Role of Na-H exchange in the inotropic action of Bay K 8644 and of ouabain in guinea-pig isolated atria

Josiane Ghysel-Burton & ¹Théophile Godfraind

Laboratoire de Pharmacodynamie Générale et de Pharmacologie, Université Catholique de Louvain, Avenue Emmanuel Mounier, 73, UCL 7350, B-1200 Bruxelles, Belgium

1 The inotropic effects of two concentrations of ouabain and of Bay K 8644 have been studied in isolated left atria of the guinea-pig in physiological solutions at pH lowered from 7.4 to 6.0 and in the presence of ethylisopropylamiloride (EIPA) an inhibitor of Na⁺/H⁺ exchange. The low concentration of ouabain (300 nM) was chosen to saturate the high affinity binding sites (it occupied about 7% of the low affinity sites). The high concentration of ouabain saturated both high and low binding sites. Bay K 8644 evoked a positive inotropic effect of a magnitude similar to ouabain (300 nM).

2 When comparing the positive inotropic effects of equi-effective concentrations of ouabain (300 nM) and of Bay K 8644 (100 nM), it was observed that extracellular acidification specifically depressed the inotropic effect of ouabain 300 nM; the positive inotropic effect of the high concentration of ouabain (3 μ M) was barely affected by extracellular acidification.

3 EIPA 10 μ M depressed the positive inotropic effect of ouabain 300 nM, but did not affect the peak response to Bay K 8644. The depressant action of EIPA on the positive inotropic effect of ouabain was concentration-dependent and was much more obvious on the effect of ouabain 300 nM than on ouabain 3 μ M.

4 An increase in diastolic tension was evoked by 3 μ M but not by 300 nM ouabain. This increase in tone was reduced dose-dependently by EIPA (10–30 μ M). It was also significantly reduced when the extracellular pH was equal to 6.4 or 6.0.

5 Ouabain (300 nM) evoked a gain in tissue Na and an equivalent loss in tissue K. Acidification of the extracellular pH down to pH 6.0 evoked a pH-dependent reduction of Na gain but left K loss unaltered. EIPA 10, 20 and 30 μ M evoked a significant reduction of Na gain without significantly affecting K loss.

6 Ouabain (3 μ M) evoked a large gain in tissue Na and an equivalent loss of K. Tissue Ca content was also increased. Acidification of the extracellular pH from pH 7.4 to pH 6.9 evoked a significant reduction of Na and Ca gain; changes in tissue K were not significant. Acidification down to pH 6.0 increased reduction of Na and Ca gain, but not that of K loss which nevertheless became statistically significant.

7 These results show that tissue Na gain observed after inhibition of the sodium pump by ouabain may be related to activation of Na-H exchange. They also indicate that blockade of Na-H exchange selectively reduced the inotropic effect of ouabain in guinea-pig atria resulting from interaction of the glycoside with high affinity binding sites. This confirms previous observations in rat heart.

Introduction

In rat heart, it is agreed by several authors that ouabain-evoked positive inotropic effects occur through an interaction with both high and low affinity binding sites (Adams *et al.*, 1982; Finet *et al.*, 1982). Only the interaction with low affinity sites appears to be responsible for the appearance of intoxication (Finet *et al.*, 1983). Electrophysiological studies show that inhibition of Na-K pump current in guinea-pig ventricular myocytes by cardioactive steroids occurs at high- and low-affinity sites (Mögl *et al.*, 1989). Enzymatic and ligand studies have shown that these binding sites may be related to two (Na + K) ATPases isoforms showing, respectively, high and low affinity for ouabain (Noël & Godfraind, 1984). Recent studies have shown that these isoforms found in heart tissue are coded by different genes and could be similar to the alpha and alpha + isoforms identified in nervous tissue (Sweadner & Farshi, 1987).

We have recently observed that ethylisopropylamiloride (EIPA), an inhibitor of Na-H exchange, selectively inhibits the positive inotropic effect evoked by low concentrations of ouabain interacting with high affinity binding sites in rat isolated ventricles (Finet & Godfraind, 1986). In view of our earlier reports that high and low affinity binding sites for ouabain may be identified in guinea-pig atria (Godfraind & Ghysel-Burton, 1977), we have examined whether the observations made in rat isolated heart could be extended to another

species. Therefore, we have measured the inotropic response of isolated left guinea-pig atria to a low (300 nM) and a high (3 μ M) concentration of ouabain and have studied its modification by EIPA and by changes in the pH of the bathing fluid. In this preparation, ouabain 300 nM evokes an increase in contractile force without change in diastolic tension whereas ouabain 3 μ M evokes changes in both systolic and diastolic tension (Ghysel-Burton & Godfraind, 1979). We have also examined the influence of these treatments on the contractile response to Bay K 8644 100 nM which evoked at pH 7.4 an increase in contractile force similar to ouabain 300 nM (for references see Godfraind *et al.*, 1986). In addition, we have estimated changes in ionic gradients evoked by these manipulations.

