

Different Effect of Ouabain on Interferon Production and Action (38753)

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Ouabain is known to inhibit specifically the function of membrane adenosinetriphosphatase (ATPase) which determines the intracellular concentration of potassium and sodium (1). The integrity of this Na/K dependent ATPase is required for the effect of several hormones on cell metabolism (2) (3). Ouabain also inhibits lymphocyte transformation by phytohemagglutinin *in vitro* (4).

In a preliminary publication (5) we reported that the antiviral effect of primate interferon was blocked by ouabain. This effect was reversed by adding K ions in increasing concentrations to tissue culture medium.

The aim of this work is to study the mechanism of the anti-interferon effect of ouabain and to determine the possible involvement of membrane ATPase in interferon induction and action.

Material and Methods. Cell lines. African Green monkey cells, BSC-1, and mouse L cells were used in this study. The ATPase of BSC-1 cells is blocked for 2×10^{-7} M amounts of ouabain (5) and that of L cells is resistant to amounts of 10^{-5} M or more (6). All cells were grown in Eagle's essential medium (MEM) supplemented with tryptose phosphate broth and 10% heat-inactivated calf serum.

A previously described somatic monkey-mouse hybrid cell system, MKCV^{III} (7) was also employed. These hybrid cells are sensitive to both murine and primate interferons.

Viruses. Vesicular stomatitis virus (VSV), Indiana strain (titer: 1×10^7 PFU/ml) or encephalomyocarditis (EMC) virus (titer: 2×10^9 PFU/ml), were propagated in mouse L cells. Viral titers were estimated by routine plaque techniques.

Newcastle disease virus (NDV), Hertfordshire strain, titering 4×10^9 egg infectious doses 50 (ED₅₀)/ml, was prepared

in 9-day-old embryonated chicken leghorn eggs.

Interferon production. Mouse interferon was obtained by infecting MSV-IF⁺ cells with NDV (8). Primate interferon was prepared using BSC-1 cells induced by NDV. After viral adsorption, the cells were washed and maintained in MEM without calf serum. The supernatants were collected 18 h after induction and stored at pH 2 for 5 days. Before utilization, the interferons were centrifuged for 15 hr at 23,000 rpm in a SW 25-1 rotor.

Interferon assay. Mouse interferon was titered in comparison with the NIH reference and primate interferon with the INSERM reference. Interferon was incubated with the sensitive cells for 18 hr. The interferon titer was expressed as the dilution which inhibited 90% of the replication of the challenge virus. In occasional experiments, the interferon titer was also estimated by the inhibition of 50% of the cytopathogenic effect (CPE) of the challenge virus.

Estimation of the ouabain effect on cellular RNA or protein synthesis. Incorporation of radioactive precursors was studied in BSC-1 cells (grown in 35×10 mm plastic petri dishes) in the presence or absence of ouabain. ³H-uridine (2 μCi) and a mixture of ¹⁴C-amino acids (1 μCi) diluted in phosphate buffered saline (PBS) were added to the cells and incubated for 30 min at 37°. The cells were then washed 3 times with PBS chilled to 4°, scraped, and treated with chilled 10% trichloroacetic acid (TCA). After filtration and three subsequent washings with 5% TCA, the acido-precipitable fraction was hydrolyzed with concentrated NH₄ OH. A sample was counted in a Tricarb liquid scintillation spectrometer using Brays fluid. These radioactive precursors were purchased from the Centre de l'Énergie Atomique (C.E.A.), Saclay, France.

Ouabain. Crystalline G strophanthin (oua-

bain) was obtained from Merck. The stock solution was dissolved in MEM at a concentration of 200 $\mu\text{g}/\text{ml}$.

Results. Establishment of antiviral resistance of BSC-1 cells treated by ouabain at different time intervals after contact with interferon. Seven sets of BSC cell preparations (grown in hemolysis tubes) were treated with 1000 reference units of primate interferon. Ouabain ($2.6 \times 10^{-7} M$) was added to the cells at the same time and at selected intervals after interferon. For each sample, interferon and ouabain were kept in contact with the cells for 5 hr, removed thereafter, and the cells fed with fresh serum-free MEM for a total of 20 hr at 37° . After a new washing, the cells were challenged with VSV at the multiplicity of infection (MOI) = 1 PFU/cell. The antiviral resistance induced was estimated by viral yield inhibition at the end of the first replicative cycle. As shown in Fig. 1, the anti-interferon activity of ouabain was highest when added to the cells simultaneously with interferon, decreased rapidly

