Cation Transport and Specificity of Ionomycin

COMPARISON WITH IONOPHORE A23187 IN RAT LIVER MITOCHONDRIA*

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Based on the effects of ionomycin upon mitochondrial respiration, ionomycin was shown to be an effective ionophore for Ca²⁺ in rat liver mitochondria. The ionomycin-induced efflux of Ca²⁺ across the inner membrane was more sensitive to loading the mitochondria with Ca²⁺ than was efflux catalyzed by A23187. At saturating concentrations of Ca²⁺, the turnover number for ionomycin was 3- to 5-fold greater than that of A23187. Ionomycin catalyzed the efflux of mitochondrial Mg²⁺ at rates comparable to those observed with A23187. Ionomycin also mediated an efflux of K⁺ provided that the mitochondria were depleted of their endogenous divalent metal ions. The apparent turnover numbers for K⁺ efflux suggest that ionomycin is more specific for divalent metal ions than A23187.

Ionomycin is a recently discovered polyether antibiotic with properties characteristic of a divalent cation ionophore (1, 2). In contrast to the other known divalent cation ionophores, ionomycin is a dibasic acid having both a carboxylic acid group and an enolized β-diketone group (3), thus readily explaining the 1:1 stoichiometry of its complexes with divalent cations. Beeler et al. have shown that ionomycin increases the permeability of sarcoplasmic reticulum vesicles and liposomes to Ca²⁺ (4).

Of the known divalent cation ionophores (for reviews, see Refs. 5 and 6), A23187 is most often employed for studies of Ca²⁺ transport across biological membranes because of its relatively high specificity for divalent over monovalent cations (7). Cation displacement studies with ionomycin indicate a similar specificity for divalent cations and, in addition, a greater affinity for Ca²⁺ over Mg²⁺ than observed with ionophore A23187 (2). Although the extent to which binding specificity determines transport specificity is not well understood for the polyether ionophores, the results of this binding study suggest that ionomycin might transport Ca²⁺ with greater specificity than does A23187. This potential advantage over other divalent metal ionophores prompted us to examine the transport properties and specificity of ionomycin in rat liver mitochondria, and to compare this novel divalent cation ionophore with A23187. Mitochondria were chosen for this study because of their extensive use in determining the transport properties of A23187 (8, and references therein) and other ionophores.

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† The abbreviations used are: CHES, 2-(cyclohexylamino)-ethanesulfonic acid; CCCP, carbonyl cyanide m-chlorophenylhydrazone; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethylether) N,N,N',N''-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

MATERIALS AND METHODS

Mitochondria were prepared as described previously (9), and protein was estimated by the Biuret reaction in the presence of 1% deoxycholate. Experiments were initiated by the addition of mitochondria to the appropriate medium (described in the figure legends). A 2-min incubation of the mitochondria at 25°C preceded any further additions.

Oxygen consumption and medium pH were monitored simultaneously with a dual channel Gilon oxigraph equipped with a Clark electrode (Yellow Springs Instruments) and a combination pH electrode (Beckman No. 39030). The concentration of K⁺ in the medium was monitored simultaneously by means of a K⁺ electrode (Beckman No. 39047). The K⁺ electrode was referenced to the combination pH electrode and the signal was amplified by a Fisher pH meter (Accumet No. 144) and a fixed range, variable window, nulling amplifier before recording. Calibration of the electrodes was accomplished by addition of known concentrations of HCl or KCl to the incubation medium.

Efflux of mitochondrial Mg²⁺ was determined by dual wavelength spectrophotometry (American Instrument Co., DW2a) in the presence of 100 μM Eriochrome Blue SE, an indicating dye for free Mg²⁺ (10). The wavelength pair 570-540 nm was employed for these studies. Alternatively, efflux of Mg²⁺ was measured by atomic absorption analysis (Varian AA-575). Aliquots of the mitochondrial suspension were rapidly sedimented in a microcentrifuge (Eppendorf No. 5412), and the resulting supernatant fluid was analyzed for Mg²⁺.

