Vol. 95, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1980 Pages 140-147

CONVERSION OF MONENSIN FROM AN IONOPHORE TO AN INHIBITOR OF Na^+ UPTAKE BY SV3T3 MEMBRANE VESICLES AS A FUNCTION OF Na^+ CONCENTRATION

Richard T. Hamilton and Marit Nilsen-Hamilton

Cell Biology Laboratory, The Salk Institute
Post Office Box 85800, San Diego, CA 92138 U.S.A.

Received May 19,1980

SUMMARY: Monensin is a Na^+ ionophore in membrane vesicles from SV3T3 cells; but its ability to stimulate Na^+ flux is inhibited by increasing concentrations of Na^+. At greater than 20-mM Na^+, monensin inhibits Na^+ uptake by the vesicles. Cs^+ and NH_4^+ also cause monensin to inhibit Na^+ uptake, but general alterations in ionic strength do not convert the ionophore to an inhibitor. Monensin does not cause Na^+ loss during collection of the vesicles on filters; nor is inhibition the result of the vesicle lumen being made alkaline by H^+ loss in exchange for Na^+. The specificity for cation and ionophore indicates that a precise interaction between the cation, ionophore, and membrane is required for inhibition.

INTRODUCTION

Although the physical properties of monensin have been much studied (see ref. 1 for a list), few investigators have examined its properties in biological systems. Those studies that have been done have generally been based on the assumption that monensin acts as it does in model systems such as a modified Pressman cell; in this system monensin catalyzes a one for one Na^+ and H^+ exchange (2). This exchange property of monensin should make it useful for determining the contribution of chemical and electrical forces used to energize uphill transport of 2-aminoisobutyric acid (3-8) and Pi (9,10) in membrane vesicles from SV3T3 cells.

We reported (10) the unusual observation that monensin inhibits Na^+ influx by SV3T3 membrane vesicles derived mainly from the plasma membrane and endoplasmic reticulum. In this paper, we add to that finding and show that monensin is a Na^+ ionophore in the vesicles, but that this activity is inhibited by increasing concentrations of the Na^+ substrate itself. At concentrations of Na^+ greater than about 20-mM, monensin is seen to inhibit Na^+ uptake. Apparently, increasing concentrations of Na^+ are converting monensin from an ionophore into an inhibitor.

Abbreviations: SV3T3 cells, mouse 3T3 cells transformed by simian virus 40; AIB, 2-aminoisobutyric acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; DMSO, dimethylsulfoxide.

0006-291X/80/130140-08$01.00/0

METHODS

Cell Culture and Preparation of Membrane Vesicles. Swiss 3T3 cells transformed by Simian Virus 40, given to us by P. Rudland (Ludwig Institute for Cancer Research, Sutton, England), were recloned. Stock cultures were kept in Dulbecco and Vogt's modification of Eagle's medium, supplemented with 10% calf serum. The stock cell cultures were grown as monolayers on plastic Petri tissue culture dishes, at 37°C, in a water-saturated atmosphere, with 10% CO_2 in air. Monthly checks for mycoplasma were made by incorporation of [^{3}H]-thymidine and autoradiography of the cells. Another test was the measurement by fluorescent microscopy of Hocchst-dye, stained DNA in the cytoplasm of the SV3T3 cells (11). A third test for mycoplasma in the culture medium and in the SV3T3 cells was made, based on the determination of adenosine phosphorylase activity (12). No mycoplasma were detected.

For the preparation of membrane vesicles, cells were seeded in half-gallon, glass roller bottles and grown to maximal cell density. Membrane vesicles were prepared as described before (10). The quality of membrane preparations was periodically tested using characteristic marker enzymes (10). The membrane vesicles were derived mainly from the plasma membrane and the endoplasmic reticulum. Protein was determined by intrinsic protein fluorescence (13). Vesicles were stored at 5 to 10 mg of protein per ml in 0.25 M sucrose, 10-mM Tris-HCl (pH 7.4), and 0.25-mM $MgCl_2$, at -70°C.

