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Mechanism of monensin-induced hyperpolarization of neuroblastoma–glioma hybrid NG108-15

([^{3}H]tetraphenylphosphonium$^+$/ouabain/Na$^+$,K$^+$-ATPase/Na$^+$ uptake/tissue culture)

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ABSTRACT **Addition of the ionophore monensin to mouse neuroblastoma–rat glioma hybrid NG108-15 cells leads to a 20 to 30-mV increase in the electrical potential across the plasma membrane as shown by direct intracellular recording techniques and by distribution studies with the lipophilic cation [^{3}H]-tetraphenylphosphonium$^+$ (TPP$^+$) [Lichtshtein, D., Kaback, H. R. & Blume, A. J. (1979) *Proc. Natl. Acad. Sci. USA* 76, 650–654]. The effect is not observed with cells suspended in high K$^+$ medium, is dependent upon the presence of Na$^+$ externally, and the concentration of monensin that induces half-maximal stimulation of TPP$^+$ accumulation is approximately 1 μM. The ionophore also causes rapid influx of Na$^+$, a transient increase in intracellular pH, and a decrease in extracellular pH, all of which are consistent with the known ability of monensin to catalyze the transmembrane exchange of H$^+$ for Na$^+$. Although ouabain has no immediate effect on the membrane potential, the cardiac glycoside completely blocks the increase in TPP$^+$ accumulation observed in the presence of monensin. Thus, the hyperpolarizing effect of monensin is mediated apparently by an increase in intracellular Na$^+$ that acts to stimulate the electrogenic activity of the Na$^+$,K$^+$-ATPase. Because monensin stimulates TPP$^+$ accumulation in a number of other cultured cell lines in addition to NG108-15, the techniques described may be of general use for studying the Na$^+$,K$^+$ pump and its regulation *in situ*.**

The Na$^+$,K$^+$-activated ATPase (Na$^+$,K$^+$ pump) (ATP phosphohydrolase, EC 3.6.1.3) is present in the membranes of excitable (1) and nonexcitable (2–4) tissues and represents a major pathway for Na$^+$ and K$^+$ transport across the plasma membrane of eukaryotic cells. Moreover, hydrolysis of ATP by this membranous enzyme is often accompanied by the simultaneous movement of three equivalents of Na$^+$ out and two equivalents of K$^+$ into the cell. This inequality in ion movements confers an electrogenic activity to the enzyme. That is, its activity results in the net outward movement of a positive current, which may lead to the generation of a membrane potential ($\Delta\Psi$, interior negative).

Although the resting $\Delta\Psi$ in nerve is due primarily to a K$^+$ diffusion gradient ($[K^+]_{in} > [K^+]_{out}$), electrogenic Na$^+$,K$^+$-ATPase activity makes a contribution to $\Delta\Psi$ in certain cells (5, 6). Moreover, enhanced activity of the pump may have important consequences, because hyperpolarization will result. Presynaptically, this will lead to reduced transmitter release and postsynaptically, to decreased sensitivity to excitatory synaptic stimulation.

Evidence has been presented indicating that the activity (7, 8) and coupling ratio—i.e., Na$^+$ efflux/K$^+$ influx (9, 10)—of the Na$^+$,K$^+$-ATPase are not constant, but are subject to regulation. In liver cells, for example, catecholamines, prostaglandin E_1, and glucagon inhibit the Na$^+$,K$^+$ pump in a manner that is blocked by insulin, and these effects are related to changes in the intracellular concentration of cyclic AMP (11–13). In contrast, in frog skeletal muscle (14), rat soleus muscle (15), sympathetic ganglia (16), mouse brain synaptosomes (17), and atrial tissue (18–20), various catecholamines stimulate the activity of the jump. Finally, microinjection of Na$^+$ into snail ganglia neurons has been shown to stimulate directly the Na$^+$,K$^+$ pump, leading to 20-mV hyperpolarization of the neuronal membrane (7, 8).

