Br. J. Pharmac. (1981), **73**, 341–345

TIME COURSE OF OUABAIN UPTAKE IN ISOLATED MYOCARDIAL CELLS: DEPENDENCE ON EXTRACELLULAR POTASSIUM AND CALCIUM CONCENTRATION

L. MELDGAARD, E. STEINESS & S. WALDORFF

Department of Pharmacology, University of Copenhagen, Denmark

1 Spontaneously beating myocardial cells isolated from newborn rats have been used to evaluate the time course of cellular ouabain uptake.

2 The rate of cellular uptake and the amount of ouabain bound at equilibrium were computed by fitting the experimental data to the conventional exponential equation for receptor binding of drugs.

3 At normal extracellular potassium and calcium concentrations a biexponential equation was the best fit to the experimental data, indicating two receptor sites of ouabain with different rates of uptake.

4 Increasing extracellular potassium or calcium concentrations decreased the amounts of ouabain bound at equilibrium.

5 High and low extracellular concentrations of potassium or calcium decreased the rate of ouabain uptake.

6 It is well known that ouabain changes ionic fluxes. Changes in the extracellular potassium and calcium concentrations also influence the amount of ouabain taken up by myocardial cells, as demonstrated in the present study.

Introduction

The positive inotropic effect of digitalis is presumably due to an inhibition of the membrane ($Na^+ + K^+$) adenosinetriphosphatase (ATPase) (Godfraind & Lesne, 1972; Godfraind & DePover, 1973), but a digitalis-induced enhancement of the calcium transport into the myocardial cell has also been claimed (Bailey & Dresel, 1968; Shine, Serena & Langer, 1971; Langer, 1977).

In intact cells a reciprocal relationship between the binding of ouabain to erythrocytes and the concentration of potassium has been demonstrated (Gardner & Conlon, 1972); a similar relationship between the cellular binding of ouabain and potassium has been demonstrated in myocardial cells (Baker & Willis, 1972; McCall, 1979).

Hypokalaemia increases the myocardial sensitivity to cardiac glycosides and it has been suggested that the binding of digitalis to membrane ($Na^+ + K^+$) ATPase and the corresponding inhibition of the enzyme are responsible for the toxic action of digitalis (Okita, 1977). The characteristics of the binding of the cardiac glycosides to this enzyme have been investigated both in fragments from cell membrane and in homogenates obtained from different tissues (Akera, Larsen & Brody, 1969). The binding of digitalis to the isolated ($Na^+ + K^+$) ATPase preparations and also the total amount of digitalis bound to intact myocardium are markedly influenced by the extracellular potassium concentration (Prindle, Skelton, Epstein & Marcus, 1969; Akera, Temma, Wiest & Brody, 1978; Steiness, 1978).

However, in contrast to the cellular study of the interaction between potassium and digitalis, the interaction between extracellular calcium and digitalis has not been elucidated.

Using isolated, spontaneously beating myocardial cells, we have investigated the influence of potassium and calcium on the rate of ouabain uptake and the amount of ouabain bound at equilibrium.

Methods

Myocardial cell preparation

Ventricles were isolated from 2–3 day old rats killed by decapitation. The tissue was cut into small pieces and washed 3–4 times with a calcium- and magnesium-free Hanks' balanced salt solution (Gibco Bio Cult), buffered with 10 mM HEPES (N-2-hydroxyethylpiperazine-*N*-ethanesulphonic acid). The tissue pieces were dissolved by a step-wise enzyme digestion according to Harary & Farley (1960), using 0.05% crude collagenase (0.15 units per mg) (Boehringer Mannheim). The primary myocardial cell culture was established with a culture medium

0007-1188/81/060341-005 $01.00

(Medium 199, with Hanks' salt solution, 25 mM HEPES and heat-inactivated foetal calf serum at 10%). The inorganic ion concentration was (mM): sodium 141.7, chloride 136.9, potassium 5.8, calcium 1.3, sulphate 0.8, magnesium 0.8 and phosphate 0.8.

The myocardial cell culture was prepared by the rotation-aggregation technique introduced by Moscona (1960) with a final population density of 0.5×10^6 cells per ml.

