AMANTADINE HYDROCHLORIDE: INHIBITOR OF CLEAVAGE AND DNA SYNTHESIS IN SEA URCHIN OVA

PIERRE SOUPART,* ROBERT A. RINALDI†
AND VIRGINIA M. PICKEL†

**Department of Obstetrics and Gynecology and †Department of Anatomy,
Vanderbilt University School of Medicine, Nashville, Tennessee 37203, U.S.A.*

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Amantadine hydrochloride (Symmetrel, E. I. Du Pont de Nemours and Co.), prevents entry of some influenza viruses into host cells (Hoffmann, Newmayer, Haff & Goldsby, 1965). Since sperm penetration into mammalian ova may be accomplished by mechanisms similar to those of virus penetration (Soupert & Noyes, 1964; Soupert & Clewe, 1965), we have investigated the effects of amantadine on a model fertilization system, the egg of the sea urchin, *Arbacia punctulata*. In these ova, a fertilization membrane is observed within 2 min after mixing spermatozoa and eggs, syngamy and first cleavage are completed within 15 and 70 min, respectively, at 23° C and pH 8.2 in artificial sea water (Harvey, 1956).

Gametes were collected by electrical stimulation, and the jelly coat of eggs was removed with HCl (Harvey, 1956). The reactivity of ova to increasing concentrations of amantadine was measured by determining the percentage of ova with fertilization membrane and the percentage of ova cleaving (on 100 ova samples). The pH of amantadine solutions in artificial sea water was adjusted to 8.2 by addition of anhydrous sodium carbonate. Ova from several females, exhibiting more than 90% fertilization in preliminary tests, were pooled and treated as follows: (1) fertilized and incubated in the presence of amantadine; (2) incubated for 10 min with amantadine, then washed three times with fresh sea water before fertilization; (3) incubated with amantadine 10 min after fertilization; (4) incubated with amantadine after parthenogenetic stimulation by hypertonic shock for 17 to 21 min (Harvey, 1956).

When fertilization occurred in presence of amantadine, the first cleavage was inhibited as a function of concentration (Table 1). Fifty per cent inhibition of cleavage was obtained for concentrations ranging from 25 to 50 µg/ml, and almost total inhibition was observed for concentrations of 200 µg/ml and higher. Percentages of fertilization membrane formation were, in all cases, slightly higher than those for cleavage. Transfer of fertilized ova to sea water containing amantadine inhibited cleavage in the same concentration range. Fertilization membrane formation and subsequent cleavage were also inhibited in ova in-

† Present address: Department of Anatomy, Georgetown University School of Medicine, Washington, D.C., 20007.

cubated 10 min with amantadine and washed three times before fertilization. Cleavage following parthenogenetic activation was inhibited by amantadine in the manner described for fertilized ova. At concentrations inhibiting more than 50% cleavage, amantadine induces chemical changes in echinochrome as evidenced by a shift from bright orange to dull brown and clumping of pigment granules. At these concentrations, the fertilization membranes that still formed were irregular, eccentric or incompletely surrounded the ova. Sperm motility and fertilizing ability were not significantly altered by amantadine treatment at any concentration tested.

TABLE 1
EFFECTS OF AMANTADINE HYDROCHLORIDE (A-HCL) ON THE CLEAVAGE OF THE EGG OF ARBACIA PUNCTULATA

Amantadine hydrochloride ($\mu\text{g/ml}$) (M)	Mean % of cleavage (and S.E.)			
	Fertilization and incubation in A-HCl (14 expts)	Incubation in A-HCl 10 min after fertilization (4 expts)	Incubation 10 min in A-HCl, wash, fertilization (11 expts)	Parthenogenetic stimulation, wash, incubation in A-HCl (6 expts)
10 0.53×10^{-4}	77 (6.40)	88 (7.02)	91 (9.03)	90 (4.38)
25 1.32×10^{-4}	54 (12.91)	83 (11.87)	88 (12.70)	69 (14.82)
50 2.65×10^{-4}	47 (8.14)	72 (22.82)	66 (10.55)	57 (14.63)
75 3.98×10^{-4}	27 (9.10)	—	39 (21.73)	36 (9.36)
100 6.3×10^{-4}	26 (7.32)	60 (17.33)	20 (7.41)	30 (8.21)
200 1.25×10^{-3}	5 (2.74)	18 (3.65)	15 (7.20)	27 (7.83)
300 1.89×10^{-3}	0	2 (1.44)	4 (2.77)	9 (4.31)
400 2.52×10^{-3}	0	0	4 (2.18)	4 (1.48)
500 3.15×10^{-3}	0	0	1.5 (1.31)	0
Controls	96 (1.44)	99 (0.71)	93 (2.22)	87 (5.86)

