Abbreviations: BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; CCP, carbonyl cyanide *m*-chlorophenylhydrazone; 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; VAL, valinomycin.

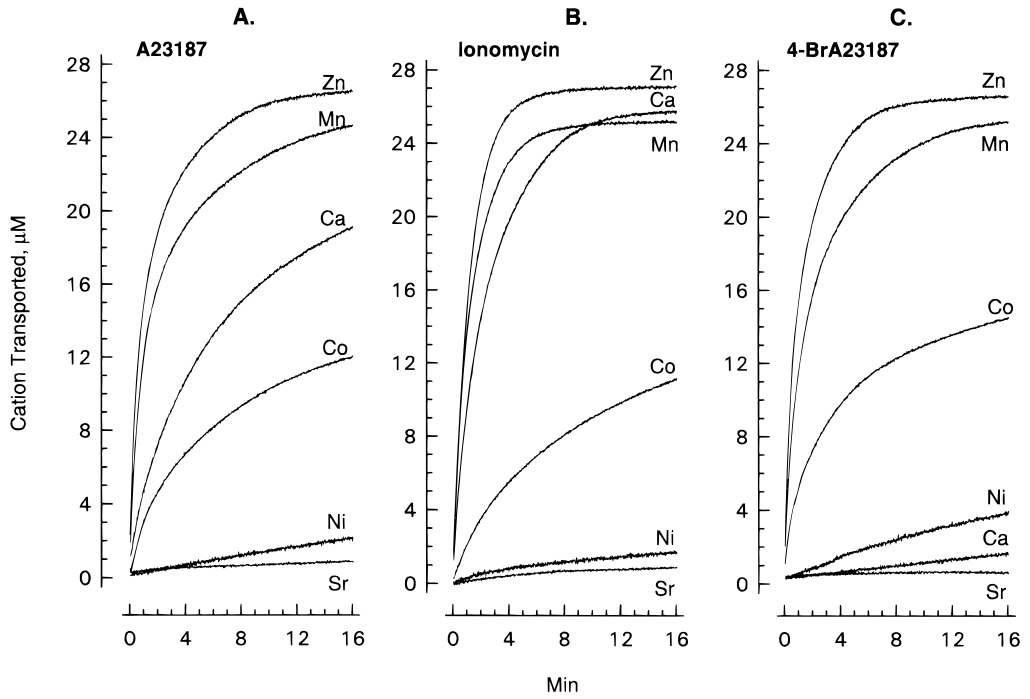

FIGURE 1: Transport of selected divalent cations by Ca^{2+} ionophores. POPC vesicles containing Quin-2 were incubated in a medium containing 50 mM CsCl plus 10 mM Hepes and 10 mM Mes. The nominal concentrations of POPC and Quin-2 were 1.0 mM and 27.5 μM, respectively. The external pH was adjusted to 7.00 using highly purified CsOH, and VAL (0.5 μM) plus CCP (5 μM) were present to maintain the internal pH at the external value. Divalent cation chlorides were present at 100 μM in all cases. Instrumentation and other conditions were as described in Experimental Procedures. Transport was initiated by the addition of 0.10 μM A23187 (A), 0.30 μM Ionomycin (B), or 0.10 μM 4-BrA23187 (C), respectively.

columns to remove contaminating divalent cations (Erdahl *et al.*, 1994). To maintain internal pH at the external value, VAL (0.5 μM) and carbonyl cyanide *m*-chlorophenylhydrazone (CCP) (5 μM) were also present (Erdahl *et al.*, 1995). Specific concentrations of ionophores, divalent cation chlorides, and pH values are given in the figure legends. Reactions were started by addition of the divalent cation ionophore, following an initial 2−3 min period which was allowed for the equilibration of internal and external pH.

Formation of the Quin-2−cation complexes was monitored by difference absorbance measurements using an Aminco DW2a spectrophotometer operated in the dual wavelength mode. An Oriel 59800 band-pass filter was used between the cuvette and the beam scambler−photomultiplier assembly to prevent detection of the fluorescent light emitted by Quin-2 and A23187. 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. These wavelengths varied slightly from cation to cation, as determined by titrations of Quin-2 in the vesicle suspension medium at pH 7.00. The following values were used: Zn^{2+} (341 nm), Mn^{2+} (338 nm), Ca^{2+} (338 nm), Co^{2+} (343 nm), Sr^{2+} (339 nm), and Ni^{2+} (345 nm). Data were collected on disk through a computer that was interfaced to the spectrophotometer, using Unkel Scope software (Unkel Software, Inc., Lexington, MA).

Data Analysis. Both external and internal methods were used when the transport data were calibrated. For the external method, vesicles containing a known amount of Quin-2 were lysed with 0.33% (w/v) Cs^{+}−deoxycholate and titrated with a standard solution of the cation under the conditions of interest. These data were compared to those obtained by an internal method in which Quin-2 was titrated without lysing the vesicles, by including an excess of an appropriate ionophore in the system. Data obtained by the two methods were coincident except for small deviations that are observed as Quin-2 approaches saturation with some of the cations [see Erdahl *et al.* (1994)]. These are not significant for the present study because the initial rate of transport is the parameter of interest.