Ouabain binding

After 1–2 days in the culture, the myocardial cell aggregates exhibited rhythmic and synchronous contractility and were used for experiments. The culture medium was replaced by one containing 2.5% serum and with an ion concentration, as described above, when the time course of ouabain uptake was examined under 'normal' conditions. When potassium-dependent ouabain uptake was investigated, potassium-free culture medium was adjusted to a potassium concentration of 1 mM, 5.8 mM, 10 mM or 100 mM. When calcium-dependent ouabain uptake was investigated, calcium-free culture medium was adjusted to a calcium concentration of 10^{-4} mM, 1.3 mM, 10 mM.

The time course of the cellular uptake of ouabain was measured using [^{3}H]-ouabain, generally labelled, and with a specific activity of 49 Ci per mmol (Amersham). The final ouabain concentration in the incubation tubes was 10 nM.

All experimental procedures were performed at 37°C and under sterile conditions. The binding reactions were stopped at different times (0.5, 2,4,6,8,10,12,14,16,18,20,30,40,60,90,120,150 and 180 min) by adding ice-cold Hanks' balanced salt solution to the incubation tubes (Tobin & Brody, 1972). To remove the non-bound [^{3}H]-ouabain, the cells were washed three times with ice-cold Hanks' balanced salt solution by alternate centrifugation at 100 *g* and resuspension.

After the final washing the cell pellets were lysed with Triton X-100 and the radioactivity was measured in a Packard Tri Carb liquid scintillation spectrometer using Insta-Gel as scintillation fluid. Differences in counting efficiency were corrected for by use of an internal standard.

To quantify the unspecific binding, simultaneous experiments on [^{3}H]-ouabain binding were carried out with ouabain 10^{-3} M, since this binding was clearly correlated to the nondisplaceable amount when digoxin (10^{-5} M) was added. The sampling procedure was similar to that described for [^{3}H]-ouabain but binding of the radioactivity was considered unspecific and subtracted from the corresponding binding data.

The amounts of cellularly bound [^{3}H]-ouabain were expressed per mg cellular protein. Protein determination was performed by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine albumin as protein standard.

Calculations

The experimental data were fitted to the following equations for time-dependent drug-receptor binding.

For one binding site:

$$A_t = A_{eq} \times (1 - e^{-kt})$$

For two binding sites:

$$A_t = A_{eq1} \times (1 - e^{-k1t}) + A_{eq2} \times (1 - e^{-k2t})$$

where A_t represents the amount of ouabain bound at the given time t, A_{eq} the amount bound at equilibrium, and k the rate constant of binding (Mackay, 1979). The calculation of A_{eq} and k values was made by a non-linear method, weighted according to the actual variances and using a suitably chosen rate constant and asymptote as primary estimate (Saab Univac 1108).

Results

At physiological concentrations of potassium and calcium, the experimental data were fitted to the conventional exponential equation for binding to one or two receptor sites (Figure 1). A statistically significant improvement was achieved, when the data were fitted to the equation for two binding sites (F-test, $P<0.05$); however, the two curves are very similar (Figure 1). The computed rates of uptake

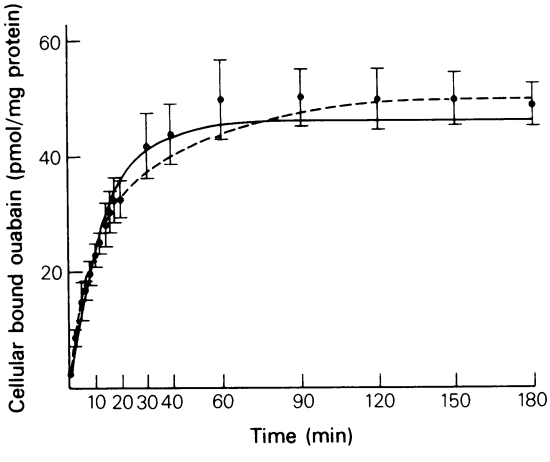

Figure 1 Time course of cellular uptake of ouabain (10 mM), the computed curves for one- (continuous line) and two- (broken line) receptor model; (●) represent the mean of 10 experiments and the vertical bars represent one s.e.mean.