We have recently demonstrated (21) that distribution studies with the lipophilic cation [^{3}H]tetraphenylphosphonium$^+$ (bromide salt) (TPP$^+$) can be used quantitatively to determine $\Delta\Psi$ in cultured NG108-15 cells. These cells are excitable and have been shown to synthesize and release the neurotransmitter acetylcholine (22). During the course of these investigations, it was demonstrated that addition of the carboxylic polyether ionophore monensin to the cells leads to an increase in TPP$^+$ accumulation, and electrophysiologic techniques confirmed the interpretation that this effect is due to hyperpolarization of the cell membrane. Because monensin should catalyze the electroneutral exchange of internal H$^+$ for external Na$^+$ under the conditions utilized (23–25), it was postulated that hyperpolarization could result from either of two effects or a combination thereof: (*i*) an increase in intracellular Na$^+$ might stimulate Na$^+$,K$^+$-ATPase activity and thus increase electrogenic pump activity; or (*ii*) by dissipating the Na$^+$ concentration gradient in an electrically neutral fashion, a force that is limiting for $\Delta\Psi$ would be removed and $\Delta\Psi$ might increase without a corresponding increase in pump activity. It was also suggested that differentiation between these alternatives might be possible by testing the effect of ouabain on the increase in TPP$^+$ accumulation induced by monensin. In this communication, the mechanism of monensin-induced hyperpolarization is investigated in detail, and evidence is presented indicating that the effect is due to activation of the Na$^+$,K$^+$ pump secondary to an increase in intracellular Na$^+$. In addition, it is demonstrated that monensin induces increased TPP$^+$ accumulation in a number of other cultured cell lines in addition to NG108-15.

MATERIALS AND METHODS

Cells. NG108-15 is a mouse neuroblastoma–rat glioma hybrid clonal line (26), N1E and NS20 are clonal lines derived from mouse neuroblastoma C1300 (27), and N115 is a clonal line derived from mouse neuroblastoma N1E (27). Starter cultures of these cells were generously provided by M. Nirenberg (National Heart, Lung, and Blood Institute, National Institutes of Health) and E. Richelson (Mayo Foundation, Roch-

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Abbreviations: $\Delta\Psi$, membrane potential; TPP$^+$, tetraphenylphosphonium$^+$ (bromide salt).

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ester, MN). The C6 rat glioma cell line (CCL107) was obtained from the American Type Culture Collection. Suspension cultures of human HeLa-S3 cells, as well as primary chicken embryo cells, were kindly donated by A. Weissbach (Roche Institute of Molecular Biology, Nutley, NJ). With the exception of NG108-15, all cells were grown and maintained in Dulbecco's modified Eagle's medium plus 10% horse serum, penicillin G (100 U/ml), and streptomycin sulfate (10 μg/ml). The medium for NG108-15 cells contained, in addition, 0.1 mM hypoxanthine, 1 μM aminopterin, and 12 μM thymidine.

Harvesting of NG108-15, N1E, and NS20 cells was accomplished by shaking the cells off of culture flasks after they had reached 100% confluency and centrifuging at 250–500 $\times$ *g* for 5 min at room temperature. HeLa-S3 cells were centrifuged directly from suspension cultures. The pellets were diluted at least 1:10 and resuspended in a medium containing 135 mM NaCl or 135 mM choline Cl, 50 mM Hepes, adjusted to pH 7.4 with Tris base, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5.5 mM glucose. The suspensions were then centrifuged again and the cells were resuspended in the same solution to a concentration of 14–50 $\times$ 10^6 cells per ml. Suspensions were incubated at 37°C for 10 min prior to use. Trypsin-detached C6 and trypsin-dissociated primary chicken embryo cells were plated into 16-mm-diameter multiwell dishes (Costar 3524) and incubated at at 37°C for 1–4 days until they became confluent. Immediately prior to use, the medium in the wells was aspirated and the cells were washed once with the choline-containing medium described above.