The results show that treatments able to evoke intracellular acidosis inhibited to a greater extent the positive inotropic action of ouabain 300 nM than that of ouabain 3 μ M or of Bay K 8644 100 nM. This inhibition was associated with an attenuation of tissue Na gain resulting from ouabain action. Such observations indicate that the role of Na-H exchange in the inotropic effect of low doses of ouabain is not restricted to the rat but may also be important in other species.

Methods

Preparation

Albino guinea-pigs weighing approximately 400 g were killed by a blow on the head, exsanguinated and the heart rapidly

¹ Author for correspondence.

removed. The atria were dissected out in physiological solution. The left atria were suspended in an organ bath between platinum electrodes under a resting tension of 5 mN. The atria were stimulated with rectangular pulses of 10 ms (strength at least twice threshold) at a rate of 3.3 Hz.

Physiological solutions

The composition of the Tyrode solution (was mM): NaCl 137, KCl 6, CaCl_2 1.82, MgCl_2 1.05, NaH_2PO_4 0.417, NaHCO_3 11.9, glucose 5.5. It was equilibrated at 30°C with a mixture of 95% and 5% CO_2 .

The composition of the HEPES-Tyrode was similar to Tyrode for KCl, MgCl_2 , CaCl_2 and glucose, it contained HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) 10 mM and NaOH added to reach the appropriate pH (6, 6.4, 6.9, 7.4, 8.4), the concentration of Na^+ being kept as in Tyrode. It was equilibrated at 30°C with 100% O_2 .

Ionic content determinations

At the end of the incubation, the atria were removed from the organ bath and each preparation was blotted on filter paper and was pressed three times with a roller weighing 350 g. After weighing, each atrium was placed in a quartz crucible and left overnight at 100°C; it was then weighed. To remove organic

material, it was left at 500°C for 18 h. The residue was dissolved in 1 ml HCl (1 N) and assayed by atomic absorption as described previously for another tissue (Godfraind, 1976).

Results are expressed in mmol kg^{-1} wet wt.

Recording of the contractions and experimental protocol

Recording of the contractile activity were made by an isometric lever using two strain gauges as part of a balanced bridge, the output of which was fed into a potentiometric recorder. The preparations were equilibrated during 30 min in Tyrode solution then the HEPES-Tyrode pH 7.4 for 30 min and, when required in HEPES-Tyrode at another pH.

Changes in systolic tension evoked by various treatments were expressed either as the difference between the actual force and the force developed before the treatment was applied or as a percentage of the tension developed before the treatment.

Statistical methods

Whenever possible, values are presented as means $\pm$ s.e.mean. Significance of differences between means was tested by Student's *t* test, a level of probability of less than 0.05 being considered as significant.

Figure 1(a) Recordings of the contractions of guinea-pig isolated left atria preincubated in Tyrode solution for 30 min and transferred for additional 30 min to HEPES-Tyrode at pH 7.4 and thereafter in HEPES-Tyrode at the pH indicated (8.4, 7.4, 6.4). The time of recording is indicated. **(b)** Contractile force of guinea-pig isolated left atria incubated in HEPES-Tyrode at pH 8.4 (■), 7.4 (□), 6.9 (▲), 6.4 (◆). Abscissa scale: time after admission of the indicated perfusion fluid in the organ chamber (min). Ordinate scale: systolic tension developed by the preparations (mN). Each point is the mean of 6 preparations; s.e.means are indicated on the last measure when they exceeded the size of the symbols.

Drugs

Ouabain (Merck) and EIPA (Ciba-Geigy) were dissolved in distilled water. 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. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) was purchased from Janssen Chimica (Belgium).

Results

Influence of pH_o on the contractile response

Figure 1 (a and b) illustrates the time-course of the contractility of atria immersed in physiological solution buffered at pH varying from 8.4 to 6.4. Transfer of preparations from pH 7.4 to pH 6.9 and 6.4 evoked a sudden decrease followed by a slow time-dependent reduction of the contractile force such that after 90 min observation, the systolic tension developed at acidic pH was about one third of the tension at pH 7.4. Figure 1a shows that immediate alterations evoked by pH changes were not washout artifacts but were related to pH changes.

