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ENHANCEMENT OF THE TOXIC EFFECTS OF VERATRINE ON GUINEA-PIG ATRIUM BY THRESHOLD INOTROPIC DOSES OF OUABAIN

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Isolated atria of guinea-pigs were treated with veratrine until the initial signs of toxicity were seen. Ouabain was then added cumulatively, starting with a threshold inotropic concentration, 50 nM, until the tissue became dysrhythmic. It was found that a concentration of ouabain which by itself gave a positive inotropic effect of only 3%, significantly enhanced the toxicity of veratrine. Veratrine had no effect on the $(Na^+ + K^+)$-adenosine triphosphatase ($(Na^+ + K^+)$-ATPase) enzyme isolated from guinea-pig ventricle. The conclusion drawn is that at threshold inotropic concentrations of ouabain it is likely that the $(Na^+ + K^+)$-ATPase is inhibited rather than stimulated.

Introduction There is widespread acceptance of the hypothesis that the cardio-toxic effects of cardiac glycosides are due to inhibition of the $(Na^+ + K^+)$adenosine triphosphatase enzyme [$(Na^+ + K^+)$-ATPase] (see Rhee, Dutta & Marks, 1976; Kass, Lederer, Tsien & Weingart, 1978). Whether the positive inotropic action of the glycosides is brought about by the same mechanism is still the subject of some debate. Concentrations of ouabain that produce a positive effect in isolated heart tissues, do inhibit the isolated enzyme (Akera, Larsen & Brody, 1969; 1970), but the work of Zachowski, Lelievre, Aubry, Charlemagne & Paraf (1977) and Lelievre, Wallick & Schwartz (1979) on $(Na^+ + K^+)$-ATPase from plastocytoma cells raises the possibility that standard isolation procedures for the enzyme, involving the use of EDTA buffers, may make the isolated enzyme more susceptible to ouabain than it is *in situ*. In addition, several studies using indirect methods to assess $(Na^+ + K^+)$-ATPase activity have suggested net stimulation of the enzyme with therapeutic doses of the glycosides (see Cohen, Daut & Noble, 1976).

In an attempt to resolve the problem of whether the ATPase is stimulated or inhibited at threshold inotropic doses of ouabain, we studied the interaction of ouabain and veratrine on isolated heart muscle. Veratrine has been shown to exert both its positive inotropic and toxic effects by inhibiting inactivation of the fast sodium channel and so increasing sodium flux into the heart cell (Horackova & Vassort, 1974). Any measure which inhibits the sodium pump should therefore enhance both inotropic and toxic effects, while stimulation of the pump might be expected to have the opposite action. We decided to see whether a threshold inotropic concentration of ouabain would potentiate the toxicity of veratrine: if it did, this would give an indication that ouabain, even at a low concentration, reduces the sodium-handling capabilities of the cell. The onset of veratrine toxicity was taken as the point at which further doses of veratrine caused a decrease in the force of contraction rather than an increase as before. The extent of this negative inotropic effect is a useful criterion of the toxicity of veratrine since it is more reproducible, and occurs within a narrower dose range, than the genesis of dysrhythmias.

The effect of veratrine on the isolated ATPase was also examined, to eliminate the possibility that it was having a direct action on this enzyme.

Methods *Force of contraction studies* Male guinea-pigs, 500 to 700 g, were killed by a blow to the head and exsanguinated. The hearts were excised, and the left atria dissected free; these were then cut in half and each half mounted in an organ bath in contact with a pair of platinum electrodes. They were stimulated at 3 Hz, 500 μs pulse width, and supramaximal voltage. The bathing medium, (mM: NaCl 94.8, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 0.12, $NaHCO_3$ 24.9, Na pyruvate 4.9, Na_2 fumarate 5.4, Na glutamate 4.9, glucose 11.5, $CaCl_2$ 2.5) was bubbled with 95% O_2 and 5% CO_2: the temperature was maintained at 30°C throughout. Isometric contractile force was monitored with a Grass FT-O3C transducer: the loading diastolic tension was 1 g. Force was expressed as a percentage of the value immediately before addition of veratrine.

After equilibration for 1 h, veratrine was added to the bath to give a concentration of 2 μg/ml and allowed to act for 5 min, by which time the maximum effect had been reached. Then equal doses of veratrine were added to the bath at 5 min intervals, each dose raising the bath concentration by 0.5 μg/ml, until the force of contraction was seen to decrease between two consecutive doses. This decrease was taken as the first sign of toxicity, and occurred in the concentration range 2.5 to 4 μg/ml.