[^{3}H]TPP$^+$ Uptake. With cell suspensions, uptake of [^{3}H]-TPP$^+$ was measured at 37°C in 500-μl reaction mixtures (total volume) as described (21). Reactions were initiated by adding 50 μl of washed cells previously equilibrated at 37°C to 450 μl of a solution containing 20 μM [^{3}H]TPP$^+$ (7.2 mCi per mmol; 1 Ci = 3.7 $\times$ 10^{10} becquerels) in one of the following mixtures: (*i*) "high Na$^+$ medium"—135 mM NaCl/5 mM KCl/50 mM Tris·Hepes, pH 7.4/1.8 mM $CaCl_2$/0.8 mM $MgSO_4$/5.5 mM glucose; (*ii*) "high K$^+$ medium"—same as high Na$^+$ medium except that 130 mM KCl was used in place of NaCl; and (*iii*) "choline$^+$ medium"—same as high Na$^+$ medium except that 135 mM choline Cl was used in place of NaCl and no KCl was present. Reaction mixtures containing these solutions were equilibrated at 37°C prior to addition of cells. After initiating the reactions, incubations were continued at 37°C for given times, and the reaction vessels were centrifuged in a Brinkman Eppendorf Microcentrifuge (Model 3200) for 1 min. The supernatants were immediately aspirated and the pellets, after resuspension in 1.0 ml of 1% Triton X-100, were transferred to counting vials containing 12 ml of Aquasol, and radioactivity was determined by liquid scintillation spectrometry. Corrections for radioactivity trapped in the extracellular space of the pellets were determined by assaying an aliquot of the supernatant and calculating the total amount trapped from measurements of the extracellular space (21). With cells attached to plastic in multiwell dishes, 1.0 ml of the assay medium containing [^{3}H]TPP$^+$ was added to each well, and, after incubation at 37°C for given times, the medium was aspirated. The contents of each well were then transferred quantitatively to scintillation vials by dissolving the material in 1.0 ml of 0.2 M NaOH containing 0.5% Triton X-100 and washing each well with an additional 1.0 ml of 1% Triton X-100. Assays on cell suspensions were performed in duplicate or triplicate, and assays on attached cells were done in quadruplicate. In either case, replicate values did not vary by more than 5%.

^{22}Na$^+$ Uptake. Reactions were initiated by adding a 50-μl aliquot of cells that were washed and suspended in high Na$^+$ medium to 450 μl of the same medium containing 0.5 μCi of ^{22}Na$^+$ and other components as indicated. Incubations were continued at 37°C for given times, and the reaction mixtures were centrifuged as described above. After we aspirated the supernatants, the pellets were rapidly resuspended in 1.0 ml of ice-cold high Na$^+$ medium and the suspensions were immediately centrifuged. The supernatants were aspirated and discarded, and the ^{22}Na$^+$ content of the pellets was assayed with a gamma counter (Nuclear Chicago Model 1185-28470).

Calculation of TPP$^+$ Concentration Gradients and $\Delta\Psi$. TPP$^+$ concentration gradients and values for $\Delta\Psi$ derived from these values were calculated as described (21) by using intracellular volumes of 2.76 and 2.16 μl per 10^6 cells for cells in high Na$^+$ and high K$^+$ medium, respectively. These values are not significantly different in the presence of monensin. Because intracellular microelectrode recordings from these cells demonstrate that $\Delta\Psi$ across the plasma membrane is completely abolished in high K$^+$ medium (21), TPP$^+$ uptake under these conditions is unrelated to the $\Delta\Psi$ across the plasma membrane. Thus, by subtracting the values obtained for TPP$^+$ accumulation at high external K$^+$ concentrations, that component of the total accumulation due to the $\Delta\Psi$ across the plasma membrane can be approximated (i.e., $[TPP^+]_{in}^{low\ K^+} - [TPP^+]_{in}^{high\ K^+} = [TPP^+]_{in}^{corrected}$). Dividing by the external TPP$^+$ concentration yields the concentration ratio, and insertion of this value into the Nernst equation gives $\Delta\Psi$ $\{\Delta\Psi = -2.3\ RT/F \log [TPP^+]_{in}^{corrected}/[TPP^+]_{out})\}$.