When preparations were transferred from pH 7.4 to pH 8.4, they showed a sudden reduction in contractile force with a return to pH 7.4 values achieved after 15 min. Further, the contractile force at pH 7.4 and 8.4 were not different.

Figure 2 shows the inotropic effect of ouabain 300 nM and 3 μ M and of Bay K 8644 100 nM at pH 7.4. As already reported (Ghysel-Burton & Godfraind, 1979), ouabain 3 μ M evoked a fast increase in the systolic tension up to a peak followed by a reduction of the contraction. The decrease in systolic tension was associated with the development of diastolic contracture. On the other hand, ouabain 300 nM evoked a progressive increase of the systolic contraction reaching a maximum after 45 min treatment; it was maintained for prolonged periods and the diastolic tension remained unchanged. The time to maximum effect was much faster with Bay K 8644 100 nM which produced an inotropic effect of a magnitude similar to ouabain 300 nM, it was also sustained as soon as the plateau was reached.

In Figure 3, the time course of the inotropic response to ouabain 300 nM and to Bay K 8644 100 nM is compared at different pH values. At pH 8.4, the inotropic effect of both drugs was slightly higher than at pH 7.4 but this was not sta-

Figure 2 The time course of the systolic tension of stimulated left guinea-pig atria bathed in HEPES-Tyrodé solution at pH 7.4 and treated with ouabain 300 nM ($n = 6$) ($\blacktriangle$), ouabain 3 μ M ($n = 4$) ($\blacklozenge$) and Bay K 8644 0.1 μ M ($n = 3$) ($\blacksquare$) or untreated controls ($n = 6$) ($\square$). Abscissa scale: time of observation (min), drugs were added at 15th min. Ordinate scale: systolic tension developed by the preparations (mN). Each point is the mean of the indicated number of observation; s.e. means are indicated on the last measure when they exceeded the size of the symbols.

Figure 3 The time course of the systolic tension of stimulated left guinea-pig atria bathed in HEPES-Tyrodé solution at pH 7.4, 8.4, 6.9 and 6.4 and treated or not treated (controls ($n = 6$) ($\square$)) by ouabain 300 nM ($n = 6$) ($\blacktriangle$) and Bay K 8644 0.1 μ M ($n = 3$) ($\blacksquare$). Abscissa scale: time of observation (min), drugs were added at 15th min. Ordinate scale: systolic tension developed by the preparations (mN); s.e. means are indicated on the last point if they exceeded the size of the symbols.

tistically significant. At pH 6.9, the inotropic effect of ouabain was obviously attenuated and even faded after 1 h ($P < 0.01$ when compared to pH 7.4). On the other hand, the effect of Bay K 8644 on contractile force was similar at pH 6.9 and 7.4. At pH 6.4, the inotropic effect of ouabain was much reduced and it was not sustained ($P < 0.01$). The effect of Bay K 8644 was reduced by 30% ($0.05 < P < 0.01$) and showed an important fade.

Figure 4 Peak increase in contractile force of stimulated left guinea-pig atria treated by ouabain 300 nM (solid columns, upper panel, $n = 6$) and by ouabain 3 μM (hatched columns, lower panel, $n = 4$) after incubation in HEPES-Tyrode solution at the pH indicated (left panel) and after pretreatment or not with ethylisopropylamiloride (EIPA) (right panel). Ordinate scale: increase in contractile force (mN); * $P < 0.05$; ** $P < 0.01$. Abscissa scale: left panel: pH of the medium; right panel: concentration of EIPA (μM).

Figure 4 illustrates a comparison of the pH-dependence of the maximum inotropic effect of the two concentrations of ouabain. Acidification of the bathing fluid had a more depressive action on peak effect of ouabain 300 nM than of ouabain 3 μM which was altered only at pH 6 ($P < 0.05$). However, acidification evoked a reduction of the magnitude of the diastolic contracture observed with ouabain 3 μM (see Figure 5).