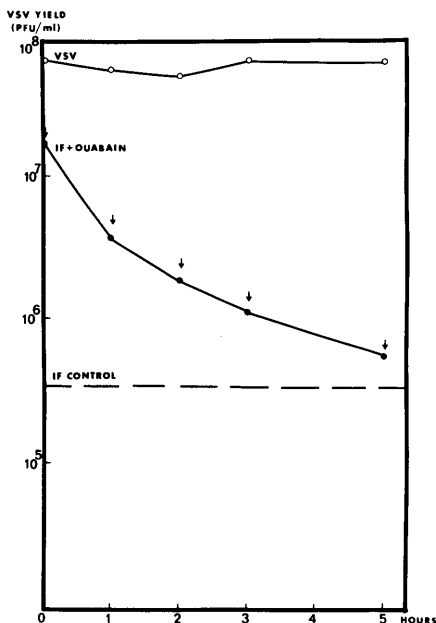


FIG. 1. Establishment of antiviral resistance of BSC cells treated by ouabain at different time intervals after contact with interferon. At each point BSC cells were challenged with VSV only 20 hr after interferon treatment. The viral yield is titrated after one replicative cycles.

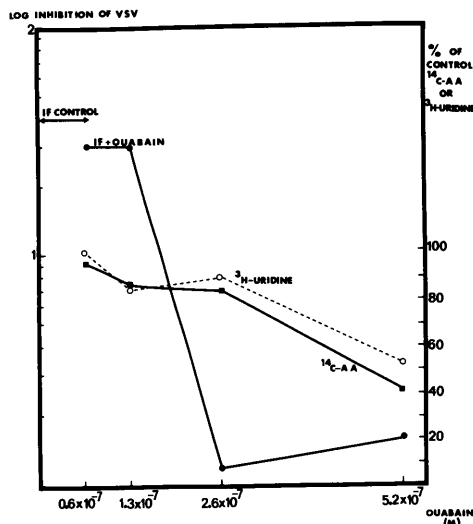


FIG. 2. Effect of ouabain on interferon action and on the incorporation of ^{14}C amino acids (■) and ^3H -uridine (○) in the acido-insoluble fraction of BSC cells. Results are expressed as the % of radioactivity in cells treated with ouabain compared to untreated cells. (●) = antiviral effect of interferon expressed by the log inhibition of VSV.

after the first hour, more slowly thereafter, and was almost nil at 5 hr. Thus, ouabain inhibits only the establishment of the antiviral state.

It is known that the antiviral effect of interferon requires the integrity of cellular protein synthesis. We therefore explored the effect of ouabain on RNA and protein synthesis using ^3H -uridine and a mixture of ^{14}C -amino acids. In parallel sets of experiments, the cells were pulse-labeled (as described in Material and Methods) in the presence of increasing concentrations of ouabain in interferon-treated or untreated cells. The results are summarized in Fig. 2. Concentrations of ouabain ($2.6 \times 10^{-7} M$), which did not significantly affect cellular RNA or protein synthesis, inhibited almost completely the antiviral effect of interferon.

Lack of effect of ouabain on primate and mouse interferon in monkey-mouse hybrid cells. MKCV^{III} Clone 4 hybrid cells, sensitive to both mouse and primate interferons, were used. Membrane ATPase activity in these cells was not blocked with high amounts of ouabain ($10^{-5} M$) (unpublished data), thus their resistance to this substance

was comparable to that of the parental murine cells. This observation is important since these hybrid cells contain detectable amounts of monkey antigens in the cell membrane, as shown by fluorescent-labeled antibodies to monkey cells (C. Grunewald-Krembel and R. Wicker, unpublished experiments). The hybrid clone contains an almost complete set of mouse chromosomes, while the number of monkey chromosomes is about 20 (7). In addition, after induction, both mouse and monkey interferons are released in the medium. Even in the presence of considerable amounts of ouabain, no anti-interferon effect was observed either with mouse or with human interferon (Table I). This experiment seems to indicate that ouabain does not compete with interferon for cell membrane receptor sites since concentrations 1000 times higher than those required to block the antiviral action of interferon in BSC cells are ineffective in these hybrids.