To extract the initial rates, an early portion of the progress curves was fit to eq 1 using standard nonlinear least-squares methods.

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

In eq 1, A_T and A_O are the observed and the initial absorbance values, respectively, B is the initial rate, C is a correction factor for nonlinearity, and t is time. Rates are expressed in micromolar per second of external cation transported into the vesicles. Transport selectivities are expressed as S values defined by eq 2.

$$S_M = \frac{\text{initial rate of } M^{n+} \text{ transport}}{\text{initial rate of } Ca^{2+} \text{ transport}} \quad (2)$$

When S is being determined, an equal concentration of the cation in question is substituted for Ca^{2+} with all other conditions held constant. Complexation and extraction selectivities are expressed in an analogous way. All data were obtained at 25.0 °C.

RESULTS

Selectivity Sequences. Figure 1 shows progress curves for the transport of several divalent cations into POPC vesicles by each of the Ca^{2+} ionophores. Considering first A23187 and Ionomycin (Figure 1A,B, respectively), the rank order

Table 1: Transport, Complexation, and Extraction Selectivities of Ca^{2+} Ionophores[a]

ionophore (cation)	*S* values: transport[b]	complexation (1:1)[c]	complexation (2:1)[d]	extraction[e]
A23187				
Zn^{2+}	8.59	195	158	3570
Mn^{2+}	5.00	3.80	32	357
Co^{2+}	0.86	372	794	964
Ca^{2+}	1.00	1.00	1.00	1.00
Ni^{2+}	0.030	1100	1000	168
Sr^{2+}	0.007	0.23	0.01	0.004
ionomycin				
Zn^{2+}	1.87	2880		
Mn^{2+}	1.98	214		
Co^{2+}	0.176	2090		
Ca^{2+}	1.00	1.00		1.00
Ni^{2+}	0.022	9550		
Sr^{2+}	0.005	0.107		0.023
4-BrA23187				
Zn^{2+}	501			
Mn^{2+}	251			
Co^{2+}	93.0			
Ca^{2+}	1.00			
Ni^{2+}	3.76			
Sr^{2+}	1.02			

[a] The values shown are selectivities, normalized to Ca^{2+}, which was assigned a value of 1.00 in all cases. They were calculated using eq 2 (transport) or analogous expressions when complexation or extraction data were considered. [b] Values are from the present work (Figure 1), and refer to the initial rate of transport, determined as described in Experimental Procedures and the legend to Figure 1. [c] Values are from Chapman *et al.* (1990a) (A23187) or from Stiles *et al.* (1991) (Ionomycin) and refer to the formation of 1:1 complexes as defined in those references. [d] Values are from Tissier *et al.* (1993) and refer to 2:1 complexes (ionophore−cation) as defined in that reference. [e] Values are from Pfeiffer and Lardy (1976) (A23187) or Liu and Hermann (1978) (Ionomycin) and are defined by reactions given in those references.

of initial rates is $Zn^{2+} > Mn^{2+} > Ca^{2+} > Co^{2+} > Ni^{2+} > Sr^{2+}$ under the conditions used to obtain the figure. Transport *S* values were calculated from these data and are shown in Table 1, where they are compared to solution complexation and extraction *S* values which were calculated from literature data using expressions analogous to eq 2. Comparing these values shows a general lack of correlation between the various measures of selectivity. In the case of transport, it is seen that selectivity is much lower than complexation selectivity and fails even to follow the same rank order. There is perhaps a closer relationship between extraction and complexation selectivity; however, these correlations are also weak. Thus, there is little indication that traditional expressions of selectivity such as relative complexation and extraction constants can be applied in a straightforward way to predict the transport selectivity of the Ca^{2+} ionophores.

In the case of 4-BrA23187, no extraction or complexation selectivity data are available for comparison. However, the transport properties of this ionophore (Figure 1C) are very similar to those of A23187 and Ionomycin, except in the case of Ca^{2+}. This cation is transported slowly by 4-BrA23187 compared to the other compounds. As a consequence, the transport *S* values of 4-BrA23187 calculated according to eq 2 differ dramatically from those of A23187 and Ionomycin (Table 1). Since transport selectivity shows no simple relationship to complexation or extraction selectivity, parameters of the vesicle transport experiments were varied systematically to help identify the basis of the former parameter. In conducting these studies, we concentrated on the activity of the compounds as ionophores for Zn^{2+}, Mn^{2+}, and Ca^{2+} because the greatest selectivity differences between the ionophores are seen among this group of cations (Table 1).