Transport Assays. Uptake was measured by the filtration technique detailed before (10). The volume was 100 µl and contained: sucrose, 200-mM; Hepes/Tris, 20-mM, pH 7.4; $MgCl_2$, 1-mM; and $CaCl_2$, 0.1-mM. Temperature was 37°C. The stopping and washing solution was 0.8 M NaCl, 10-mM Hepes/Tris HCl, pH 7.4, at 2°C.

MATERIALS

New England Nuclear supplied ^{22}NaCl; 2-amino[methyl-^{3}H]-isobutyric acid was from ICN. Samples of monensin, nigericin (Na salts), and antibiotic A23187 were gifts from Dr. J. W. Chamberlin of the Lilly Research Laboratories; FCCP was a gift from Dr. P. G. Heytler of du Pont de Nemours; valinomycin and gramicidin D were from Sigma.

RESULTS

Monensin's Effect on the Time-course of Na^+, 100-mM, Uptake

Opposite to monensin's acceleration of Na^+ uptake in a model system (2), monensin inhibits Na^+ uptake by membrane vesicles from SV3T3 cells (Fig. 1). In the presence of monensin (20 µM, 12 nmol/mg protein), uptake remains time dependent, but is reduced, at 5 s, to about 20% of the control. Also, Na^+ uptake does not reach the level of uptake of the control, even after 120 min. At 120 min, Na^+ uptake in the absence of monensin appears to have leveled off; it might be still rising slightly in the presence of 20 µM monensin. At 120 min, Na^+ uptake in the presence of 20 µM monensin is 57% of the control; it is 74% of the control in the presence of 10 µM monensin. Our results so far (manuscript in preparation) suggest that Na^+ bind to the vesicles and that monensin inhibits this binding; this would account for the reduced accumulation of Na^+ with monensin present.

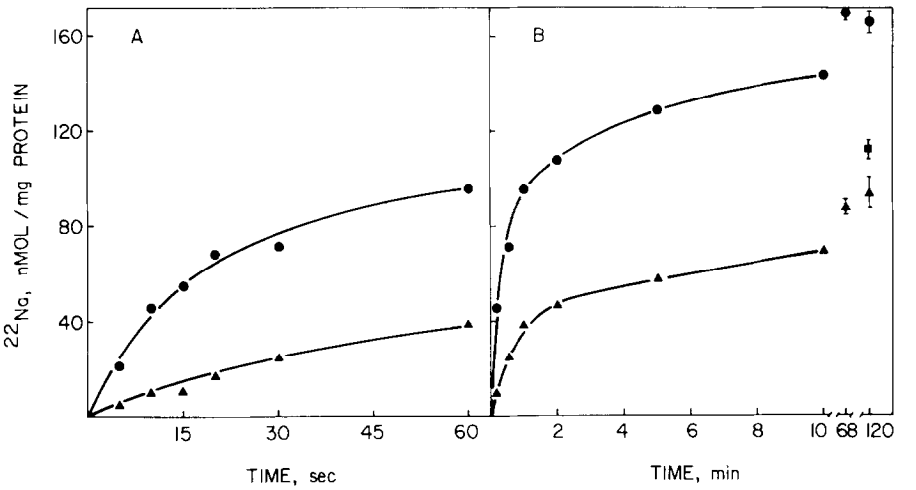

Fig. 1. The effect of monensin on the time-course of uptake of $^{22}Na^+$. For each time point, 167 µg membrane protein were used. The concentration of $^{22}NaCl$ was 100-mM (S.A. = 128.7 cpm/nmol). (●) 2% DMSO was incubated with the vesicles before uptake; (▲) 2% DMSO and 20 µM monensin (12 nmol/mg); (■) 2% DMSO and 10 µM monensin. A: early time points up to 60 s; B: time points up to 120 min.