Interferon production in the presence of ouabain. Interferon was induced in several sets of BSC cells with NDV at the MOI = 100 ED 50/cell. After 1 hr of adsorption, the virus was eliminated and the cells maintained in MEM with increasing doses of ouabain (1.3×10^{-7} – 10^{-5} M). Controls consisted of BSC cells induced without ouabain and BSC cells noninfected by NDV and treated or not with ouabain. After an 18-hr incubation period at 37°, the cell supernatants were collected and dialyzed for 5 days at pH 2 to inactivate the residual inducing virus and to eliminate the ouabain from the preparation.

TABLE I. LACK OF INHIBITORY EFFECT OF OUABAIN ON MURINE AND PRIMATE INTERFERON ACTIVITIES IN MONKEY-MOUSE HYBRID CELLS.

	Ouabain (M)	EMC ^c (PFU/ml)
Mouse IF ^a	—	1.3×10^5
Mouse IF	1.3×10^{-5}	1.6×10^5
Primate IF ^b	—	6×10^5
Primate IF	1.3×10^{-5}	7×10^5
MEM	—	1×10^7
MEM	1.3×10^{-5}	1.3×10^7

^a Mouse interferon = 500 reference units.

^b Primate interferon = 1000 reference units.

^c The viral yield is measured at the end of the first replicative cycle.

TABLE II. LACK OF INHIBITORY EFFECT OF OUABAIN ON INTERFERON PRODUCTION IN BSC CELLS.^a

Cell line	Ouabain (M)	Log VSV yield
		Control IF-treated cells
BSC + NDV	0	3.22
BSC + NDV	1.3×10^{-7}	3.27
BSC + NDV	1.3×10^{-6}	3.25
BSC + NDV	1.3×10^{-5}	3.25
BSC + MEM	1.3×10^{-6}	0.52
BSC + MEM	1.3×10^{-5}	0.57
BSC + MEM	0	0

^a BSC cells (5×10^6) were infected with NDV at the MOI = 100 ED 50/cell. The cells were then kept in contact with MEM and ouabain for 18 hr and the amount of interferon eliminated in the supernatants was titrated by VSV yield inhibition. The results are expressed as the log of the viral yield in control cells/the log of the viral yield in interferon-treated cells.

The results are presented in Table II. Ouabain did not decrease interferon production in BSC cells at a concentration 100 times greater than the one used to block interferon action in the same cells. In addition, it had equally no effect on interferon production when added before induction. As shown in Table III, ouabain (1.3×10^{-6} M), kept in the tissue culture medium for 18 hr, did not affect interferon production when NDV was added to the cells 1 h before, at the same time, or 1 hr after ouabain. These results are important in view of the possible secondary effect of ouabain on cellular protein synthesis.

Diminution of the antiviral state in NDV-induced BSC cells in the presence of ouabain. Since interferon production is not affected by ouabain, whereas interferon action is sensitive to this substance, it was of interest to explore the interferon sensitivity of cells induced to produce interferon.

BSC cells were infected with NDV at the MOI = 100 ED 50/cell in the presence of increasing concentrations of ouabain. After 18 hr the supernatants were harvested and assayed for interferon activity. The antiviral state induced in the interferon-producing cells was estimated using EMC at the MOI = 1 PFU/cell. The viral yield was assayed in mouse L cells (Table IV). Interferon produc-

tion was not affected by ouabain; however, the antiviral state in these interferon-producing cells was diminished almost 100-fold. The different concentrations of ouabain employed had no detectable effect on the viral yield.

Discussion. Ouabain inhibits the establishment of the antiviral state in BSC cells. In somatic monkey-mouse hybrid cells (sen-

sitive to primate interferon) no such effect was observed since mouse ATPase, insensitive to 10^{-5} M of ouabain, has probably compensated for the sensitive primate variant. It can be concluded, therefore, that the inhibitory effect of ouabain could not be explained by a competition between ouabain and interferon for the same cell receptor sites. It could also not be due to an inhibition of cellular protein synthesis. Moreover, in the presence of ouabain and interferon, the protein (s) involved in the establishment of the antiviral state is (are) apparently not synthesized. This is based on the fact that after the removal of ouabain and interferon, followed by further incubation of the cells for 15 hr, no antiviral resistance appears in the cells. It seems, therefore, that the inhibition of cell membrane ATPase by ouabain is followed by the blockage of an early metabolic step which occurs after the interaction of interferon with the cell receptors. This step is probably needed for the development of the antiviral state.

The role of the membrane ATPase in the interferon cell receptor system is not understood. Ouabain could change the structural configuration of interferon receptors by

TABLE III. INTERFERON PRODUCTION IN BSC CELLS PRETREATED WITH OUABAIN (1.3×10^{-6} M).