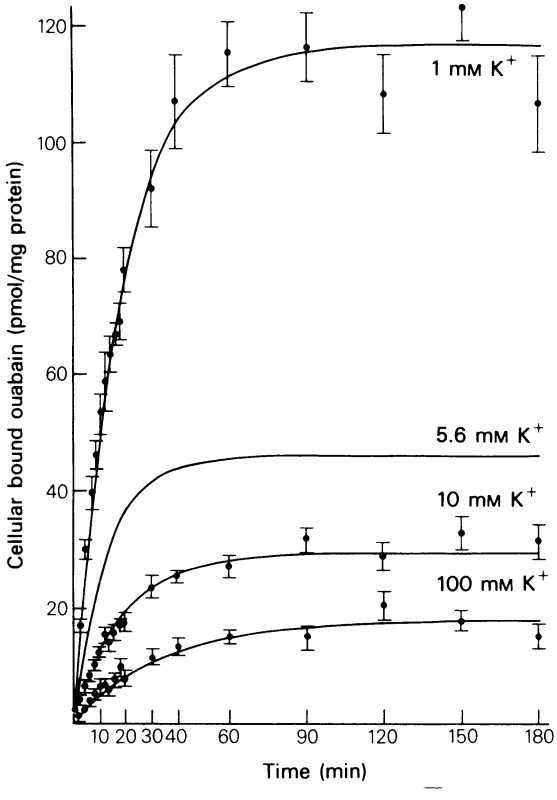

Figure 2 The time course of cellular uptake of ouabain at different extracellular potassium concentrations (computed curves); (●) represent the mean of 8 experiments and the vertical bars represent one s.e.mean.

were 0.023 min^{-1} and 0.16 min^{-1} for two receptors and the corresponding amounts of ouabain bound at equilibrium were 28.2 pmol/mg protein and 22.9 pmol/mg protein.

When extracellular potassium or calcium were changed, no significant difference was obtained when the data were fitted to the one- or the two-receptor model. All our experimental data are therefore fitted to a one-receptor model.

Potassium

The effects of different extracellular potassium concentrations on the time course of ouabain uptake to myocardial cells are depicted in Figure 2. The computed amount of bound ouabain at equilibrium decreased significantly with increasing extracellular potassium concentration. The observed quantities of cellularly bound ouabain after 180 min ($A_{180\ min}$) correspond with the computed amount at equilibrium (A_{eq}) (Table 1). Both high (10 mM, 100 mM) and low (1 mM) concentrations of potassium decreased significantly the rate of ouabain uptake (Table 1).

Calcium

The influence of changes in extracellular calcium concentration on the time course of ouabain uptake to isolated myocardial cells is shown in Figure 3. The computed amount of bound ouabain at equilibrium increased signficantly with decreasing calcium concentration. The observed quantities of cellularly bound ouabain at 180 min ($A^{180\ min}$) correspond with the computed amount of ouabain bound at equilibrium (Table 2). Low (10^{-4} mM) and high (10 mM) calcium concentrations increased the rate of ouabain uptake significantly (Table 2).

Discussion

Two different, specific, myocardial binding sites for digitalis glycosides have been suggested (Godfraind & Lesne, 1972). Accordingly, one might suspect that two binding sites would have different rate constants for uptake of ouabain. In the present experiments two different rate constants for ouabain binding were revealed. However, the difference between the two rates was minor (Riggs, 1976). When the potassium and calcium concentrations were changed, no

Table 1 Influence of extracellular potassium concentration on the amount of cellularly bound ouabain at 180 min ($A_{180\ min}$); the computed amount at equilibrium (A_{eq}) and the corresponding rate constant for binding reaction (k) are shown

Extracellular potassium concentration (mM)	n	$A_{180\ min}$ *(pmol/mg protein)*	A_{eq} *(pmol/mg protein)*	P	*Rate constant* (k min^{-1})	*P*
1	8	107.03 ± 16.40	116.57 ± 7.00	0.001	0.055 ± 0.0054	0.001
5.8	16	49.20 ± 12.03	46.04 ± 2.68	0.001	0.075 ± 0.0080	0.001
10	8	34.63 ± 3.09	29.07 ± 2.20	0.001	0.055 ± 0.0074	0.001
100	8	15.15 ± 0.49	17.67 ± 1.72		0.031 ± 0.0054	

Values are mean ± s.d.