The free acid of ionomycin was formed by repeatedly washing a solution of the sodium salt, 5.3 μM, in chloroform with 2 mM aqueous HCl. Cation complexes were prepared by equilibrating equal volumes of the free acid at 5.3 μM in chloroform with 2 mM aqueous HCl. The aqueous phase was buffer at pH 9.02 ± 0.01 with 2 mM CHES. After separating the two phases by centrifugation, the ultraviolet spectrum of the compound in the chloroform phase was determined on a Cary 118 spectrophotometer at 25°C. The path length of the cell was 1 cm and the reference cuvette contained chloroform.

Ionophores were added to mitochondrial suspensions in negligible volumes of ethanol; control experiments for solvent addition were routinely performed. The sodium salt of ionomycin was kindly supplied by Dr. J. Westley of Hoffman-LaRoche. Ruthenium red (approximately 20%; nominal concentrations reported), valinomycin, CCCP, and Eriochrome Blue SE (plasmocornith B) were obtained from Sigma. A23187 was a gift from Dr. Robert Hamill of Eli Lilly Co.

RESULTS

The Effects of Ionomycin on Oxygen Consumption and Net Proton Movements in Rat Liver Mitochondria—The effects of ionomycin on succinate oxidation demonstrate that the compound is an effective ionophore for Ca²⁺ in mitochondria. Ionomycin stimulates succinate oxidation by rat liver mitochondria (Fig. 1, Trace 1) and this stimulation is abolished by EGTA, a chelator of Ca²⁺, or ruthenium red, an inhibitor of electron transport (11).
The effects of ionomycin upon succinate oxidation and net proton movements in rat liver mitochondria. Oxygen consumption and H+ movements were monitored as described under "Materials and Methods." Mitochondria, 2.5 mg of protein/ml, were incubated in a medium consisting of 207 mM mannitol, 63 mM sucrose, 10 mM sodium succinate, and 5 mM sodium Hepes, pH 7.4, in a total volume of 4.5 ml. Ionomycin (IONO), ruthenium red (RuRed), 2 nmol/mg of protein, EGTA, 1 mM, valinomycin (VAL), 0.08 nmol/mg of protein, and CaCl2, 50 nmol/mg of protein, were added where indicated by the arrows. The addition of ionomycin was 0.43 nmol/mg of protein except in Experiment 5 in which the amount was 0.026 nmol/mg of protein. The dashed line in Experiment 1 is a continuation of the linear portion of the ionomycin-stimulated respiration. It is included to aid in visualizing the time-dependent decrease in the stimulated rate as discussed in the text.

The effects of ionomycin upon net proton movements are also presented in Fig. 1. The ability of EGTA or ruthenium red to prevent the respiratory stimulation indicates that 1) ionomycin neither acts as a protonophore uncoupler nor does it promote a nonspecific stimulation and 2) an energy-dependent uptake of Ca2+ by mitochondria is necessary for the respiratory stimulation by the compound. Thus, the stimulation of succinate oxidation results from an energy-dissipating flux of Ca2+ across the inner membrane brought about by the ionophore releasing Ca2+ and the energy-dependent Ca2+ importer producing reaccumulation.

Similar to previous observations with A23187 (12), ionomycin plus valinomycin stimulated succinate oxidation in the presence of divalent metal chelators (Fig. 1, Experiment 2). Valinomycin alone had little effect on the rate of respiration under these conditions (12, and data not shown). These observations indicate that ionomycin plus valinomycin can establish an energy-dissipating cycling of K+ across the inner membrane analogous to nigericin plus valinomycin (16, 17). These data suggest that ionomycin, like the monocarboxylic acid-K+ ionophores, can mediate a K+ for H+ exchange across the inner membrane. The transport by ionomycin of cations other than Ca2+ will be examined in further detail below.