Effects of pH and Cation Concentration on Transport Selectivity. Figure 2 shows the effect of pH on the absolute initial rates of Zn^{2+}, Mn^{2+}, and Ca^{2+} transport by each of the ionophores. Widely varying behaviors are seen. With A23187 (panel A), Zn^{2+} transport is inhibited, Ca^{2+} transport is enhanced, while Mn^{2+} transport is relatively unaffected as the medium pH is increased from 6 to 8. With Ionomycin (panel B), a rising pH first increases the transport rate of all three cations, but above a pH of ~7.4, the rates of Zn^{2+} and Mn^{2+} transport decline slightly while Ca^{2+} transport continues to increase. 4-BrA23187 is a low-activity ionophore for Ca^{2+} across the entire pH range investigated (panel C) and shows little dependence on pH when the data are viewed on an expanded scale [not shown, see also Erdahl *et al.* (1995)]. With Zn^{2+} and Mn^{2+}, the rates of transport by 4-BrA23187 are relatively insensitive to pH between 6 and 7 and then decline progressively at higher values (panel C).

With regard to transport selectivity, the somewhat complex initial rate data reduce to a relatively simple pattern when they are normalized to the Ca^{2+} data by calculating *S* values as described by eq 2. In this form, it is seen that with 4-BrA23187 S_{Zn} and S_{Mn} reach maximal values of about 700 and 400, respectively, at the optimal pH of ~6.7 (Figure 4A). A23187 is most selective at acidic pH and much less selective than its derivative regardless of the pH considered. Ionomycin is clearly the least selective of the three compounds, showing *S* values less than 10 across the entire range of pH values examined.

At the pH of optimal selectivity (6.7), the absolute rates of Zn^{2+}, Mn^{2+}, and Ca^{2+} transport by the three ionophores are dependent upon the cation concentration in ways which also vary with the cation and the ionophore in question (Figure 3). Again the patterns observed are complex but reduce to relatively straightforward behavior when the data are expressed as *S* values (Figure 4B). In this form, it is seen that S_{Zn} and S_{Mn} for 4-BrA23187 rise progressively to values near 2400 and 1200, respectively, as the absolute divalent cation concentration is lowered to the 10 μM range. The selectivities of A23187 and Ionomycin also increase inversely with the cation concentration but never approach the high values seen with 4-BrA23187 (Figure 4B).

A23187 and 4-BrA23187 Transport Divalent Cations via Two or More Complex Species. In the case of Ca^{2+} and A23187, it is generally thought that the 2:1 complex (ionophore−cation) is the transporting species, exclusively (Erdahl *et al.*, 1995), and that diffusion of this species across the membrane is rate-limiting with regard to the overall process (Kolber & Haynes, 1981). This model predicts that a plot of the log of the rate *vs* the log of the A23187 concentration would display a slope of 2.0, and experimental values near this have been reported (Blau *et al.*, 1984; Erdahl *et al.*, 1994). The relationship between transport rate and ionophore concentration was further investigated during this study, over a range of conditions, as illustrated in Figure 5. As expected, on the basis of the previous reports, plots of the log of the rate of Ca^{2+} transport *vs* the log of the concentration of A23187 or 4-BrA23187 have slopes near 2.0 when the medium pH is 7.00, and the Ca^{2+} concentration

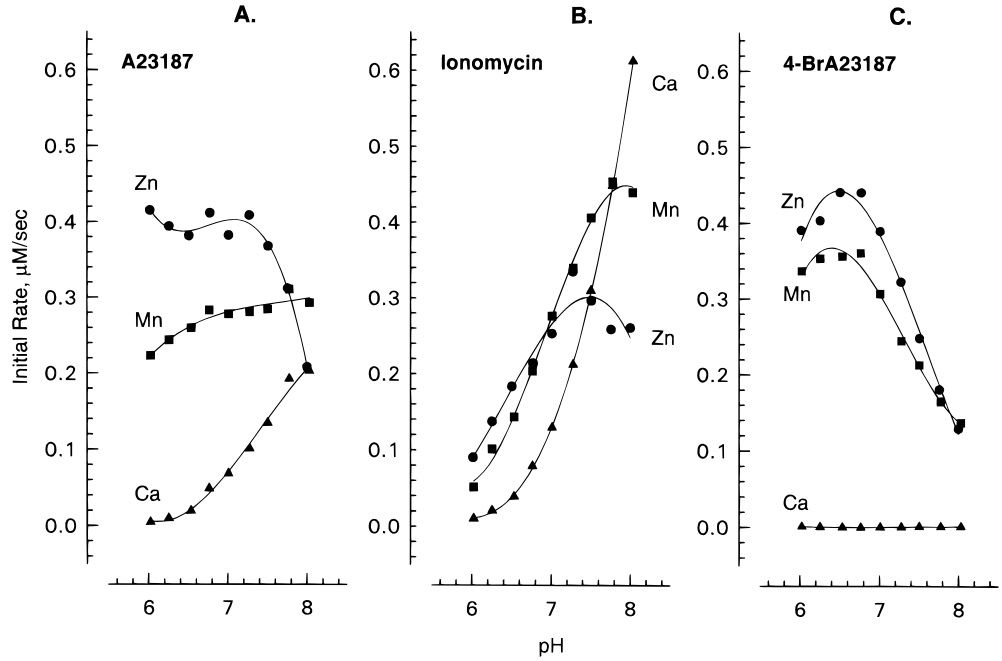

FIGURE 2: Effect of pH on the initial rate of transport. Experiments were conducted as described in Experimental Procedures and the legend to Figure 1, except that the medium pH was adjusted to the values shown. The concentration of divalent cation chlorides was 100 μM. Ionophores were present as follows: (A) A23187 (0.10 μM), (B) Ionomycin (0.30 μM), and (C) 4-BrA23187 (0.10 μM). For all panels: (●) Zn^{2+}, (■) Mn^{2+}, and (▲) Ca^{2+}.