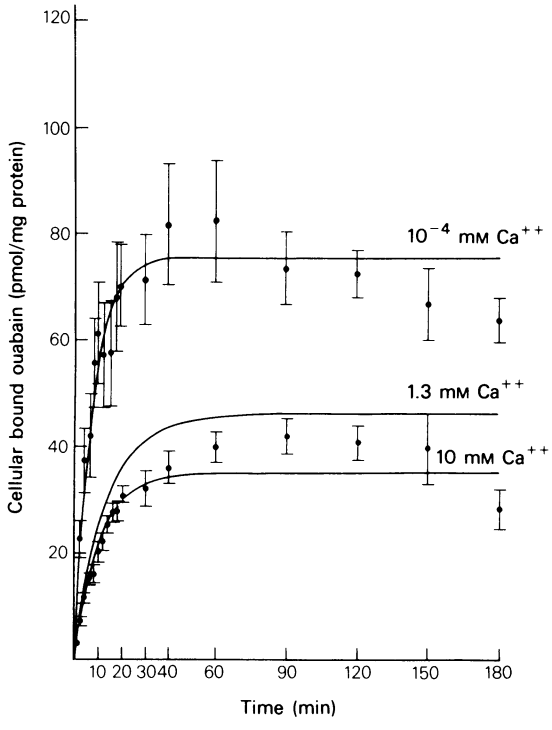

Figure 3 Time course of cellular uptake of ouabain at different extracellular calcium concentrations (computed curves); (●) represent the mean of 8 experiments and the vertical bars represent one s.e.mean.

marked improvement was achieved when fitting the binding data to the two-receptor model. However, time-dependent uptake of drug to one or two different binding sites is not a suitable method to reveal different types of binding sites. Accordingly, our results are fitted to the one-receptor model.

In the present study it was shown that changes in extracellular potassium concentration influence the amount of ouabain bound to myocardial cells in an inverse manner. The same relationship between extracellular potassium and digitalis content in the myocardial tissue has been found by Cohn, Kleiger & Harrison (1967) and Steiness (1978). It seems that a pharmacodynamic effect of digitalis is influenced by potassium so that the lower potassium concentration increases the positive inotropic response considerably (Prindle *et al.*, 1969). Furthermore, it is known that hypokalaemia increases the risk of myocardial digitalis toxicity (Steiness & Olesen, 1976). The digitalis toxicity is supposed to be correlated with the inhibition of the membrane ($Na^+ + K^+$) ATPase (Okita, 1977). Both the increase in inotropic response and the increased risk of digitalis toxicity during hypokalaemia could therefore be explained partly by an increased digitalis binding to the membrane ($Na^+ + K^+$) ATPase and not only by a synergistic effect of hypokalaemia and digitalis glycosides (Akera *et al.*, 1978). It is well-known that a synergistic effect exists between extracellular calcium concentration and digitalis glycosides with respect to inotropy and toxicity (Lee & Klaus, 1971). In our experiments an increased ouabain binding was found at a low calcium concentration, and the corresponding inotropic effect may not be great enough to overrule the marked negative inotropism of calcium *per se*.

The rate of onset of ouabain action is enhanced by both low and high extracellular calcium concentrations (Park & Vincenzi, 1976). In the present study a corresponding relationship between extracellular calcium concentration and rate constant was revealed in the cellular uptake of ouabain.

It can be concluded that the kinetics of ouabain uptake in isolated myocardial cells are markedly influenced by changes in extracellular potassium and calcium concentration.

This work was supported by grants from Hjerteforeningen, Danish Medical Research Council and Weimanns Legat.

Table 2 Influence of extracellular calcium concentration on the amount of cellularly bound ouabain at 180 min ($A_{180\,min}$); the computed amount at equilibrium (A_{eq}) and the corresponding rate constant for binding reaction (k) are also shown.

Extracellular calcium concentration (mM)	n	$A_{180\,min}$ *(pmol/mg protein)*	A_{eq} *(pmol/mg protein)*	P	*Rate constant* (k min^{-1})	P
10^{-4}	8	63.78 ± 10.38	75.20 ± 3.24		0.138 ± 0.013	
1.3	16	49.20 ± 12.03	46.04 ± 2.68	0.001	0.075 ± 0.008	0.001
10	8	28.20 ± 9.04	35.18 ± 2.32	0.001	0.090 ± 0.014	0.005

Values are mean ± s.d.

