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Selective inhibition by ethylisopropylamiloride of the positive inotropic effect evoked by low concentrations of ouabain in rat isolated ventricles

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1 The biphasic positive inotropic effect of ouabain has been studied in rat ventricular strips in the presence of ethylisopropylamiloride (EIPA) an inhibitor of Na^+/H^+ exchange.

2 EIPA (10–20 μM) depressed dose-dependently the high affinity component of the ouabain inotropic effect, whereas it did not significantly modify the low affinity inotropic effect related to high concentrations of ouabain.

3 At the EIPA concentrations studied, there was no observable modification of the inotropic effect of Bay K 8644 (10 nM, 0.3 μM) or of isoprenaline (10 nM, 1 μM).

4 These results indicate that the inotropic effect of ouabain resulting from its interaction with high affinity sites is selectively sensitive to EIPA.

Introduction

The membrane Na^+/H^+ exchange system that has been described in epithelial cells (Cuthbert, 1976) is present in rat cultured cardiac cells (Frelin *et al.*, 1984). This system is a major pathway for Na^+ uptake by these cultured cardiac cells and it may regulate their internal pH by exchanging incoming Na^+ for outgoing H^+. This system is inhibited by the diuretic drug amiloride and more potently by its N-5 disubstituted derivatives, dimethylamiloride and ethylisopropylamiloride (EIPA) (Frelin *et al.*, 1984).

In the guinea-pig isolated atria, it has been observed that amiloride inhibits the positive inotropic effect of both ouabain and dihydroouabain, whereas EIPA inhibits the effect of ouabain but not that of dihydroouabain (Ghysel-Burton & Godfraind, 1986). In guinea-pig atria, the inotropic effect of dihydroouabain has been attributed to an interaction with low affinity binding sites, whereas the inotropic effect of ouabain has been ascribed to an interaction with both low and high affinity binding sites (Godfraind *et al.*, 1982).

In the present experiments, we have studied the action of EIPA on the inotropic effect of ouabain in rat ventricular strips where two different Na/K-ATPase activities with different affinities for ouabain have been demonstrated (Noel & Godfraind, 1984). In this tissue, the dose-effect curve of ouabain shows two components related to those high and low affinity binding sites (Finet *et al.*, 1983). The present results show that the inotropic effect resulting from the interaction of ouabain with high affinity sites is selectively inhibited by EIPA.

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Methods

Protocol

Right ventricular strips ($n = 228$) (length: 11.2 ± 0.2 mm; width: 1.70 ± 0.02 mm; weight: 22.6 ± 0.1 mg) from 57 Wistar rats weighing 250–300 g were dissected perpendicularly to the axis of the heart. They were suspended in 25 ml organ baths and stretched at a length which allowed the maximum systolic contraction to be recorded (i.e. a resting tension of about 500 mg). They were bathed in Tyrode solution containing (mM): NaCl 137, KCl 6, $CaCl_2$ 1.82, $MgCl_2$ 1.05, NaH_2PO_4 0.417, $NaHCO_3$ 11.5 and glucose 5.5, at 30°C, aerated with 95% O_2 and 5% CO_2.

Whole left atria were dissected, stretched at the maximum systolic contraction and maintained under the same conditions as the ventricles.

The tissues were electrically stimulated by field electrodes with rectangular 10 ms pulses (voltage twice threshold) at 1 Hz. Contractile responses were measured with an isometric strain gauge (Gould Statham) coupled to a potentiometric recorder (Elema Mingograf 81, Schonander).

After their dissection and their suspension in the organ baths the tissues were allowed to equilibrate for one and a half hours under electrical stimulation. The contractility recorded after this equilibration period was taken as control and was monitored for 90 min in the absence and presence of EIPA.

The positive inotropic effects of ouabain, Bay K 8644 and isoprenaline have been studied in two groups of tissues: one group pretreated, and the other not pretreated with EIPA during a 30 min pre-incubation period. Both groups were compared to control preparations (untreated with inotropic agents).

The contractility of the electrically driven right ventricular strips was monitored until the maximum inotropic effect produced by the different agents had been observed.

Contractions at any given time were measured and expressed as a percentage of the initial contraction in the same tissue. The inotropic concentration-effect curve was calculated by taking the maximum effect at each concentration of ouabain and comparing it with the controls at the same time.