Electrophysiological Measurements. Intracellular recordings were performed on NG108-15 cells by using high-resistance microelectrodes as described (21).

Materials. [^{3}H]TPP$^+$ (2.5 Ci per mmol) was synthesized by the Isotope Synthesis Group of Hoffmann-La Roche under the direction of Arnold Liebman according to methods of S. Ramos, L. Patel, and H. R. Kaback (unpublished results). Carrier-free ^{22}Na$^+$ was purchased from New England Nuclear. Unlabeled TPP$^+$ was obtained from K & K; ouabain was from Calbiochem; and veratridine was from Aldrich. Monensin was generously supplied by J. Berger of Hoffmann-La Roche and batrachotoxin was supplied by J. Daly, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health. All other materials were of reagent grade obtained from commercial sources.

RESULTS

Effect of Monensin on $\Delta\Psi$ in NG108-15 Cells. As shown previously (21), NG108-15 cells suspended in a medium containing physiological concentrations of Na$^+$ and K$^+$ (i.e., high Na$^+$ medium) take up TPP$^+$ rapidly for 5–10 min and achieve a steady-state level of accumulation at 15–20 min that is maintained for at least 50 min. On the other hand, when the cells are suspended in high K$^+$ medium, the steady-state level of TPP$^+$ accumulation is depressed by 50–60%. By using this differential, a $\Delta\Psi$ of -72 ± 4 mV can be calculated from the data shown at "0 time" in Fig. 1*A*. When 20 μM monensin was added to cells that had accumulated TPP$^+$ to a steady state under physiological conditions, the cells immediately began to accumulate more TPP$^+$, and within 10–20 min a new steady state was achieved that was 54% greater than that obtained in the absence of the ionophore (Fig. 1*A*). In contrast, when monensin was added to cells suspended in high K$^+$ medium, there was only a marginal increase in TPP$^+$ uptake (i.e., the steady state was increased by 5%). With cells in physiologic medium, half-maximal stimulation of TPP$^+$ uptake was observed at about 1 μM monensin, and a stable plateau level was observed at concentrations higher than 10 μM (Fig. 1*B*). Alternatively, addition of monensin at concentrations up to 10 μM had no significant effect on TPP$^+$ accumulation by cells in high K$^+$

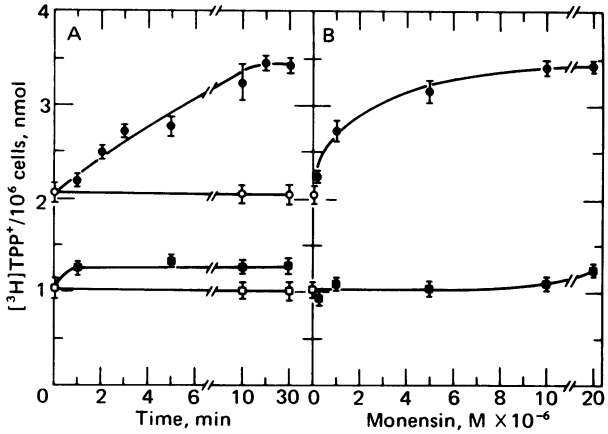

FIG. 1. Effect of monensin on TPP^+ accumulation. NG108-15 cells were incubated in high Na^+ medium (○, ●) or high K^+ medium (□, ■) with 20 μM [^{3}H]TPP^+ for 20 min at 37°C. At this time (t = 0), monensin was added (●, ■) and the incubations were continued at 37°C. (*A*) Increasing time of incubation after addition of 20 μM monensin. (*B*) Effect of given concentrations of monensin during a 20-min incubation. Results are presented as the mean ±SD of four experiments.

medium, and only 5% stimulation was observed at 20 μM. By using values for TPP^+ accumulation obtained in the presence of 10 μM monensin with cells in high Na^+ and high K^+ media, a $\Delta\Psi$ of −93 mV was calculated (i.e., there was apparent hyperpolarization to the extent of −21 mV). Based on similar measurements at various temperatures, monensin-induced hyperpolarization appeared to be relatively constant from 30°C to 37°C (i.e., −20 to −30 mV), but the ionophore had no significant effect on $\Delta\Psi$ at 22°C (Table 1).