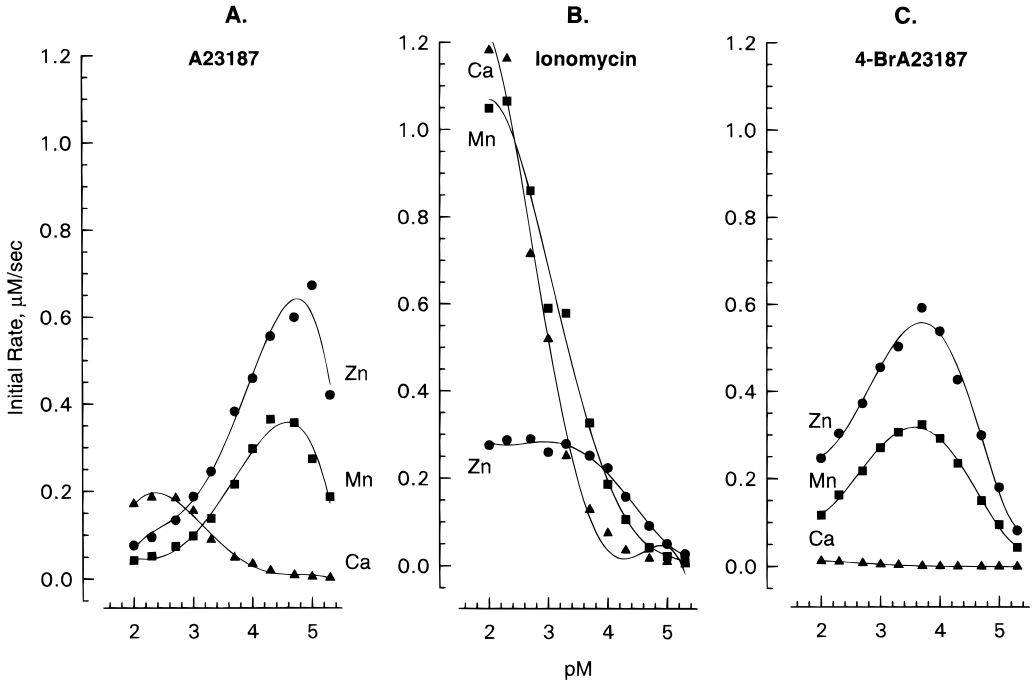

FIGURE 3: Effect of cation concentration on the initial rate of transport. Experiments were conducted as described in Experimental Procedures and the legend to Figure 1 except that the medium pH was 6.7 and the concentration of divalent cation chlorides was varied as shown. Ionophores were present as follows: (A) A23187 (0.10 μM), (B) Ionomycin (0.30 μM), and (C) 4-BrA23187 (0.10 μM). For all panels: (●) Zn^{2+}, (■) Mn^{2+}, and (▲) Ca^{2+}.

is 100 μM (Figure 5). However, under the same conditions, when Zn^{2+} is the transported cation, the slope obtained with either ionophore is ~1.4. This value is well less than 2 and taken alone could indicate either that Zn^{2+} is transported in part by 2:1 and in part by 1:1 complexes or that a 3:2 complex is the predominate species. Since solution equilibrium studies have identified 2:1 and 1:1 complexes between A23187 and Zn^{2+}, but given no evidence of a 3:2 complex with any of the cations studied here [see Chapman *et al.* (1990a) and references therein], the former interpretation is preferred.

A broader set of experiments like those shown in Figure 5 is summarized in Table 2. These data suggest that both Zn^{2+} and Mn^{2+} are transported, in part, through 1:1 complexes with A23187 and 4-BrA23187 and that a smaller fraction of Ca^{2+} transport may also occur through a 1:1 complex, particularly with the latter compound. Thus, attempts to explain the differences in transport selectivity