Action of ethylisopropylamiloride on the contractile response

Ethylisopropylamiloride (EIPA) 10 μM , 20 μM and 30 μM evoked a transient increase in contractile force followed by a

Figure 5 Maximal diastolic tension developed in the presence of ouabain 3 μM by stimulated guinea-pig left atria incubated, (a) in Tyrode without ($n = 6$) or with ethylisopropylamiloride (EIPA) 10, 20, 30 μM ($n = 4$) and (b) in HEPES-Tyrode solution at various extracellular pH ($n = 4$). Ordinate scale: increase in diastolic tension (mN), * $P < 0.05$; ** $P < 0.01$. Abscissa scale: (a) EIPA concentration; (b) pH of the HEPES-Tyrode solution.

slow decrease of contractility (Figure 6); at higher concentrations, it evoked a fast intoxication of the preparation. Ouabain was added after a preincubation of 30 min with EIPA. Figure 4 shows that the inotropic action of ouabain 300 nM was reduced by 60% with EIPA 10 μM and by 80% with EIPA 20 μM and blocked by EIPA 30 μM ($P < 0.01$). The action of ouabain 3 μM was reduced by EIPA, but 55% of the response was still present with EIPA 30 μM . As Figure 5 shows, the diastolic contracture evoked by ouabain 3 μM was reduced dose-dependently by EIPA. Figure 6 illustrates the action of EIPA 10 μM on equi-effective concentrations of ouabain and Bay K 8644. It shows that by contrast with ouabain, the time to peak and the peak effect of Bay K 8644 were not affected by EIPA treatment; however, the plateau of systolic response to Bay K 8644 was not sustained as it was in controls.

Figure 6 The time course of the changes in systolic tension of stimulated left guinea-pig atria pretreated (■) or not pretreated (□) with ethylisopropylamiloride (EIPA) 10 μM and treated, upper panel (a), with Bay K 8644 0.1 μM (without Δ and with EIPA Δ) and, lower panel (b), with ouabain 300 nM (without Δ and with EIPA Δ). Drugs were added after a pretreatment of 30 min. Abscissa scale: time after the addition of EIPA to the Tyrode solution (min). Ordinate scale: changes in systolic tension (mN). Each point is the mean of 12 observations during the pretreatment and of 6 observations after. The limits of the s.e. means did not exceed the size of the symbols.

Figure 7 Changes in the ionic composition of stimulated left guinea-pig atria bathed in HEPES-Tyrod solution at various pH and treated by ouabain 300 nM (a) and ouabain 3 μ M (b). Ordinate scale: upper panels, mmol kg^{-1} wet wt. gain in Na (hatched columns) and loss in K (open columns); lower panels, mmol kg^{-1} wet wt. gain in Ca (solid columns); each column represents the mean of 6 observations, * $P < 0.05$; ** $P < 0.01$.

Action of pH changes and of ethylisopropylamiloride on the ionic composition of isolated atria

The ionic content of atria was estimated after an incubation of 3 h in the various experimental conditions described here. Bay K 8644 lowering pH_o down to 6.4 and EIPA 10 μ M, 20 μ M and 30 μ M did not change the ionic composition of the tissue (data not shown). A small reduction of tissue K content was

Figure 8 Changes in the ionic composition of stimulated guinea-pig left atria bathed in Tyrod solution and not pretreated or pretreated by ethylisopropylamiloride (EIPA) 10, 20 and 30 μ M, then, treated with ouabain 300 nM (a) and ouabain 3 μ M (b). Ordinate scale: upper panels, mmol kg^{-1} wet wt. gain in Na (hatched columns) and loss in K (open columns); lower panels, mmol kg^{-1} wet wt. gain in Ca (solid columns); each column represents the mean of 6 observations; * $P < 0.05$; ** $P < 0.01$.

observed when pH_o was reduced down to pH 6 ($P < 0.01$); however, there was no perceived change in Na and Ca content. As shown in Figure 7, ouabain 300 nM and 3 μ M evoked the classical dose-related change in ionic content: gain of Na, loss of K and gain of Ca. Ouabain 300 nM evoked a net gain in Na and an equivalent loss of K ($P < 0.01$), but no change in Ca content. The action of ouabain 3 μ M on monovalent cations content was more pronounced than that of ouabain 300 nM and it was accompanied by a gain in Ca ($P < 0.01$). Decrease of pH_o attenuated significantly the gain of Na and Ca evoked by ouabain, (pH 6.9, $P < 0.05$; pH 6.4, $P < 0.01$); the loss of K was unchanged with ouabain 300 nM and reduced with ouabain 3 μ M.

As Figure 8 illustrates, Na and Ca gain and K loss evoked by ouabain 3 μ M were attenuated in the presence of EIPA. In the absence of EIPA, the gain of Na evoked by ouabain 300 nM was balanced by an equivalent loss of K; with EIPA 10 μ M, the Na content of ouabain-treated atria was reduced ($P < 0.01$) but the loss of K was unchanged. Thus, in the two conditions, reduced pH_o and presence of EIPA, the action of ouabain on the tissue content of Na and Ca was selectively attenuated, the ouabain-dependent reduction of tissular K being much less affected.