Ouabain was then added to the bath every 15 min in increments calculated to raise the bath concen-

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tration by 50 nM each time, until the tissue became completely dysrhythmic. This occurred at a concentration of 250 nM, that is, after 75 min treatment with ouabain. As a control, some tissues were treated with veratrine in the same way, but ouabain was not added at the first sign of toxicity; instead the tissues were allowed to remain in contact with the veratrine alone for 75 min. In these controls it was possible to observe, over the time course of an experiment, the negative inotropic effect of a threshold toxic dose of veratrine in the absence of ouabain. Dose-response curves to ouabain alone were carried out on other tissues again, to determine the positive inotropic effect of concentrations between 50 and 250 nM.

$(Na^+ + K^+)$-ATPase studies The ATPase was isolated by the method of Akera *et al.* (1969) from guinea-pig ventricle tissue. The ventricles were frozen in the sucrose/EDTA buffer after the atria had been removed for force experiments, and the isolation procedure was carried out some weeks later. This freezing of the hearts before isolation of ATPase was found by Akera *et al.* (1969) to have no detectable effect on the subsequent activity of the enzyme. The enzyme was incubated in a medium containing (mM): NaCl 100, KCl 15, $MgCl_2$ 5, Tris HCl 50, adjusted to pH 7.4. The reaction was started by adding 10 mM Tris ATP, also at pH 7.4, to a final concentration of 5 mM in the medium; inorganic phosphate release was measured by the method of Lebel, Poirier & Beaudoin (1978). The ouabain-sensitive ATPase activity, using tissue pooled from eight hearts, was 9.6 ± 0.6 μmol inorganic phosphate liberated mg protein^{-1} h^{-1} (mean ± s.e. mean): this is lower than the figure of 17 μmol mg protein^{-1} h^{-1} obtained by Akera's group. Of this activity, 26.6 ± 3.9% was inhibited by 500 nM ouabain, which compares reasonably well with the finding of Akera *et al.* (1969) of a 25% inhibition by 400 nM ouabain. The effects on the isolated enzyme of both veratrine, 3.5 μg/ml, and the ethanol in which it was dissolved, 0.175% final concentration, were also determined.

Data were analysed statistically by Student's *t* test.

Veratrine (a mixture of alkaloids including veratridine) was obtained from Sigma Chemical Company.

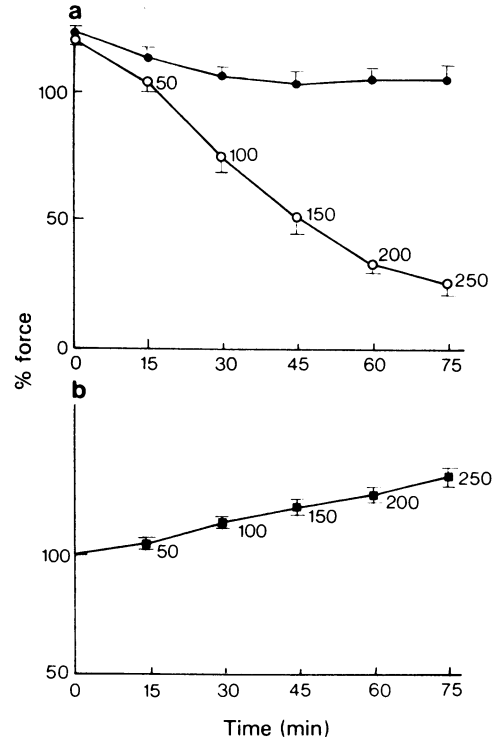

Figure 1 The interaction of ouabain and veratrine on guinea-pig isolated atria. (a) Comparison of responses to veratrine (3 to 3.5 μg/ml) plus increasing concentrations of ouabain (○), against those to veratrine alone over the same time period (●). The ordinate scale is force expressed as a percentage of that immediately before addition of veratrine. (b) Dose-response curve to ouabain alone (■). The ordinate scale is force expressed as a percentage of that immediately before addition of ouabain. In both cases the abscissa scale is time in min, and the numbers beside the points give the concentration of ouabain (nM) in the bath, at that time. The vertical lines show s.e. mean; $n = 7$ for both (a) and (b).