Analysis of the results

Data were analysed as previously described (for details see Finet *et al.*, 1983) assuming that the relationship between the ouabain concentration and the inotropic effect could be considered either as being due to one saturable component (1) or to the sum of two saturable components (2):

$$E = \frac{E_{max}C}{EC_{50} + C} \quad (1)$$

$$E = \frac{E_{max_H}C}{EC_{50H} + C} + \frac{E_{max_L}C}{EC_{50L} + C} \quad (2)$$

where E is the inotropic effect (increase in contraction); E_{max}, maximum inotropic effect; EC_{50}, concentration of ouabain producing a half-maximal effect; C, concentration of ouabain in the bath; H or L, contribution of high or low sensitivity inotropic components.

Data were analysed using a computerized non-linear regression program based on the steepest descent technique which adjusts the parameters to minimize the sum of the squared errors. The two models (1 and 2) were discriminated by using an F test for comparison of total variances; the model with the lowest variance has been assumed to be the most representative of the experimental data (Noel & Godfraind, 1984).

Data are presented as means ± s.e.mean. Significance of differences between means were tested by Student's t test, a level of probability of less than 0.05 being considered significant.

Drugs

Ouabain (Merck) and EIPA (Ciba-Geigy) were dissolved in distilled water. Isoprenaline sulphate (Boehringer Ingelheim) was dissolved in distilled water containing 11.2 mM Na_2SO_3 and 35 mM HCl as a stock solution of 10 mM. Bay K 8644 (methyl 1,4-dihydro-2,6 dimethyl-3 nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, Bayer) was dissolved in acetone as a stock solution of 10 mM; all dilutions were made in the physiological solution.

Results

After the equilibration period, the contractile force of ventricular strips was equal to 0.87 ± 0.03 g ($n = 62$) for the strips incubated without EIPA, and, respectively, to 0.89 ± 0.03 g ($n = 52$) and to 0.85 ± 0.03 g ($n = 54$) for the strips incubated subsequently with EIPA 10 μM and 20 μM (these values are not significantly different).

Concentrations of EIPA lower or equal to 6 μM did not modify the ventricular contraction. EIPA concentrations equal to or higher than 10 μM evoked a negative inotropic effect that reached its maximum effect after about 30 min. After this 30 min incubation period, the contractile force was decreased by 8 ± 2% ($n = 6$) in the presence of 10 μM EIPA and by 22 ± 5% ($n = 6$) in the presence of 20 μM EIPA. In rat atria, EIPA 10 and 20 μM evoked a transient positive inotropic effect that was followed by a continuous decrease of the contractile force which did not allow the study of the effect of EIPA on the inotropic action of ouabain, so this was done in guinea-pig atria (Ghysel-Burton & Godfraind, 1986).

Concentration-effect curves obtained with ouabain in rat ventricles untreated or pretreated with EIPA are illustrated in Figure 1a. The effect of ouabain was measured when the peak inotropic effect had been reached. In both the absence and presence of EIPA, the time to peak inotropic effect was reduced when the concentration was increased. For concentrations lower than 10 μM, the time to peak effect was about 20 min, whereas it was reduced to 10 min at 30 μM and to 2 min at 600 μM ouabain. Concentrations of ouabain higher than 10 μM produced a toxic effect on the tissues marked by an increase of the resting tension. This effect was observed once the maximum inotropic effect had been reached, except with concentrations of ouabain of 1 mM and above. In those cases,