In the absence of added Ca2+, the rate of oxygen consumption due to Ca2+ cycling was a linear function of the ionomycin level (Fig. 2, open circles), eventually saturating at the protonophore-stimulated rate. Previous work has shown that the mitochondrial Ca2+ level had only a slight effect upon the stimulation of respiration by A23187 (7). In contrast, the data in Figs. 2 and 3 show that the cycling of Ca2+ induced by...
ionomycin is very sensitive to the amount and the chemical state of mitochondrial Ca\(^{2+}\). The levels of the ionophore at which maximal and half-maximal rates of respiration occurred were decreased significantly when the mitochondria were loaded with 50 ng ion Ca\(^{2+}\)/mg of protein (Fig. 2, closed circles). The double reciprocal plot of oxygen consumption versus ionophore level in the presence of added Ca\(^{2+}\) was linear (Fig. 2, inset) as might be expected for a mechanism in which a 1:1 complex of ionomycin with Ca\(^{2+}\) is responsible for Ca\(^{2+}\) efflux. Furthermore, the rate of oxygen consumption induced by ionomycin was first order with respect to the amount of added Ca\(^{2+}\) at lower cation levels and could be saturated at higher levels (Fig. 3A). The amount of Ca\(^{2+}\) required to saturate the respiration rate decreased as the level of ionophore was increased, further supporting this mechanism.

Further information about the effect of Ca\(^{2+}\) accumulation on the ionomycin-induced cycling rate is provided in Fig. 3B. The ability of accumulated Ca\(^{2+}\) to enhance the stimulation of respiration by a given amount of ionophore could be due either to an increase in the amount of mitochondrial Ca\(^{2+}\) or to an increase in the transmembrane pH gradient, thereby promoting deprotonation of the carrier. Accordingly, experiments were carried out in the presence of the permeant anions acetate or inorganic phosphate to further investigate the mechanism by which added Ca\(^{2+}\) alters the Ca\(^{2+}\) cycling rate. Permeant anions are weak acids that are accumulated together with the divalent cation during energy-dependent uptake of Ca\(^{2+}\). By donating protons to the matrix, they reduce the increase in the transmembrane pH gradient that occurs during Ca\(^{2+}\) uptake (21). An additional effect with inorganic phosphate is the sequestration of mitochondrial Ca\(^{2+}\) through formation of amorphous calcium phosphate deposits in the matrix. The effects of these agents upon the stimulation of respiration induced by ionomycin are presented in Fig. 3B. In these experiments, the mitochondria were loaded with Ca\(^{2+}\), 50 ng ion/mg of protein, prior to the addition of the ionophore. Acetate had a slight stimulatory effect, while increasing concentrations of inorganic phosphate decreased the rate of cycling by 75%. These data indicate that the predominant effect of Ca\(^{2+}\) loading on the ionomycin-dependent cycling rate is related to the availability of the cation rather than an effect on matrix pH and will be further discussed below.

**Transport of Mg\(^{2+}\) and K\(^{+}\) Mediated by Ionomycin—Mitochondria contain significant amounts of Mg\(^{2+}\) and K\(^{+}\) (20 to 25 and 100 to 130 ng ion/mg of protein, respectively) allowing an examination of the specificity of cation transport by ionophores. Ion binding is a prerequisite for carrier-mediated ion transport. Previous studies on the ability of ionomycin to bind monovalent cations were equivocal (2). The data in Fig. 4, however, provide spectroscopic evidence for the complexation of monovalent cations. Although the extent of complexation in each case may not be identical, the changes in the spectrum that occur when the free acid is equilibrated with different cations demonstrate formation of Na\(^{+}\) and K\(^{+}\) as well as Ca\(^{2+}\) and Mg\(^{2+}\) complexes. Since the absorbance by ionomycin in this range is due to the enolized \(\beta\)-diketone group (3, 18), the data suggest that the cations are coordinated to this functional group in their complexes, as has been shown for Ca\(^{2+}\) (3).

Eriochrome Blue SE, a Mg\(^{2+}\)-indicating dye, was employed to demonstrate ionomycin-induced efflux of Mg\(^{2+}\) from mitochondria (Fig. 5A). The initial rate of efflux was a linear function of the amount of ionophore and appeared to saturate at higher levels of ionomycin (Fig. 5B). The efflux of Mg\(^{2+}\) induced by ionomycin was confirmed by atomic absorption (data not shown).

The ability of ionomycin plus valinomycin to produce a sustained stimulation of respiration in the presence of EGTA (Fig. 1) indicated the ability of the ionophore to catalyze K\(^{+}\) transport. The experiments in Fig. 6 were designed to demonstrate K\(^{+}\)-efflux more directly. The results show that both ionomycin and A23187 catalyzed a net efflux of K\(^{+}\) from mitochondria when sodium EDTA, 1 mm, was included in the medium. Significant aspects of these data are the following: 1) the rate of efflux was dependent upon the concentration of the ionophore, 2) a lag period that was inversely related to the concentration of the ionophore was evident prior to the

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**Fig. 4. Spectrophotometric detection of cation complexation by ionomycin.** The free acid of ionomycin and its cation complexes with Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\), and Mg\(^{2+}\) were prepared and their absorption spectra in chloroform were recorded as described under "Materials and Methods." The labeling of the spectra in the figure indicates the cation bound to the compound.