Amantadine seems to attach firmly to some egg structure(s) or immediately causes irreversible damage, since its action was not reversed by extensive washing before fertilization. The drug acts at least on two different mechanisms: fertilization membrane formation and cleavage. This is indicated by: (1) inhibition of both fertilization membrane formation and cleavage in eggs treated with this drug before activation; (2) inhibition of cleavage following amantadine treatment of eggs in which activation was triggered either by spermatozoa or parthenogenetic stimulation. Inhibition of cleavage suggested that the effects of amantadine are comparable to those of a mitotic blocking agent such as colchicine. In a series of six experiments, the mean percentage (S.E.) cleavage in the presence of colchicine was as follows: 93 (3.29), 90 (3.87), 9.2 (3.81), 0.2 (0.05), 0% for concentrations of 5.0×10^{-5} , 7.5×10^{-5} , 1.0×10^{-4} , 2.5×10^{-4} and 5.0×10^{-4} M, respectively. No colour change in echinochrome was observed in presence of colchicine. With amantadine, 50% cleavage inhibition was obtained for a concentration of about 2.65×10^{-4} M; whereas with colchicine, the same degree of inhibition was obtained at about 1.75×10^{-4} M as deduced from the data. When cleavage inhibition curves for colchicine and amantadine were compared, it was found that the effect of amantadine was gradual whereas that of colchicine was sudden. Colchicine blocks cleavage

PLATE 1

Representative autoradiographs of fertilized ova sampled after 2 hr continuous incubation in [^3H]thymidine.

FIG. 1(a). No drug treatment, two-cell stage, active DNA synthesis in nuclei.

FIG. 1(b). Colchicine (1.0×10^{-3} M), cleavage block, active DNA synthesis in pronuclei.

FIG. 1(c). Amantadine hydrochloride (100 $\mu\text{g/ml}$), two-cell stage, depressed DNA synthesis in nuclear centre.

FIG. 1(d). Amantadine hydrochloride (300 $\mu\text{g/ml}$), cleavage block, strongly inhibited DNA synthesis in pronuclear centre and periphery.

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by disruption of the spindle proteins of the mitotic apparatus (Inoue & Sato, 1967) but does not affect DNA synthesis (Zimmerman & Zimmerman, 1967).

In a further comparison of the effects of amantadine and colchicine, DNA synthesis was evaluated by autoradiography. Ova were continuously incubated with [^3H]thymidine (2.0 $\mu\text{C}/\text{ml}$, specific activity 6.7 $\text{C}/\text{m-mole}$) in the presence of amantadine or colchicine, beginning 10 min before fertilization. Ova were sampled 2 hr following fertilization, fixed in Bouin's solution, dehydrated, embedded in Araldite, and sectioned at 2 μ . The sections were washed in cold 5% trichloroacetic acid, thoroughly rinsed in water, coated with Kodak's NTB2 emulsion, exposed for 28 days at 10° C and stained with thionine. Ova fertilized in the absence of amantadine or colchicine exhibited normal DNA synthesis and cleavage (Pl. 1, Fig. 1a). Although colchicine-treated ova ($1.0 \times 10^{-3} \text{ M}$) did not cleave, there was active DNA synthesis in the pronuclei (Pl. 1, Fig. 1b). In amantadine-treated ova ($6.3 \times 10^{-4} \text{ M}$), there was an inhibition of cleavage and a decrease in DNA synthesis in both non-cleaving and cleaving ova (Pl. 1, Fig. 1c). At higher concentrations (1.9 to $3.1 \times 10^{-3} \text{ M}$), there was an arrest of cleavage with a striking inhibition of DNA synthesis (Pl. 1, Fig. 1d). The inhibition of DNA synthesis was characterized by the tracer being localized at the periphery of the nucleus and not within its central portion. This may indicate that DNA synthesis in sea urchin eggs is initiated at the nuclear membrane as recently shown for human heteroploid amnion cells (Comings & Kakefuda, 1967). Amantadine inhibition could be achieved therefore by considerably retarding DNA synthesis.

The original purpose of our investigation was to test the effects of amantadine on sperm penetration into sea urchin ova. The data did not indicate a block to sperm penetration but revealed a definite antizygote effect in this model fertilization system.

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