Although data will not be presented in detail, similar results were obtained by using direct intracellular recording techniques with NG108-15 cells. With cells impaled at 33°C, local application of 20 μM monensin induced 20–30 mV hyperpolarization (interior negative). On the other hand, the time course of the monensin-induced hyperpolarization measured electrophysiologically was considerably faster than that observed by TPP^+ distribution (i.e., $t_{1/2} = 50 \pm 10$ sec electrophysiologically versus 5 min with TPP^+—see Fig. 1*A*). This discrepancy is not surprising, because the permeability of the membrane to TPP^+ limits the rate at which the cation passively equilibrates with $\Delta\Psi$ (i.e., lipophilic ions are not kinetic probes for $\Delta\Psi$).

Action of Monensin on NG108-15 Cells. The internal Na^+ concentration of NG108-15 cells was approximately 9 mM (unpublished data), and influx of $^{22}Na^+$ under physiologic conditions was slow (Fig. 2). In the presence of ouabain, there was a significant increase in $^{22}Na^+$ influx; however, even by 120 min, the Na^+ concentrations in the intracellular and extracellular compartments had not equilibrated. These observations are consistent with previous findings (22) demonstrating that NG108-15 cells are relatively impermeable to Na^+ and contain a Na^+,K^+-ATPase that catalyzes Na^+ efflux. In contrast to the effect of ouabain, addition of monensin at 22–37°C caused much more rapid uptake of $^{22}Na^+$, and by 20–30 min the intracellular Na^+ concentration approximated that in the external medium. It is important to note that addition of monensin to NG108-15 cells also results in changes in internal and external pH. When the permeant weak acids 5,5′-dimethyloxazolidine-2,4-dione and acetylsalicylic acid were used for monitoring internal pH (28), there was an increase in intracellular pH from pH 7.6 to pH 8.2 that occurred within 1 to 2 min after addition of the ionophore. Within 5 to 7 min, however, intracellular pH returned to pH 7.6 for reasons that are not apparent. When extracellular pH was measured with a rapidly responding pH electrode, monensin caused an instantaneous (within 5 sec) decrease in pH that remains constant for at least 30 min. Finally, the data presented in Table 2 demonstrate that the increase in TPP^+ accumulation induced by monensin is dependent upon the presence of Na^+ in the external medium. Under conditions at which external Na^+ was 121 mM, monensin produced about −22 mV hyperpolarization, whereas in the absence of Na^+ this effect was almost completely abolished (i.e., $\Delta\Psi$ increased by only −2 mV on addition of monensin). Taken as a whole, the observations are consistent with the known ability of monensin to catalyze the transmembrane exchange of H^+ for Na^+.

Because the contention that monensin catalyzes an electrically neutral exchange is important for some of the arguments to be presented, we also compared the effect of the ionophore with the effects of veratridine and batrachotoxin, two agents that induce electrogenic Na^+ influx and thus depolarize NG108-15 cells (22, 29). Clearly, treatment of the cells with either agent resulted in complete depolarization, as evidenced by TPP^+ distribution studies (Table 2). In contrast, although monensin caused a marked increase in Na^+ uptake (Fig. 2), the ionophore did not depolarize NG108-15 cells, even in the presence of ouabain (see Fig. 3).