Sodium Ion Uptake as a Function of Na^+ Concentration

At 0.5-mM Na^+, Na^+ uptake, at 9 s, is accelerated at least 3.5-fold by monensin (Fig. 2). This acceleration declines almost as a linear function of the logarithm of Na^+ concentration. At concentrations below about 20-mM Na^+, the uptake rate is lower with monensin present. No more inhibition occurs beyond 50-mM Na^+; we have confirmed this saturability in other experiments. As a control for general changes in permeability, we show that monensin slightly inhibits the rate of AIB transport, but this inhibition is not appreciably influenced by NaCl (Fig. 2).

Salt Specificity of Monensin's Inhibition of Na^+ Uptake

We measured the uptake of 0.5-mM Na^+ as a function of choline-chloride concentration, with and without monensin. At no concentration of choline chloride, from 1-mM to 75-mM, did this salt appreciably alter monensin's acceleration of Na^+ uptake (data not shown). Other chloride salts (at 75-mM) were tested for their ability to bring about inhibition of 0.5-mM Na^+ uptake by monensin (Table I). In the presence of CsCl and NH_4Cl, monensin inhibited Na^+ uptake by 76% and 53%; uptake of 75.5-mM Na^+ was inhibited by 54%. In the presence of LiCl and $MgCl_2$, monensin accelerated Na^+ uptake, slightly more than in the control. In the presence of KCl and RbCl, monensin accelerated Na^+ uptake, but acceleration was less than in the control. The degree to which the various cations bring about inhibition of Na^+ uptake by monensin does not correlate with their complexation order with monensin (2,15-18).

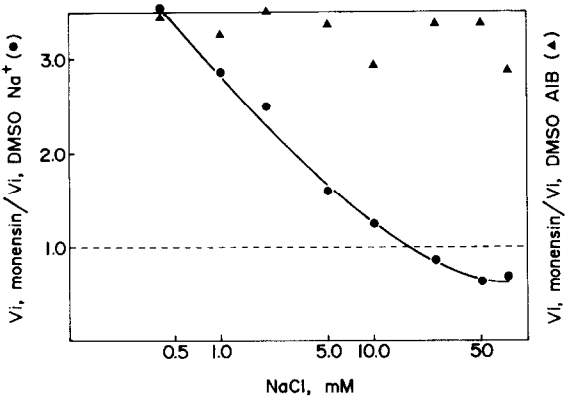

Fig. 2. Ionophoric activity of monensin on $^{22}Na^+$ as a function of the NaCl concentration. The uptake of $^{22}Na^+$ and [^{3}H]-AIB was measured in the same reaction mixture. The specific activity of [^{3}H]-AIB was 31.0 cpm/pmol; the concentration was 0.2-mM. In each reaction there were 5.28 X 10^5 cpm of ^{22}Na; there were 230 µg protein. The monensin concentration was 10 µM (4.4 nmol/mg protein), DMSO was at 1%. Monensin and DMSO were incubated with the membranes for 10 min before the uptake, and uptake was for 9 s (Vi). The line was drawn by eye. For each value, duplicate determinations were made. All values are accurate to within 7% standard error. ^{22}Na (●); [^{3}H]-AIB (▲).

The Effect of Buffering Capacity and Protonophores on the Inhibition

We also tested the idea that an increase in alkalinity of the vesicle lumen was inhibiting monensin's activity; an increase in alkalinity would arise from a Na^+/H^+ exchange (2). Changing the buffering capacity of the uptake reaction, by raising the buffer concentration from the usual concentration of

Table I. Effect of various chloride salts on monensin's ionophoric activity

Cation Chloride	Vi, DMSO (nmol/min/mg)	Vi, monensin (nmol/min/mg)	Vi, monensin / Vi, DMSO
None	1.20	2.60	2.2
Li^+	0.64	1.67	2.6
Na^+	118	53.7	0.46
K^+	0.82	1.43	1.7
Rb^+	1.03	1.71	1.7
Cs^+	0.99	0.24	0.24
NH_4^+	0.43	0.20	0.47
Mg^{2+}	1.07	2.95	2.8