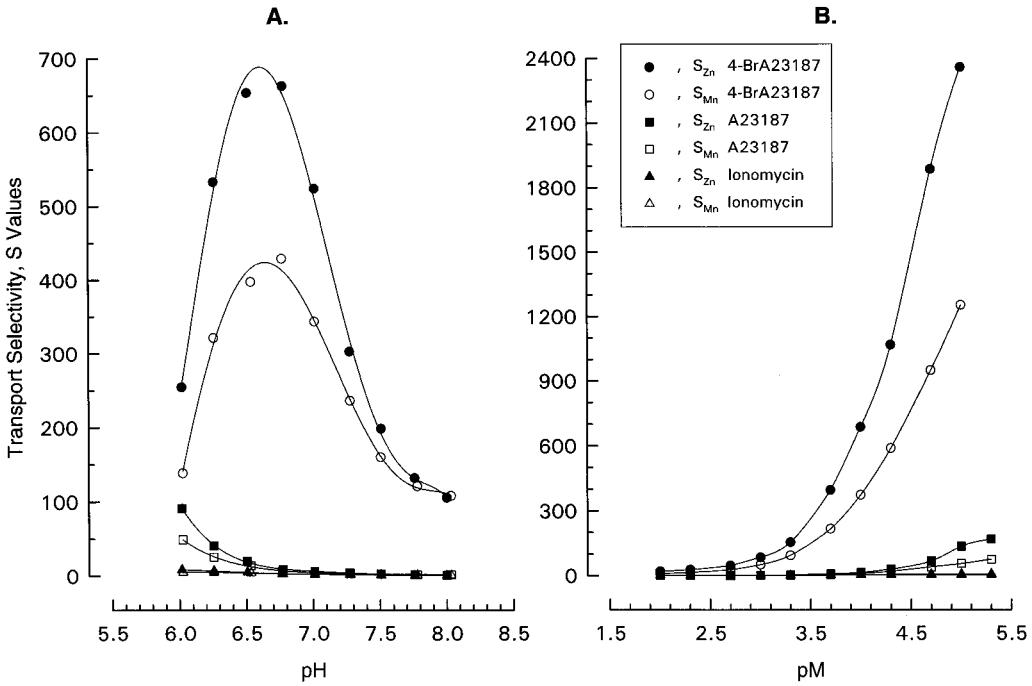

FIGURE 4: Optimum conditions for the selective transport of Zn^{2+} and Mn^{2+} over Ca^{2+}. Panel A shows the variation of S_{Zn} and S_{Mn} as a function of medium pH for the three Ca^{2+} ionophores. The values were calculated according to eq 2, using the initial rate data shown in Figure 2. Panel B shows the variation of S_{Zn} and S_{Mn} as a function of divalent cation concentration. The values were also calculated according to eq 2, using the initial rate data shown in Figure 3. The symbol key inset shown in panel B applies to both panels.

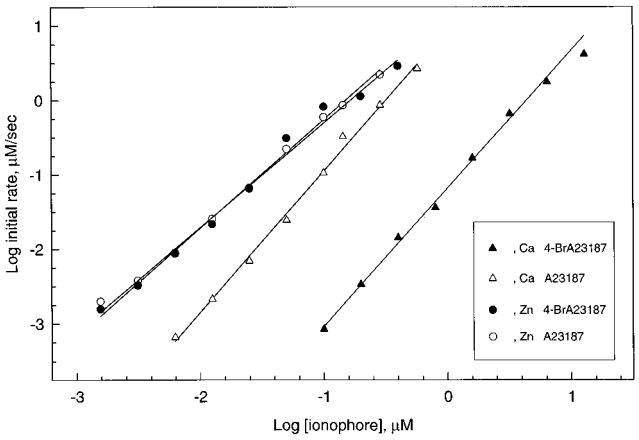

FIGURE 5: Effect of ionophore concentration on the rate of transport. Transport experiments like those illustrated in Figure 1 were conducted using Zn^{2+} or Ca^{2+} and various concentrations of either A23187 or 4-BrA23187. The pH was 7.00; the concentration of the divalent cation was 100 μM, while other conditions were as described in Experimental Procedures and the legend to Figure 1. The data are expressed as the log of the initial rate of cation transport *vs* the log of the ionophore concentration. The specific cation and ionophore referred to by the symbols are as follows: (▲) Ca^{2+} and 4-BrA23187, (△) Ca^{2+} and A23187, (●) Zn^{2+} and 4-BrA23187, (○) Zn^{2+} and A23187.

Table 2: Stoichiometries of Transporting Species[a]

ionophore (cation)	slope, log *v*s log plot		
	pH 6.00	pH 7.00	pH 8.00
A23187			
Zn^{2+}	1.42	1.46	1.31
Mn^{2+}	1.52	1.56	1.54
Ca^{2+}	1.96	1.98	1.80
ionomycin			
Zn^{2+}	0.91	0.97	0.87
Mn^{2+}	0.94	0.95	0.90
Ca^{2+}	0.96	0.95	0.97
4-BrA23187			
Zn^{2+}	1.37	1.40	1.12
Mn^{2+}	1.42	1.46	1.25
Ca^{2+}	1.65	1.85	1.71

[a] Slopes were obtained from experiments like those shown in Figure 5 using the medium pH values specified in the table. The concentration of divalent cations was 100 μM in all cases.

between these ionophores should account for the apparent existence of multiple transporting species. In contrast to A23187 and 4-BrA23187, Ionomycin appears to transport all three cations only through 1:1 complexes over the range of conditions considered in Table 2, although some of the slopes observed with this compound are noticeably below 1, allowing for a minor involvement of other species.