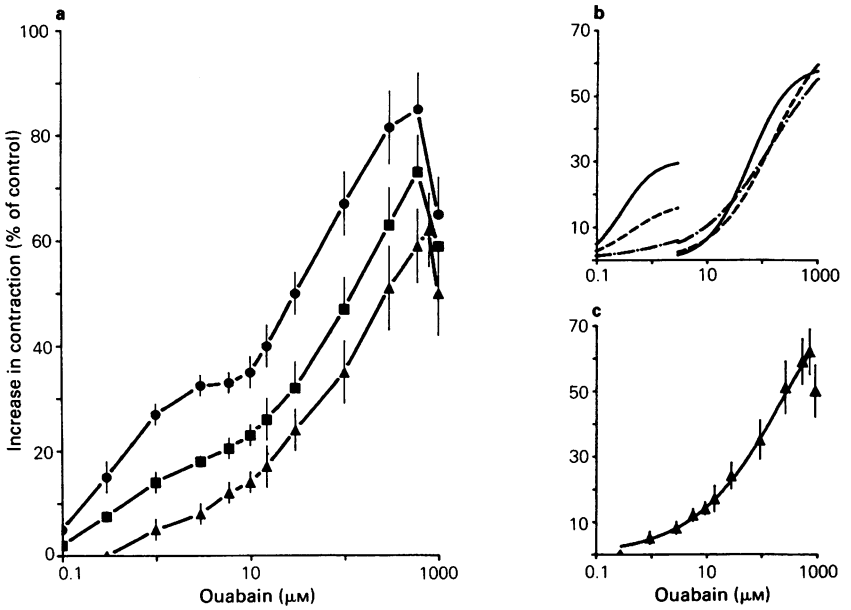

Figure 1 Increased contractility of rat ventricular strips evoked by ouabain in the absence of ethylisopropylamiloride (EIPA, ●) and in the presence of EIPA 10 μM (■), 20 μM (▲). Each point is the mean of at least 4 experiments, vertical lines show s.e.mean. (a) Experimental concentration-effect curves. (b) Theoretical curves drawn assuming two saturable components: ouabain alone (————), with EIPA 10 μM (— — — —), 20 μM (—.—.—). (c) Continuous line: theoretical curve drawn for ouabain in the presence of EIPA 20 μM, assuming one saturable component. The data obtained with ouabain 1 mM have been excluded for the analysis. (▲) Experimental data.

the toxic effect appeared during the development of the inotropic effect of ouabain and therefore reduced its magnitude.

Confirming previous observations (Finet *et al.*, 1983, and see Discussion), in the absence of EIPA the positive inotropic concentration-effect curve of ouabain appeared to be biphasic. In the presence of EIPA at concentrations lower or equal to 6 μM, the positive inotropic effect of ouabain was not affected. In the presence of EIPA 10 and 20 μM, the inotropic effect evoked by ouabain was depressed in a concentration-dependent manner. This depression was most apparent at low concentrations of ouabain. For instance, in the absence of EIPA, 3 μM ouabain evoked an increase in contractility of 0.29 ± 0.02 g ($n = 6$). In the presence of 10 and 20 μM EIPA, the increase was equal to 0.15 ± 0.02 g ($n = 6$) ($P < 0.01$) and 0.08 ± 0.02 g ($n = 6$) ($P < 0.01$) respectively. The maximal increase in contraction evoked by 0.6 mM ouabain was equal to 0.82 ± 0.1 g ($n = 4$) in the absence of EIPA, but to 0.72 ± 0.07 g ($n = 4$) ($P > 0.1$) and 0.60 ± 0.07 ($n = 4$) ($P > 0.1$) in the presence of EIPA 10 and 20 μM, respectively.

In view of the biphasic nature of the concentration-effect curve evoked by ouabain, the observed responses were analysed as previously, by comparing two models describing the results as shown in Methods. Data analysis for controls (ouabain alone) and preparations treated with EIPA 10 μM are presented in Table 1. They show that the biphasic model (equation 2) has a lower variance than the monophasic model (equation 1) ($P < 0.01$ and $P < 0.05$). This is consistent with previous findings (Finet *et al.*, 1983) assuming that ouabain interacts with two different types of receptor to produce a positive inotropic effect. On the other hand, in the presence of EIPA 20 μM, the monophasic model shows a lower variance than the biphasic one indicating that in this condition ouabain interacts with one type of receptor to produce a positive inotropic effect.

Figure 1b illustrates the theoretical curves drawn to include the experimental data with the exception of those for ouabain 1 mM, a concentration producing a rapid toxic effect. It shows the high affinity and the low affinity components of inotropy. EIPA 10 μM depressed selectively the high affinity component related to low concentrations of ouabain (0.1 to 10 μM): it reduced E_{maxH} and increased EC_{50H}. EIPA had little or

Table 1 Variance analysis of models 1 and 2 for the description of the ouabain action in the absence and presence of ethylisopropylamiloride (EIPA)

Experimental conditions	*Model one component*	*Model two components*	F *test*
Control (ouabain alone)	53.68	1.90	$P<0.01$
EIPA 10 μM	5.50	0.86	$P<0.05$
EIPA 20 μM	0.57	1.01	NS

NS = no significant difference.

no significant effect on the low affinity component of the inotropic action related to high concentrations of ouabain (10 μM to 1 mM). EIPA 20 μM abolished the high affinity component, the calculated parameters E_{max} and ED_{50} for the high affinity sites of ouabain were in this case apparently nonexistent. EIPA 20 μM did not produce a significant change in the low affinity component of the inotropic curve.

Figure 1c shows that the experimental data for the positive inotropic effect of ouabain in presence of EIPA 20 μM are fitted by a monophasic curve that has the same characteristics as the low affinity inotropic component computed in the absence of EIPA (ED_{50} equal respectively to 0.07 ± 0.02 mM and 0.06 ± 0.01 mM; $P > 0.1$). This confirms that EIPA affected only the high affinity component.