The salts were added with 0.5 mM $^{22}NaCl$; each salt was at a final concentration of 75 mM. The specific activity of ^{22}Na, at 0.5 mM Na^+, was 9735 cpm/nmol and at 75.5 mM Na^+ was 64.47 cpm/nmol. The uptake time was 9s (Vi). Reaction mixtures contained 126 µg protein. The monensin concentration was 10 µM (7.9 nmol/mg protein). DMSO was at 1%. Monensin was incubated with the membranes for 10 min at 37°C before the uptake was begun. For each value, duplicate determinations were made. All values are accurate to within 10% standard error.

Table II. Effect of ionophores on ^{22}Na uptake at low and high Na^+ concentrations

Ionophore	Vi, nmol/min/mg protein		Vi, ionophore / Vi, DMSO	
	0.5 mM NaCl	50 mM NaCl	0.5 mM NaCl	50 mM NaCl
DMSO	1.02	37.8	1.0	1.0
FCCP	0.80	36.5	0.78	0.97
Valinomycin	0.88	35.4	0.86	0.94
Gramicidin D	0.32	0	0.31	0
Nigericin	0.93	16.1	0.91	0.43
Monensin	1.38	14.4	1.35	0.38

The uptake time was 9s (Vi). Specific activity of $^{22}NaCl$, at 0.5 mM, was 17,760 cpm/nmol and, at 50 mM, was 177.6 cpm/nmol. Reaction mixtures contained 238 µg protein. The concentration of each ionophore was 10 µM (4.2 nmol/mg protein), except for FCCP, which was at 10 µg/ml. DMSO was at 1%, and the ionophores were added in DMSO. Each ionophore was incubated with the membranes for 10 min before the uptake. For each value, triplicate determinations were made. All values are accurate to within 6% standard error.

20-mM Hepes/Tris, pH 7.4, to 100-mM, or lowering the concentration to 5-mM Hepes/Tris, had no appreciable effect on inhibition of the rate of uptake of 50-mM Na^+, with monensin present. Inhibition also took place in the presence of FCCP (10 µg/ml) and carbonyl cyanide *m*-chlorophenylhydrazone (10 µM) (data not shown). These two compounds are protonophores in other systems and should immediately dissipate H^+ gradients and, therefore, increased alkalinity of the lumen. Apparently, an increase in alkalinity of the vesicle lumen is not the cause of inhibition.

The Structure of the Ionophore Correlates With its Ability to Inhibit Na^+ Uptake

To determine whether some structural feature of the ionophore could be correlated with its ability to inhibit Na^+ uptake rates, we tested a number of ionophores of similar and diverse structures to monensin on Na^+ uptake (Table II). At 0.5-mM Na^+, FCCP, valinomycin, and nigericin inhibited Na^+ uptake rates slightly (from 9% to 21%); gramicidin D inhibited Na^+ uptake by 68%; monensin, as usual, accelerated Na^+ uptake, but in this experiment, by only 35%. Valinomycin and nigericin are K^+ ionophores, gramicidin D is a Na^+ ionophore, and FCCP is a protonophore. At 50-mM Na^+, FCCP and valinomycin were without appreciable effects on Na^+ uptake, but gramicidin D eliminated Na^+ uptake. This last result can be explained by assuming that the Na^+ ionophoric activity of gramicidin D is not blocked by high concentrations of Na^+, and, therefore, Na^+ leaches from the vesicles during collection and washing with cold 0.8 M NaCl - the stopping and washing solution. Nigericin, like monensin, potently inhibited Na^+ uptake (both by about 60%); nigericin and monensin are close structural analogs (14); both are carboxylic ionophores. The structural and ion specificity of the ionophore for inhibition is evidence that a specific interaction is required.