DISCUSSION

The transport selectivity of a carboxylic acid ionophore is typically assumed to reflect complexation or extraction selectivity and to be a property of the compound which will hold over a range of conditions when it is employed as a research tool in cell biology. The results presented here show that both of these assumptions are suspect, at least with regard to the commonly used Ca^{2+} ionophores A23187, 4-BrA23187, and Ionomycin. When the available data on these compounds are compared, little correspondence is apparent between transport, complexation, and extraction selectivity (Table 1). In addition, transport selectivity is seen to be strongly dependent on conditional parameters such as pH, cation concentration, and the concentration of the ionophore considered (Figures 4 and 5). The latter characteristics, in particular, have not been recognized previously and raise several questions, including the following. (1) What is the most useful way to determine and express transport selectivity? (2) What is the origin of the sequences which are seen? (3) What are the conseqences of these sequences and their dependence on conditional parameters for the use of Ca^{2+} ionophores as research tools? Regarding

the first question, when selectivity must be known to interpret the actions of an ionophore on cells or other biological structures, the data from which it is determined clearly should be actual transport data obtained under conditions which bracket those expected in the biological system, and which take account of as many conditional parameters as possible. Comparing Figures 2 and 3 to Figure 4 shows that the practice of normalizing the data to one of the cations of interest aids in the recognition of trends and the selection of conditions which maximize selectivity, particularly when several cations are involved.

Regarding the basis of the transport selectivity data presented here, the low activity of 4-BrA23187 as a Ca^{2+} ionophore, with retention of activity for other cations, is the property of greatest interest for reasons that will be pointed out below. The absence of solution chemical data pertaining to cation complexation and protonation of this compound precludes the identification of a unique explanation. However, bromination of A23187 at position 4 (see Figure 6A for structures) may decrease its activity as a Ca^{2+} ionophore, relative to the parent compound, by decreasing the stability of the $(ionophore)_2Ca$ complex while having less effect on the stability of alternative species which can transport the other cations. The $(ionophore)_2Ca$ complex is the species primarily responsible for Ca^{2+} transport by both of these compounds (Blau *et al.*, 1984; Erdahl *et al.*, 1994, 1995). The formation of this species occurs in a stepwise manner, as illustrated by reactions 3 and 4 of the model shown in Figure 7. Solution equilibrium studies have shown that, among these two reactions, when A23187 is considered, the value of the second stepwise stability constant exceeds or is equal to that of the first reaction (Chapman *et al.*, 1987; Tissier *et al.*, 1993). This is an unusual circumstance from a chemical perspective, which favors formation of the transporting A_2Ca complex over the ACa^+ complex, which is a species that does not transport Ca^{2+} (Erdahl *et al.*, 1994, 1995). Structural studies indicate that two interligand, intramolecular hydrogen bonds (Figure 6B) contribute to the enhanced stability of the A_2Ca complex (Deber & Pfeiffer, 1976; Smith & Duax, 1976). Thus, if bromination at position 4 weakened these hydrogen bonds, the result would be destabilization of the 2:1 complex relative to the 1:1 complex, giving a reduction in Ca^{2+} transport activity which could be dramatic. It is reasonable to expect that bromination alters the strength of these hydrogen bonds because introduction of an electronegative substituent at position 4 would tend to decrease the electron density (or partial negative charge) on the carboxylate oxygen atoms, decreasing their hydrogen-bond acceptor capabilities. In addition, the bromine substitute is likely to alter the hydrogen bond between the 3-methylamino and carboxylate moieties (Figure 6A), further affecting the hydrogen-bonding properties of the latter group [see also Prudhomme *et al.* (1986a)]. Thus, electronic, inductive, or steric effects associated with the 4-bromo substitute should alter the potential of the carboxylic acid moiety to form hydrogen bonds that stabilize the 2:1 complex (Pfeiffer & Deber, 1976; Smith & Duax, 1976).

While the above considerations rationalize the low activity of 4-BrA23187 as a Ca^{2+} ionophore, they do not explain why activity as a ionophore for Zn^{2+} and Mn^{2+} would be retained simultaneously. Previous solution chemical studies on A23187 and the data in Table 2 show how this property can be explained. When A23187 is titrated with any of the