**Fig. 5. Efflux of mitochondrial Mg\(^{2+}\) mediated by ionomycin.** Efflux of mitochondrial Mg\(^{2+}\) was followed by the dual wavelength technique as described under "Materials and Methods." Mitochondria were incubated at 2.5 mg of protein/ml in 184 mM mannitol, 56 mM sucrose, 10 mM sodium succinate plus rotenone at 0.5 nmol/mg of protein. A, the addition of ionomycin, indicated by the aq.

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efflux of K+, and 3) A23187, at least less than 0.2 nmol/mg of protein, catalyzed a 10-fold faster rate of K+ efflux than did ionomycin on a mole per mole basis (Fig. 6, inset). An increase in the acidity of the medium occurred at the time of ionophore addition, and the onset of K+ efflux occurred at the completion of this acidification (Fig. 7). The rate but not the extent of H+ appearance was a function of the amount of ionophore; further, the extent was the same for A23187 and ionomycin (624 ± 0.0.1 ± 0.1 ng ion H+/mg of protein, respectively). Based on these observations, the appearance of H+ in the medium can be explained most readily by the complexation of released divalent cations by EDTA (at pH 7.4, the reaction produces approximately 2H+/divalent metal complexed). Indeed, the release of essentially all the mitochondrial Mg2+ (22 nmol/mg protein) was complete at the time K+ efflux began (Fig. 7). The presence of a lag, therefore, supports and extends to ionomycin the conclusion of Pfeiffer and Lardy (12) that binding of free A23187 by endogenous divalent metal ions effectively prevents an ionophore-induced efflux of K+ in mitochondria. Ionomycin also catalyzes K+ transport in the absence of EDTA, although the efflux was substantially slower and did not occur until anaerobiosis (data not shown). Since a passive efflux of mitochondrial Ca2+ occurs at anaerobiosis, the fact that significant K+ efflux did not take place until anaerobiosis is also consistent with the above conclusion.

**FIG. 6.** Efflux of mitochondrial K+ mediated by ionomycin and A23187. Efflux of mitochondrial K+ was monitored with a K+ electrode as described under “Materials and Methods.” No attempt was made to reproduce the noise in the electrode tracings. Mitochondria, 2.5 mg of protein/ml, were incubated in total volume of 4.5 ml, with 2 mM sodium succinate, 20 mM mannitol, 70 mM sucrose, 2 mM sodium succinate, plus rotenone at 0.5 nmol/mg of protein and did not occur until anaerobiosis (data not shown). Since a passive efflux of mitochondrial Ca2+ occurs at anaerobiosis, the fact that significant K+ efflux did not take place until anaerobiosis is also consistent with the above conclusion.

**FIG. 7.** H+ appearance and Mg2+ efflux prior to the onset of K+ efflux induced by ionomycin. K+ efflux, medium pH, and Mg2+ efflux (atomic absorption) were monitored as described under “Materials and Methods.” Experiments were conducted as in Fig. 6A. Ionomycin (2.0 nmol/mg of protein) was added to the mitochondrial suspension at 2 min as indicated. Inset, by varying the amount of ionophore, the time elapsed from the addition of ionophore to the onset of K+ release (C) or to the completion of the H+ appearance (D) was varied and is plotted as a function of the level of ionophore.
Although initially linear, the enhanced rate of succinate oxidation induced by ionomycin eventually decreased with time. Under certain conditions (low level of ionophore or low protein concentration), the decrease in the stimulated rate was more extensive than shown in Fig. 1, Experiment 1. As the amount of added Ca\(^{2+}\) was increased, succinate oxidation remained linear for longer periods of time until, at saturation of the Ca\(^{2+}\) cycling rate, oxidation was linear until anaerobiosis (data not shown). Furthermore, as the concentration of Pi, in the medium was increased, the nonlinearity of oxygen consumption gradually returned. These data suggest that free ionomycin is slowly inactivated during incubations with mitochondria and that complexation with Ca\(^{2+}\) results in protection from inactivation. Although direct evidence is not yet available, it is tempting to propose that the \(\beta\)-diketone group of ionomycin undergoes a chemical reaction during incubations with mitochondria. The possibility of a time-dependent decrease in the activity of ionomycin should be considered in other biological systems, especially during long term incubations.