Mechanism of Monensin-Induced Hyperpolarization. The experiments presented in Fig. 3 provide direct evidence that

Table 1. Effect of monensin on steady-state levels of TPP^+ accumulation at different temperatures

Incubation temperature, °C	Medium	Monensin (20 μM)	TPP^+ uptake, nmol/10^6 cells	$\Delta\Psi$*, mV
	High Na^+	−	1.16	−41
22	High K^+	−	0.69	0
	High Na^+	+	1.19	−45
	High Na^+	−	2.28	−61
30	High K^+	−	1.35	0
	High Na^+	+	3.31	−89
	High Na^+	−	2.33	−70
37	High K^+	−	1.20	0
	High Na^+	+	3.65	−99

NG108-15 cells washed in high Na^+ medium were resuspended in a given medium. [^{3}H]TPP^+ uptake was assayed after a 20-min incubation at the temperature indicated.

* $\Delta\Psi$ was calculated from the difference in TPP^+ uptake by cells in high Na^+ and high K^+ media as described (21) and in *Materials and Methods*.

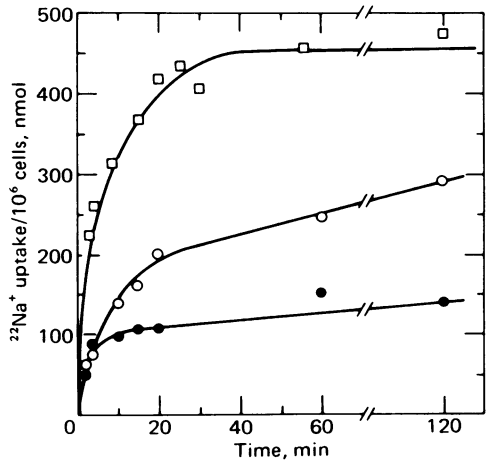

FIG. 2. Effect of ouabain and monensin on $^{22}Na^+$ uptake by NG108-15 cells. The reaction mixtures contained no additions (●), 0.1 mM ouabain (○), or 20 μM monensin (□).

Table 2. Effect of monensin, veratridine, and batrachotoxin on steady-state levels of TPP^+ accumulation by NG108-15 cells in various media

Medium	Additions	TPP uptake, nmol/10^6 cells	$\Delta\Psi$*, mV	Change in $\Delta\Psi$, mV
Na^+_{121} $K^+_{4.5}$ $choline^+_{13.5}$	—	2.33 ± 0.03 (8)	−76.7 ± 1	
	Monensin	3.65 ± 0.02 (8)	−99.0 ± 0.5	−22.3
	Veratridine	1.21 ± 0.08 (4)	0 ± 1.0	+76.7
	Batrachotoxin	1.18 ± 0.05 (4)	0 ± 0.6	+76.7
Na^+_{121} $choline^+_{13.5}$	—	2.43 ± 0.03 (6)	−79.3 ± 1	
	Monensin	3.10 ± 0.10 (6)	−91.9 ± 3	−12.6
$Choline^+_{13.5}$	—	2.45 ± 0.07 (4)	−79.8 ± 2.3	
	Monensin	2.56 ± 0.17 (4)	−82.2 ± 5.5	−2.4

NG108-15 cells were washed and resuspended in a given medium with indicated final concentrations of the major monovalent cations (in millimolar). In all cases, the anion used was chloride. Where indicated, the reaction mixtures also contained monensin, veratridine, or batrachotoxin at final concentrations of 20 μM, 500 μM, and 1.8 μM, respectively. [^{3}H]TPP^+ uptake was assayed after a 20-min incubation at 37°C. The results are presented as the mean ±SD, with the number of experiments given in parentheses.