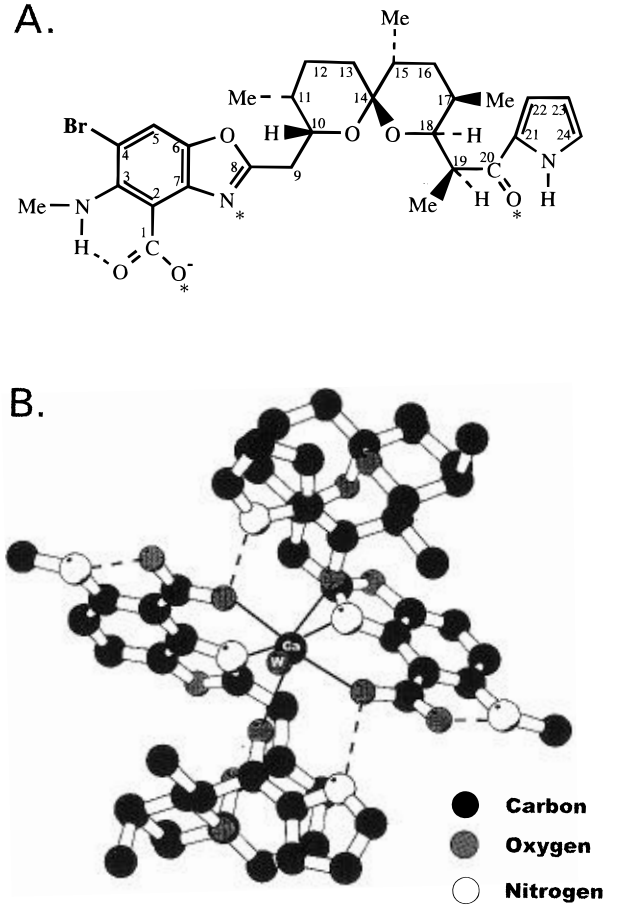

FIGURE 6: Structure of 4-BrA23187 and the $(A23187)_2Ca$ complex. (A) The bond-line structure of 4-BrA23187 (A23187) in the carboxylate form showing the numbering system referred to in the text. Cation-liganding atoms are marked with an asterisk. An intramolecular hydrogen bond between the hydrogen of the *N*-methylamino group at position 3 and the nonliganding carboxylic acid oxygen, discussed in the text, is also shown. (B) The crystal structure of the $(A23187)_2Ca$ complex, as determined by Smith and Duax (1976), which includes a single water molecule coordinated to the cation. The panel was prepared using Molscript, a molecular graphics program (Kraulis, 1991). Coordinate-covalent bonds between the cation and the ionophore molecules are shown as solid lines. Hydrogen bonds, including the two intermolecular hydrogen bonds which stabilize the 2:1 complex, relative to the 1:1 complex, are shown as dashed lines. These are formed between the liganding carboxylic acid oxygen and the hydrogen of the pyrrole nitrogen from the adjacent ionophore molecule. Bromination at position 4 is thought to destabilize the 2:1 complex by weakening these hydrogen bonds.

cations considered here, the A_2M species persist in the presence of some excess cation due to the relative magnitudes of the stepwise stability constants mentioned above. However, when the cation is in substantial excess, the 2:1 complexes disproportionate according to the following reaction.

$$A_2M + M^{2+} \rightleftarrows 2AM^+ \qquad (3)$$

The 1:1 complexes so formed are subject to interaction with OH^- (and possibly other anionic species) to form charge neutral, mixed complexes of the type AM·OH (Chapman *et al.*, 1987, 1990a). Thus, there is the potential for A23187 and 4-BrA23187 to transport divalent cations as 1:1 complexes through either electrogenic (the AM^+ complex) or electroneutral (the AM·OH complex) modes (see Figure 7).

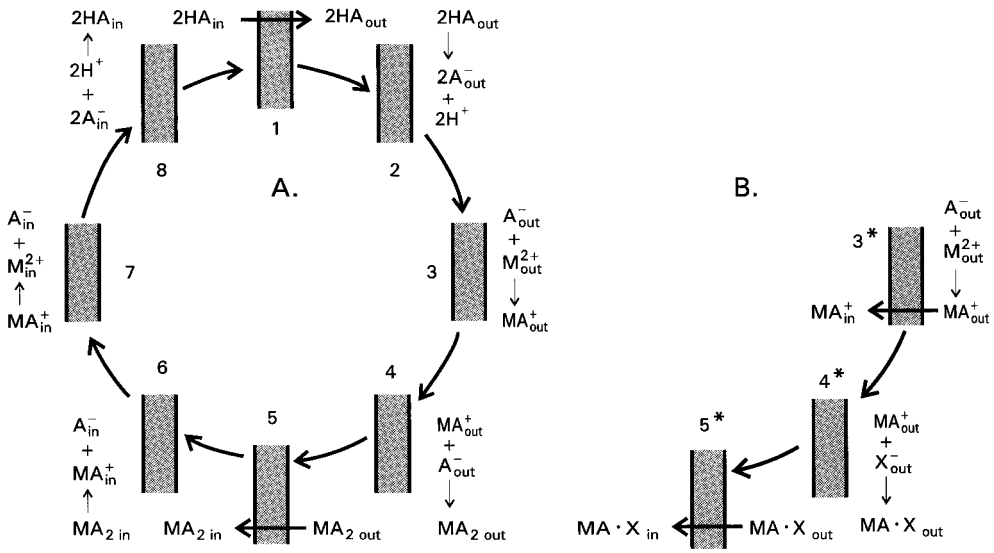

FIGURE 7: Potential transporting species. (A) The eight component reactions which comprise the electroneutral cycle by which A23187 or 4-BrA23187 (AH and A^-) transports Ca^{2+} (M^{2+}) *via* the 2:1 complex (see Figure 6). (B) Alternate component reactions which may allow the ionophores to transport Zn^{2+} and Mn^{2+} as 1:1 complexes. If the charged species formed by reaction 3* were membrane permeant, transport would be electrogenic. If only the mixed complexes formed by reaction 4* were permeant, transport would be electroneutral. Under the conditions of this study, X^- might be OH^- or possibly Cl^-.