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., TEMMA, K., WIEST, S.A. & BRODY, T.M. (1978). Reduction of the equilibrium binding of cardiac glycosides and related compounds to $(Na^+ + K^+)$ ATPase as a possible mechanism for the potassium-induced reversal of their toxicity. *Naunyn Schmiedebergs Arch. Pharmac.*, **304,** 1957–1965.

BAILEY, L.E. & DRESEL, P.E. (1968). Correlation of contractile force with a calcium pool in the isolated cat heart. *J. gen. Physiol.*, **52,** 969–982.

BAKER, P.F. & WILLIS, J.S. (1972). Binding of the cardiac glycoside ouabain to intact cells. *J. Physiol.*, **224,** 441–462.

COHN, K.E., KLEIGER, R.E. & HARRISON, D.C. (1967). Influence of potassium depletion on myocardial concentration of tritiated digoxin. *Circulation Res.*, **20,** 473–476.

GARDNER, J.G. & CONLON, T.P. (1972). The effects of sodium and potassium on ouabain binding by human erythrocytes. *J. gen. Physiol.*, **60,** 609–629.

GODFRAIND, T. & LESNE, H. (1972). The uptake of cardiac glycosides in relation to their actions in isolated cardiac muscle. *Br. J. Pharmac.*, **46,** 488–497.

GODFRAIND, T. & DEPOVER, A. (1973). The inhibition of the Na-K-ATPase of guinea-pig heart microsomes by cardiac glycosides. *Archs int. Pharmacodyn. Thér.*, **201,** 197–200.

HARARY, I. & FARLEY, B. (1960). In vitro studies of single isolate beating heart cells. *Science*, **131,** 1674–1675.

LANGER, G.A. (1977). Ionic basis of myocardial contractility. *A. Rev. Med.*, **28,** 13–20.

LEE, K.S. & KLAUS, W. (1971). The subcellular basis for the mechanism of inotropic action of cardiac glycosides. *Pharmac. Rev.*, **23,** 193–261.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.*, **193,** 265–275.

MACKAY, D. (1979). A critical survey of receptor theories of drug action. In *Kinetics of drug action. Handb. exp. Pharmac.*, Vol. 47, ed. Van Rossum, J.M. pp. 255–321. Berlin: Springer-Verlag.

MCCALL, D. (1979). Cation exchange and glycoside binding in cultured rat heart cells. *Am. J. Physiol.*, **236,** C87–C95.

MOSCONA, A. (1960). Rotation-mediated histogenic aggregation of dissociated cells. *Exp. Cell Research*, **22,** 455–475.

OKITA, G.T. (1977). Dissociation of $(Na^+ + K^+)$ ATPase inhibition from digitalis inotropy. *Fedn Proc.*, **36,** 2225–2230.

PARK, M.K. & VINCENZI, F.F. (1976). Influence of calcium concentration on the rate of onset of cardiac glycoside and aglycone inotropism. *J. Pharmac. exp. Ther.*, **198,** 680–686.

PRINDLE, K.H., SKELTON, C.L., EPSTEIN, S.E. & MARCUS, F.I. (1969). Influence of extracellular potassium concentration on myocardial uptake and inotropic effect of tritiated digoxin. *Circulation Res.*, **28,** 337–345.

RIGGS, D.S. (1976) *The Mathematical Approach to Physiological Problems.* New York: The Williams & Wilkins Company.

SHINE, K.I., SERENA, S.D. & LANGER, G.A. (1971). Kinetic localization of contractile calcium in rabbit myocardium. *Am. J. Physiol.*, **221,** 1408–1417.

STEINESS, E. (1978). Digoxin toxicity compared with myocardial digoxin and potassium concentration. *Br. J. Pharmac.*, **63,** 233–237.

STEINESS, E. & OLESEN, K.H. (1976). Cardiac arrhythmias induced by potassium loss during maintenance digoxin therapy. *Br. Heart J.*, **38,** 167–172.

TOBIN, T. & BRODY, T.M. (1972). Rates of dissociation of enzyme-ouabain complexes and $K_{0.5}$ values from different species. *Biochem. Pharmac.*, **21,** 1553–1560.

(Received April 22, 1980. Revised January 16, 1981.)