Hours	0	1	2...	IF yield ^a 18
	Ouabain	NDV	MEM	200/400
	MEM	NDV Ouabain	MEM	400
	MEM	NDV	MEM Ouabain	400
	Ouabain	—	—	<2
	MEM	NDV	MEM	200/400

^a The interferon titer is expressed in reference units and estimated by the inhibition of 50% of the CPE of the challenge virus.

TABLE IV. DECREASE OF ANTIVIRAL RESISTANCE IN INTERFERON-YIELDING BSC CELLS IN THE PRESENCE OF OUABAIN.

	Ouabain Concentration (M)	Exp. 1		Exp. 2		Exp. 3	
		IF titer ^a	EMC ^b (PFU/ml)	IF titer	EMC (PFU/ml)	IF titer	EMC (PFU/ml)
BSC + NDV	1.3×10^{-8}	400	6×10^4	ND ^c	ND	ND	ND
BSC + NDV	1.3×10^{-7}	400	1.2×10^6	200	1.5×10^7	200	8×10^6
BSC + NDV	2.6×10^{-7}	400	3.2×10^6	200	1.5×10^7	200	1×10^7
BSC + NDV	5.2×10^{-7}	400	2.5×10^6	200	1×10^7	ND	ND
BSC + NDV	0	400	8×10^4	200	1×10^5	200	3×10^5
BSC	1.3×10^{-8}	<2	2×10^8	ND	ND	ND	ND
BSC	1.3×10^{-7}	<2	2.7×10^8	<2	1.5×10^8	<2	1.8×10^8
BSC	2.6×10^{-7}	<2	3×10^8	<2	1×10^8	<2	2.3×10^8
BSC	5.2×10^{-7}	<2	3.6×10^8	<2	2×10^8	ND	ND
BSC	0	<2	2.6×10^8	<2	8×10^7	<2	1.5×10^8

BSC cells (2×10^6) were induced with NDV at the MOI = 100 ED 50/cell. The cells were then kept in contact with MEM and ouabain for 18 hr and the interferon yield was titrated using VSV as the challenge virus.

^a The interferon titer is expressed in reference units.

^b The antiviral state in interferon-yielding cells is evaluated by EMC viral yield.

^c ND = not done.

reversing the Na/K flux. It could also affect membrane adenylyl-cyclase, since the stimulation of a thyroid adenylyl-cyclase by T.S.H. is not obtained after ouabain treatment of the cells (3).

If necessary for interferon action, the integrity of the Na/K pump is not required, on the contrary, for the induction of this substance. Indeed, when BSC cells are infected by NDV (at a MOI = 100 ED 50/cell) ouabain does not affect interferon production. However, the antiviral state in these same interferon-producing cells is greatly decreased.

It is yet an unsolved problem whether the interferon-producing cells are themselves protected or not by the interferon they release. Our experiments are in favour of the second possibility. It is likely that the antiviral state in the cells is mainly induced after extracellular diffusion of interferon followed by the triggering of a specific cell membrane receptor system.

Summary. Ouabain, which inhibits specifically membrane-bound ATPase activity, also inhibits the establishment of the antiviral state induced by interferon. Once the antiviral state is established, ouabain is in-

effective. This inhibitory effect is reversed by adding Na/K ions to the cells. On the contrary, interferon production is unaffected by the same concentrations of ouabain. It is of interest that in such interferon-yielding cells, ouabain decreases the antiviral state.

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1. Schatzmann, H. J., *Helv. Physiol. Pharmacol. Acta* **11**, 346 (1953).
2. Blatt, L. M., *J. Biol. Chem.* **247**, 6551 (1972).
3. Burke, G., *Biochim. Biophysica Acta* **220**, 30 (1970).
4. Quastel, M. R., and Kaplan, J. G., *Nature (London)* **219**, 198 (1968).
5. Lebon P., and Moreau, M. C., *C. R. Acad. Sci. Paris* **276**, 3061 (1973).
6. Lamb, J. F., and MacKinnon, M., *J. Physiol.* **213**, 665 (1971).
7. Cassingena, R., Chany, C., Vignal, M., Suarez, H., Estrade, P., and Lazar, P., *Proc. Nat. Acad. Sci. U.S.A.* **68**, 580 (1971).
8. Chany, C., and Vignal, M., *C. R. Acad. Sci. Paris* **267**, 1798 (1968).

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