DISCUSSION

Monensin accelerates the uptake of Na^+ by membrane vesicles from SV3T3 cells. The acceleration is inhibited by the substrate itself, in a concentration dependent manner. At high substrate concentrations, monensin inhibits Na^+ uptake into these vesicles. In agreement with our results, it was found that monensin decreased the transport of Na^+ in frog skin (23) and in mitochondria (24).

We considered the possibility that monensin's surfactant properties were causing an apparent inhibition of Na^+ uptake, since all ionophores are surfactants at high enough concentrations (19); the surfactant effect would cause Na^+ to leach from the vesicles during washing on filters and it could lyse the vesicles. It is unlikely that increasing Na^+ concentrations increase the surfactant effect of the ionophore, since: 1) We have shown that monensin does not increase the loss of 100-mM $^{22}Na^+$ and 0.2-mM [^{3}H]-AIB from the vesicles during washing with the stopping solution of 0.8 M NaCl, at 2°C (about 12% loss). 2) If the surfactant properties of monensin were increased, then we would expect to see an increase in the initial rate of Na^+ uptake, which we do not observe (Fig. 1). 3) Also, monensin, at the concentrations used (10 μM - 20 μM), is not making the vesicles more permeable to other compounds, such as Pi, 3-O-methyl glucose, and AIB, at 100-mM NaCl. 4) If high concentrations of NaCl were not blocking the Na^+-ionophoric activity of monensin and causing monensin to inhibit Na^+ uptake, we would expect to see results like those obtained with gramicidin D; that is, we would see little or no Na^+ uptake - the Na^+ presumably being lost during washing of the vesicles on filters.

Of the cations tested, Na^+, Cs^+, and NH_4^+ were the only ones to cause appreciable inhibition of Na^+ uptake by monensin; therefore, inhibition is not the result of general changes in ionic strength. Ammonium ion, Li^+, and K^+ substantially inhibit basal Na^+ uptake; this indicates that Na^+ are reacting with specific sites on the membrane. The acceleration of Na^+ uptake is unaffected by 75-mM $MgCl_2$; this concentration of $MgCl_2$ causes aggregation of the vesicles; therefore, the ability of Na^+ to cause inhibition by monensin is probably not associated with aggregation of vesicles.

Of the ionophores tested, only nigericin, whose structure is almost identical to monensin's, inhibited Na^+ uptake, at 50-mM. Both monensin and nigericin have a carboxyl group; that the carboxyl group itself is not responsible for inhibition is indicated by our observation that antibiotic A23187 - also a carboxylic ionophore - does not inhibit Na^+ uptake, at 100-mM (data not shown). The specificity for cation and ionophore indicates that a precise interaction between the cation, ionophore, and membrane is required to inhibit Na^+ uptake.

Recently, two groups reported that monensin stimulated Na^+ uptake by mammalian cells: Monensin increased Na^+ influx by 3-fold in a neuroblastoma-glioma hybrid (20) and at the same time caused a transient increase in cell pH - consistent with monensin's ability to catalyze an exchange of H^+ for Na^+ in a model system (2). Monensin increased the total, cellular Na^+ of 3T3 cells by more than 5-fold (21); but Na^+ uptake was slow and incomplete even by 30 min. Equilibrium should be reached much sooner than 30 min if monensin is acting as an ionophore. We also found that monensin increased Na^+ flux into SV3T3 cells by about 2-fold (data not shown). We do not know yet why monensin acts differently in SV3T3 cells and vesicles. Because monensin is hydrophobic and consequently reacts with all membranes, it is difficult to find out how monensin is working in the cell. It is no doubt changing the ionic balance of the cell (19), and it could be changing metabolism (22). It is likely that - at least in 3T3 and SV3T3 cells - monensin is increasing Na^+ uptake, not as an ionophore, but as a result of its effect on the ionic balance and metabolism of the cell.