* $\Delta\Psi$ was calculated from the difference in TPP^+ uptake by cells in high Na^+ and high K^+ media as described (21) and in *Materials and Methods*.

the hyperpolarization induced by monensin resulted from activation of the Na^+,K^+-ATPase. Ouabain at concentrations known to inhibit Na^+,K^+-ATPase in NG108-15 cells (22) had little or no effect on TPP^+ accumulation during short-term incubations (i.e., 10–20 min; see ref. 25). Thus, the resting $\Delta\Psi$ in these cells resulted almost entirely from a K^+ diffusion gradient that was maintained for relatively long periods of time in the absence of pump activity (21). On the other hand, the cardiac glycoside completely blocked the increase in TPP^+ accumulation observed in the presence of monensin (Fig. 3). In the experiment shown, NG108-15 cells were allowed to accumulate TPP^+ to a steady state, at which time various concentrations of ouabain were added in the absence or presence of 10 μM monensin. In confirmation of previous results (21), ouabain had little or no effect on the steady-state level of TPP^+ accumulation in the absence of the ionophore, and, as expected, addition of monensin in the absence of ouabain led to enhanced TPP^+ accumulation (see Fig. 1 also). However, this enhancement was gradually and completely abolished as the ouabain concentration was increased to 10 mM with half-maximal inhibition at about 0.1 mM.

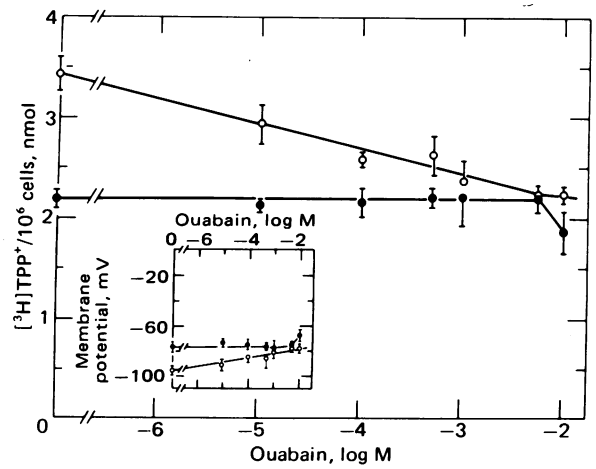

FIG. 3. Effect of ouabain on monensin-induced increase in [^{3}H]TPP^+ accumulation. NG108-15 cells were incubated in high Na^+ medium with 20 μM [^{3}H]TPP^+ for 20 min at 37°C. Monensin (10 μM final concentration) was then added to indicated samples (○) with given concentrations of ouabain. Incubations were continued at 37°C for 10 min. ●, Samples incubated with given concentrations of ouabain in the absence of monensin. (*Inset*) Data plotted in millivolts. Results are presented as the mean ±SD of four experiments.

In this context, it is interesting that monensin induced a significantly smaller increase in TPP^+ accumulation when NG108-15 cells were suspended in a medium devoid of K^+ (Table 2). This observation was also consistent with the argument that monensin-induced hyperpolarization involved the Na^+,K^+-ATPase, because the activity of this enzyme is sensitive to external K^+ as well as internal Na^+ (4). An absolute dependence on external K^+ is probably impossible to demonstrate because K^+ would be expected to leak from the cells continuously during the course of the experiments.

Effect of External K^+ and Monensin on TPP^+ Accumulation by Cells Other Than NG108-15. As shown by the data presented in Table 3, high external K^+ caused a significant decrease in TPP^+ accumulation in three different mouse neuroblastoma cell lines, rat glioma C6 cells, human HeLa-S3 cells, and chicken embryo fibroblasts, in addition to NG108-15 cells. Moreover, in each case, addition of monensin resulted in increased TPP^+ accumulation under appropriate conditions (i.e., when Na^+ was present externally). The results imply that a K^+ diffusion gradient makes a significant contribution to $\Delta\Psi$ in each of these cell lines and that monensin may lead to hyperpolarization in a manner similar to that described for NG108-15 cells.

DISCUSSION

The results presented here confirm and extend previous findings (21) showing that addition of the ionophore monensin to NG108-15 cells leads to an increase in the electrical potential across the plasma membrane. Moreover, the evidence presented suggests that this phenomenon can be elicited in various other cultured eukaryotic cells.