Results It can be seen that for the lowest concentration of ouabain used, 50 nM (which alone gave a positive inotropic effect of only 3%) the negative inotropic action of veratrine was greater in ouabain-treated preparations than in controls, Figure 1. This difference was not significant when group data were used, but when matched pairs were considered, the tissue incubated with both veratrine and ouabain showed a significantly greater drop in force than that with veratrine alone, ($P < 0.05$).

With higher concentrations of ouabain, the difference in force between control and test tissues increased still more, and there was a significant correlation, ($r = 0.978$, $P < 0.001$), between the positive inotropic effect of ouabain alone and the negative inotropic effect of ouabain plus veratrine, over the range of doses of ouabain studied. In all tissues treated with veratrine and ouabain, occasional dysrhythmias were seen when the ouabain concentration had reached 150 nM: at this point the force of contraction had fallen to 50% of that immediately before addition of veratrine. In tissues that had not been treated with veratrine, a concentration of ouabain greater than 450 nM was

necessary to produce a comparable negative inotropic effect.

Neither veratrine nor the ethanol in which it was dissolved had any significant effect on the activity of the ATPase ($n = 5$).

Discussion It is clear from these results that even at a threshold inotropic concentration of ouabain there is enhancement of the toxic effects of veratrine. Since we have ruled out an effect of veratrine, at the concentrations used, on the ATPase itself, the basis of both its inotropic and toxic actions can be assumed to be an increase in sodium entering the cell. Therefore, at these concentrations, ouabain is potentiating the effect of an increased sodium influx: if this potentiation is due to an action on the ATPase, then it is likely that ATPase inhibition, not stimulation, is involved. However, it must be added, that we have not eliminated the possibility of some unexpected interaction between ouabain and veratrine at a stage before their synergistic effect on internal sodium accumulation.

In conclusion, these experiments suggest that in whole tissues there is, at the threshold inotropic concentration of ouabain, an inhibition rather than a stimulation of the $(Na^+ + K^+)$-ATPase and that this inhibition increases in a dose-dependent manner with further addition of ouabain.

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References

AKERA, T., LARSEN, F.S., & BRODY, T.M. (1969). The effect of ouabain on sodium- and potassium-activated adenosine triphosphatase from the hearts of several mammalian species. *J. Pharmac. exp. Ther.*, **170,** 17–26.

AKERA, T., LARSEN, F.S. & BRODY, T.M. (1970). Correlation of cardiac sodium- and potassium-activated adenosine triphosphatase activities with ouabain induced inotropic stimulation. *J. Pharmac. exp. Ther.*, **173,** 145–151.

COHEN, I., DAUT, J. & NOBLE, D. (1976). An analysis of the actions of low concentrations of ouabain on membrane currents in Purkinje fibres. *J. Physiol*, **260,** 75–103.

HORACKOVA, M. & VASSORT, G. (1974). Excitation–contraction coupling in frog heart. Effect of veratrine. *Pflügers Arch.*, **352,** 291–302.

KASS, R.S., LEDERER, W.J., TSIEN, R.W. & WEINGART, R. (1978) Role of calcium ions in transient inward currents and after contractions induced by strophanthidin in cardiac Purkinje fibres. *J. Physiol.*, **281,** 187–208.

LEBEL, D., POIRIER, G.G. & BEAUDOIN, A.R. (1978). A convenient method for the ATPase assay. *Analyt. Biochem.*, **85,** 86–89.

LELIEVRE, L., WALLICK, E.T. & SCHWARTZ, A. (1979). Inhibition of murine Na, K-ATPase by ouabain. Involvement of Ca^{2+} and membrane protein ouabain sensitivity factor. *Fedn Proc.*, **38,** 372.

RHEE, H.M., DUTTA, S. & MARKS, B.H. (1976). Cardiac Na, K-ATPase activity during positive inotropic and toxic actions of ouabain. *Eur. J. Pharmac.*, **37,** 141–153.

ZACHOWSKI, A., LELIEVRE, L., AUBRY, J., CHARLEMAGNE, D. & PARAF, A. (1977). Role of proteins from the inner face of plasma membranes in susceptibility of Na^+/K^+-stimulated Mg^{2+}-ATPase to ouabain. *Proc. natn. Acad. Sci., U.S.A.*, **74,** 633–637.

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