The simplified system of membrane vesicles from SV3T3 cells has enabled us to identify an unusual property of monensin; that is, Na^+, Cs^+, and NH_4^+ convert monensin from a Na^+ ionophore to an inhibitor of Na^+ uptake. This could also be true for other ionophores and their substrates.

ACKNOWLEDGMENTS: We thank Michael Taggart for preparing membranes and Dr. Mauricio Montal for advice. This work was supported by Grant 1-RO1-CA19523 from the National Cancer Institute, DHEW.

REFERENCES

1. Briggs, R. W., and Hinton, J. F. (1978) *Biochemistry 17*, 5576-5582.
2. Ashton, R., and Steinrauf, L. K. (1970) *J. Mol. Biol. 49*, 547-556.
3. Lever, J. E. (1977) *Biochemistry 16*, 4328-4334.
4. Lever, J. E. (1977) *J. Biol. Chem. 252*, 1990-1997.
5. Hamilton, R. T., and Nilsen-Hamilton, M. (1976) *Proc. Natl. Acad. Sci. USA 73*, 1907-1911.
6. Nilsen-Hamilton, M., and Hamilton, R. T. (1976) *J. Cell. Physiol. 89*, 795-800.
7. Quinlan, D. C., Parnes, J. R., Shalom, R., Garvey III, T. Q., Isselbacher, K. J., and Hochstadt, J. (1976) *Proc. Natl. Acad. Sci. USA 73*, 1631-1635.
8. Nishino, H., Schiller, R. M., Parnes, J. R., and Isselbacher, K. J. (1978) *Proc. Natl. Acad. Sci. USA 75*, 2329-2332.
9. Lever, J. E. (1978) *J. Biol. Chem. 253*, 2081-2084.
10. Hamilton, R. T., and Nilsen-Hamilton, M. (1978) *J. Biol. Chem. 253*, 8247-8256.
11. Chen, T. R. (1977) *Exp. Cell Res. 104*, 255-262.
12. Uitendaal, M. P., de Bruyn, C. H. M. M., Hatanaka, M., and Hösli, P. (1979) *In Vitro 15*, 103-108.

13. Udenfriend, S. (1962) in *Fluorescence Assay in Biology and Medicine*, Vol. 3, p. 206, Academic Press, New York.
14. Pressman, B. C. (1976) *Annu. Rev. Biochem. 45*, 501-530.
15. Lardy, H., Graven, S. N., and Estrado-O., S. (1967) *Fed. Proc. Fed. Am. Soc. Exp. Biol. 26*, 1355.
16. Pressman, B. C. (1968) *Fed. Proc. Fed. Am. Soc. Exp. Biol. 27*, 1283.
17. Henderson, P. J. F., McGivan, J. D., and Chappell, J. B. (1969) *Biochem. J. 111*, 521.
18. Lutz, W. K., Wipf, H.-K., and Simon, W. (1970) *Helv. Chim. Acta 53*, 1741.
19. Reed, P. W. (1979) *Methods in Enzymology 55*, 435-454.
20. Lichtshtein, D., Dunlop, K., Kaback, H. R., and Blume, A. J. (1979) *Proc. Natl. Acad. Sci. USA 76*, 2580-2585.
21. Smith, J. B., and Rozengurt, E. (1978) *Proc. Natl. Acad. Sci. USA 75*, 5560-5564.
22. Knapp, H. R., Oelz, O., Roberts, L. J., Sweetman, B. J., Oates, J. A., and Reed, P. W. (1977) *Proc. Natl. Acad. Sci. USA 74*, 4251-4255.
23. Martinez Larranga, M. R., and Navarro, A. A. (1975) *Arch. Farmacol. Toxicol. 1*, 111-124.
24. Estrado-O, S., Rightmire, G., and Lardy, H. A. (1968) *Antimicrob. Agents Chemotherap. 1967*, 279-288.