In the case of Ca^{2+}, there is strong evidence against electrogenic transport by any of the ionophores considered here (Erdahl *et al.*, 1994, 1995), and the slopes of the log of the rate of Ca^{2+} transport *vs* the log of the A23178 (4-BrA23187) concentration are near 2.0, indicating that the charge neutral, mixed complexes which are 1:1 (ionophore−cation) provide at best a minor fraction of the Ca^{2+} transport activity (Table 2). These properties are consistent with expectations because Ca^{2+} has a coordination number of 7−9 while only three or four sites would be occupied by liganding atoms from the ionophore, or by OH^-, in the ACa^+ and ACa·OH complexes, respectively. The remainder would be occupied by water, which would be interactive with bulk solvent water, resulting in slow permeation of the membrane by either of the 1:1 species. Zn^{2+} and Mn^{2+} have lower coordination numbers (4−6) than Ca^{2+}, however, and so 1:1 complexes between these cations and A23187 or 4-BrA23187 would be less subject to hydration and able to cross the membrane more readily. Indeed, the slopes of log *vs* log plots obtained with these cations are well less than 2.0, indicating that both 1:1 and 2:1 complexes contribute significantly to the transport of these cations (Table 2). To the extent that Zn^{2+} and Mn^{2+} transport can occur *via* a 1:1 complex, the effect of destabilizing the 2:1 complex on the rate of transport would clearly be diminished. The exact contributions from the various pathways envisioned (Figure 7) are hard to predict because the distribution of ionophore between 2:1 and the various 1:1 complexes, as well as their stabilities and membrane permeability coefficients, are unknown. Nevertheless, significant transport of Zn^{2+} and Mn^{2+}, but not Ca^{2+}, as 1:1 complexes with A23187 and 4-BrA23187 may explain why the transport activity for these cations is retained when the hydrogen bonds which stabilize the 2:1 complexes are weakened by bromination of A23187 at position 4.

Regardless of whether the points considered above prove to be a full explanation for the differing transport selectivities of A23187 and 4-BrA23187, the high selectivity of the latter compound for Zn^{2+} and Mn^{2+} over Ca^{2+} transport suggests new applications for the latter compound as a research tool. Zn^{2+} and Mn^{2+} play numerous roles in biological systems which may include cell signaling/regulatory roles, analogous to those of the well-studied systems which are Ca^{2+} dependent (Wedler, 1993; Vallee & Falchuk, 1993; Berg & Shi, 1996). Control of the cell content and free concentrations of Zn^{2+} and Mn^{2+} are not well-understood (Wedler *et al.*, 1994; Reyes, 1996). Thus, to some extent, those investigating actions of these cations in cells face difficulties in manipulating and quantitating them *in vivo*. Analogous problems once faced by those investigating the regulatory roles of Ca^{2+} were solved, in part, by the discovery and application of Ca^{2+} ionophores. 4-BrA23187 may prove to be of a similar value with regard to the actions of Zn^{2+} and Mn^{2+}, since it should allow the manipulation of these cations in cells with no or minimal perturbation of the regulatory systems controlled by Ca^{2+}. On the basis of the present findings, such applications would best be attempted at slightly acidic pH, using low concentrations of ionophore and cations. Under these conditions, particularly with Zn^{2+}, the transport selectivity compared to Ca^{2+} is only a few-fold below the value of 10 000, which is the selectivity of valinomycin for K^+ over Na^+. As is well-known, valinomycin is fully useful for manipulating K^+ without interference from an altered Na^+ distribution.

A final point also relates to the possible roles of Zn^{2+} and Mn^{2+} in cell regulation. The free concentration of these cations in cells is controversial but is placed at near 1 μM by recent reports (Wedler & Ley, 1994; Brand & Kleineke, 1996). This value is above the concentration of free Ca^{2+} in a typical cell, except when strongly stimulated. Given the low transport selectivity of A23187 and Ionomycin (Table 1), it is then probable that the levels of Zn^{2+} and Mn^{2+} are altered when ionophore is used to either deplete or otherwise manipulate cell Ca^{2+}. Thus, some actions of these ionophores on cells are likely to reflect the perturbation of regulatory systems which are dependent on Zn^{2+} or Mn^{2+}. 4-BrA23187 is a member of a halogenated derivative set which consists of 10 or more compounds (Debono *et al.*, 1981). Other derivatives of A23187 [see Prudhomme *et al.* (1986b)] and of Ionomycin (Hu & Weiler, 1994a,b) are also

known. Given the present results, and the apparent need for divalent cation ionophores with improved transport selectivity, it appears worthwhile to further characterize the properties of these derivatives.

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