We also studied the effect of EIPA on other inotropic interventions producing an increase in contraction similar to low and high concentrations of ouabain, respectively. The Ca agonist Bay K 8644 (Schramm *et al.*, 1983) produces a positive inotropic effect in rat ventricular strips (Finet *et al.*, 1985), and we studied the effect of EIPA on the responses induced by 10 nM and 0.3 μM Bay K 8644. This latter concentration produced a maximal inotropic effect. EIPA (10 and 20 μM) did not significantly modify either of these two effects (Table 2). The β-adrenoceptor agonist isoprenaline evokes a positive inotropic effect that is attributed to an increase in slow calcium inward current (Scholz, 1984). EIPA pretreatment did not alter the inotropic effect evoked by isoprenaline 10 nM and 1 μM (Table 2).

Table 2 Contractile force of ventricular strips (as % of controls) treated by several inotropic agents

Agents concentration	*Alone*	*+ EIPA 10 μM*	*+ EIPA 20 μM*
Ouabain 1 μM	127 ± 3	114 ± 4*	105 ± 3*
Ouabain 0.6 mM	184 ± 8	174 ± 8	160 ± 8
Isoprenaline 10 nM	115 ± 4	118 ± 6	111 ± 4
Isoprenaline 1 μM	180 ± 8	177 ± 7	173 ± 8
Bay K 8644 10 nM	123 ± 5	119 ± 6	117 ± 6
Bay K 8644 0.3 μM	160 ± 7	161 ± 7	156 ± 8

Before addition of these agents, the tissues were incubated during a 30 min period without or with ethylisopropylamiloride (EIPA) 10 and 20 μM. The contractile force was measured at the peak inotropic effect and was expressed as percentage (± s.e.mean) of the initial contractility and corrected for controls. The values represent the mean of at least 4 experiments. *$P < 0.05$.

Discussion

The present experimental data confirm that the dose-effect curve of ouabain in rat ventricles is biphasic and is due to the interaction of the glycoside with high and low affinity binding sites (Finet *et al.*, 1983; Herzig & Mohr, 1984; Grupp *et al.*, 1985; Katano *et al.*, 1985). It has been demonstrated that under normal conditions, the interaction of ouabain with high affinity binding sites does not induce an increase in the resting tension (diastolic). Only the interaction of ouabain with low affinity receptors produces such toxic effects (Finet *et al.*, 1983).

The present results show that the positive inotropic effect evoked by low and high concentrations of ouabain are not equally affected after pretreatment with EIPA; EIPA 10 and 20 μM depressed the positive inotropic effect of ouabain that is related to an interaction with high affinity binding sites whereas it did not significantly modify the positive inotropic effect of ouabain related to the low affinity binding sites. The effect of EIPA at the concentrations studied here was selective for low concentrations of ouabain. EIPA did not affect the inotropic action evoked by isoprenaline or by the calcium agonist Bay K 8644. The present results are in good agreement with those recently found with guinea-pig isolated atria: an inhibition by EIPA of the positive inotropic effects of low concentrations of ouabain (Ghysel-Burton & Godfraind, 1986).

Several actions of the diuretic parent compound, amiloride have been demonstrated: an inhibition of Na^+/H^+ and of Na^+/Ca^{2+} exchange, an inhibition of adenylate cyclase activity (Mahe *et al.*, 1985), a reduction of the affinity of ouabain for its binding sites and an inhibition of Na/K-ATPase activity (Kennedy *et al.*, 1985). In rat and chick cultured cardiac cells, EIPA has been shown to be more specific than amiloride as an inhibitor of Na^+/H^+ exchange (Frelin *et al.*, 1984). By an inhibition of Na^+/H^+ exchange, EIPA could reduce intracellular sodium activity and pH (Frelin *et al.*, 1984; Lazdunski *et al.*, 1985) and this could account for the negative inotropic effect of this agent. In the present experiments, reduction of the inotropic effect produced by low doses of ouabain was noticed only with EIPA concentrations producing a negative inotropic effect during the pre-incubation period and may therefore be related to changes in intracellular sodium activity and pH. It has been found that an elevation of intracellular sodium activity stimulates glycoside binding to its receptors and that a decrease in intracellular sodium activity could reduce this binding (Akera & Brody, 1985). The present observations suggest that the binding of ouabain to high affinity sites might be more sensitive to changes in intracellular sodium activity and pH than the binding of ouabain to low affinity sites.

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