Turnover numbers of Ca\(^{2+}\) transport by ionomycin may be calculated by assuming that the calcium/site ratio (number of Ca\(^{2+}\) ions taken up per pair of electrons per energy-conserving site) remains at 2.0 in the presence of the ionophore (for a review, see Ref. 26) and that the 1:1 calcium-ionomycin complex is responsible for efflux of the divalent metal ion from mitochondria. In the absence of added Ca\(^{2+}\), an apparent turnover number of 18/s/ionomycin molecule was obtained from the slope of the linear portion of the curve in Fig. 1 (open circles). A more accurate turnover number was obtained from the data in the presence of added Ca\(^{2+}\) (Fig. 3, closed circles) since under these conditions, the ionophore appeared to be saturated with respect to Ca\(^{2+}\)-binding (Fig. 3A). The turnover number calculated from the linear portion of the curve was 130/s/ionomycin molecule. If, however, the turnover number is obtained from the slope of the double reciprocal plot of the data (Fig. 2, inset), the result is 210/s/ionomycin molecule. The latter method for obtaining the turnover number includes State 4 respiration in the calculation of the Ca\(^{2+}\) cycling rate, whereas the former method does not. Regardless of the method of calculation, the turnover number in the presence of saturating concentrations of Ca\(^{2+}\) was 3- to 5-fold greater than the turnover number for A23187 (44/s/pair of A23187 molecules) (8).

Ionomycin catalyzed the net efflux of Mg\(^{2+}\) from mitochondria (Fig. 5). The apparent turnover number was determined to be 1.4/s/ionomycin molecule. On a mole per mole basis, this number approximates the turnover numbers for Mg\(^{2+}\) transport by A23187 (5). In the absence of added Ca\(^{2+}\), therefore, the specificities of ionomycin and A23187 for Ca\(^{2+}\) versus Mg\(^{2+}\) are similar. With Ca\(^{2+}\) added to the medium, the specificity of ionomycin for Ca\(^{2+}\) over Mg\(^{2+}\) would appear to be greater than that of A23187; however, since the ionophore is probably not saturated by endogenous Mg\(^{2+}\), such a comparison of the specificities of these ionophores for divalent cations must be considered preliminary. Nevertheless, it is important to realize that transmembrane fluxes of Mg\(^{2+}\) must be considered when either ionophore is used to perturb ion transport in cellular or subcellular systems.

The demonstration of K\(^{+}\) efflux from mitochondria mediated by ionomycin and A23187 (Fig. 6 and Ref. 12) and transport of other monovalent cations by A23187 in red blood cells (27) and chloroplasts (28) points out that the specificity of these ionophores for divalent metal ions is not absolute.


Apparent turnover numbers for K\(^{+}\) transport of 0.14 and 1.6/s/molecule of ionophore were calculated from the data in Fig. 6 for ionomycin and A23187, respectively. The dependence upon the concentration of ionophore and the differential abilities of A23187 and ionomycin to promote efflux (Fig. 6) provide strong evidence for the conclusion that the efflux of K\(^{+}\) from mitochondria is mediated by the ionophores rather than by an endogenous K\(^{+}\) for H\(^{+}\) exchanger as has been proposed by others (29). The 10-fold difference in the rates of K\(^{+}\) transport catalyzed by ionomycin and A23187 (Fig. 6, inset) may aid in determining the extent to which monovalent cation movements contribute to the effects of divalent ionophores until compounds with higher charge discrimination become available.

Clearly, further work is required with well defined artificial membranes and biological systems in order to determine the factors underlying the rates and specificity of cation transport by ionomycin and, more generally, the carboxylic ionophores as a class. With such knowledge at hand, it may become possible to predict or improve these transport properties and so enhance the use of these ionophores as biochemical tools.

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