Clearly, the effects of monensin on NG108-15 cells are consistent with its ability to catalyze the transmembrane exchange of H^+ for Na^+. Monensin-induced hyperpolarization requires the presence of Na^+ in the medium, and the ionophore causes a marked increase in Na^+ uptake, a transient increase in intracellular pH, and a decrease in extracellular pH. Although the stoichiometry of the H^+ and Na^+ movements catalyzed by monensin in this system have not been determined, the exchange is probably electrically neutral (i.e., one intracellular H^+ is exchanged for one extracellular Na^+) for the following reasons: (*i*) monensin has no effect on $\Delta\Psi$ in the presence of ouabain; and (*ii*) unlike electrogenic Na^+ uptake induced by the depolarizing agents veratridine and batracho-

Table 3. Effect of high external K^+ and monensin on TPP^+ uptake by various cultured cells

Cells	Culture conditions	TPP^+ uptake, nmol/mg protein			Accumulation ratios	
		High Na^+			High K^+	High Na^+ + monensin
		Control	+ Monensin	High K^+	High Na^+	High Na^+
Hybrid NG108-15	Suspension	2.39	3.97	0.93	0.39	1.66
Mouse neuroblastoma						
N1E	Suspension	1.40	2.06	0.90	0.64	1.47
N115	Suspension	1.44	2.33	0.73	0.51	1.62
NS20	Suspension	2.29	4.00	1.00	0.44	1.75
Rat glioma C6	Attached	5.31	7.46	1.95	0.37	1.41
Human HeLa-S3	Suspension	1.20	2.16	0.74	0.62	1.82
Chicken embryo fibroblasts	Attached	3.98	5.26	1.45	0.36	1.32

[^{3}H]TPP^+ uptake was assayed after a 20-min incubation at 37°C with indicated cells in a given medium. Where indicated, monensin was added to the reaction mixtures at a final concentration of 20 μM.

toxin, monensin-catalyzed Na^+ uptake does not lead to depolarization. Finally, the observation that the effect of monensin on TPP^+ accumulation is almost completely abolished when the external K^+ concentration is raised makes it highly improbable that intracellular mitochondria play a significant role in the phenomenon.

Because monensin causes an increase in Na^+ uptake by an apparently electroneutral mechanism and its effect on TPP^+ accumulation is blocked by ouabain, it is reasonable to suggest that monensin-induced hyperpolarization is mediated by an increase in intracellular Na^+ that acts on the Na^+,K^+-ATPase. The precise nature of the increased electrogenic contributions of the pump may be complicated, however. Although ouabain has no immediate effect on $\Delta\Psi$, the Na^+,K^+-ATPase is apparently functional under resting conditions because ouabain causes a significant increase in Na^+ influx (Fig. 2) as well as a decrease in K^+ influx (unpublished data). This apparent absence of electrogenic activity could be due either to compensating anion movements or to the coupling ratio of the pump under resting conditions (i.e., Na^+ efflux/K^+ influx might be 1:1). Thus, hyperpolarization induced by increased intracellular Na^+ could result from either an increase in the absolute activity of the Na^+,K^+ pump or a change in the coupling ratio.

During the preparation of this manuscript, a paper by Smith and Rozengurt (30) appeared in which it was demonstrated that monensin and gramicidin, and in addition, serum and purified growth factors cause increased $^{86}Rb^+$ uptake by 3T3 mouse fibroblasts in a manner that is inhibited by ouabain. Because increased Na^+ uptake was also observed, the authors concluded that these agents stimulate the Na^+,K^+ pump by increasing intracellular Na^+ and postulated that this effect may be responsible for the proliferative response of 3T3 cells to serum and growth factors. The results presented here are in complete accord with these observations and suggest, moreover, that the effects of serum and growth factors on certain cells may result from an increase in $\Delta\Psi$ rather than an increase in Na^+,K^+-ATPase activity *per se*.

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