Discussion

Deitmer & Ellis (1980) have shown in sheep cardiac Purkinje fibres that the steady-state pH_i changed linearly with the extracellular pH. Data presented in this paper show that extracellular acidification from pH 7.4 to pH 6.4 affected the contractility of guinea-pig isolated atria and their response to the inotropic agents Bay K 8644 and ouabain. The inotropic response to ouabain was more sensitive to pH changes than was the inotropic response to Bay K 8644. It is likely that the reduced contractility may be attributed to intracellular acidosis (Vaughan-Jones *et al.*, 1983). As reported by Fabiato & Fabiato (1978), decrease in pH_i reduces the calcium sensitivity of contractile proteins and therefore, the tension development for a given level of activator Ca_i .

When compared to Bay K 8644, the higher sensitivity to pH_o reduction of the ouabain inotropic action could be related to the inhibition of the Na pump. It has indeed been shown that ouabain causes an increase in Na_i and a progressive intracellular acidification. There is a time-lag between these two events of about 10 min (Deitmer & Ellis, 1980). Such a time-lag could explain the difference in sensitivity between the inotropic responses of low and high doses of ouabain to reduction of pH_o , since the time to peak of the response to ouabain 3 μ M is less than 10 min. Experimental data obtained with EIPA are consistent with this interpretation. EIPA has been shown to block selectively the Na-H exchanger, leading to intracellular acidification (Frelin *et al.*, 1984). In the presence of this agent, we observed a transient positive inotropic effect followed by a long-lasting negative inotropic effect, a reduction of the inotropic action of ouabain and a fade of the inotropic response to Bay K 8644.

We have also observed that the ionic composition of the tissue was highly sensitive to pH_o and to EIPA when the sodium pump was blocked by ouabain. This indicates that tissue Na gain observed in the presence of ouabain is not only due to blockade of the pump but also the stimulation of Na-H exchange since blockade of this process by EIPA selectively reduced the Na gain. Such a conclusion is in agreement with other authors (Deitmer & Ellis, 1980; Frelin *et al.*, 1984; Kim *et al.*, 1987). As reported by Vaughan-Jones *et al.* (1983), acidification of the cardiac cell may be related to change in $[\text{Ca}_i]$, allowing a Ca-H interaction responsible for this acidification. Ouabain 300 nM and Bay K 8644 100 nM produced a similar increase in contractile force, but a net increase in tissue calcium was only observed with ouabain 3 μ M. Blockade of Na-H exchange with EIPA or acidification of the bathing

fluid barely affected ouabain-evoked K loss but significantly attenuated both Na and Ca gain evoked by ouabain. This indicates that a resultant of Na-H exchange-blockade was a secondary reduction of Na-Ca exchange stimulated by increase of Na_i. Therefore, it is likely that Na entering through Na-H exchange is responsible for activation of Na-Ca exchange when the pump is blocked by ouabain. This conclusion is at variance with Kim *et al.* (1987) who have proposed that the activation of Na-Ca exchange could produce a Ca_i increase sufficient to cause an intracellular acidification responsible in turn for stimulation of Na-H exchange. More information is needed to establish definitely this sequence of events. Nevertheless, blockade of Na-Ca exchange resulted in a marked attenuation of the diastolic contracture evoked by ouabain 3 µM.

The existence of a difference between the action of low and high doses of ouabain has been reported by several authors (see Noble, 1980). The low concentration of ouabain used in this study was high enough to saturate the high affinity binding sites (Godfraind & Ghysel-Burton, 1977; Mögöl *et al.*, 1989). From earlier measurements of specific binding to intact tissues (Godfraind & Ghysel-Burton, 1977; Ghysel-Burton & Godfraind, 1979), it may be estimated that the low affinity sites were completely saturated with ouabain 3 µM and that at the concentration of 100 nM, ouabain interacted with about 7% of these sites. This is consistent with the observation that ouabain 100 nM evoked a sustained positive inotropic effect but left unchanged the diastolic tension. Indeed, as shown in rat preparations (Finet *et al.*, 1983; Finet & Godfraind, 1986), increase in diastolic tension is evoked by ouabain when the low affinity ATPase is inhibited. Our present observations in guinea-pig heart show that the positive inotropic effect resulting from the interaction of ouabain with high affinity sites was highly sensitive to extracellular acidification and that the action resulting from the inhibition of the low affinity ATPase was reduced only at pH 6 which also reduced the

effect of Bay K 8644. On the other hand, EIPA was obviously less selective as an inhibitor of the effect of the low dose of ouabain in guinea-pig atria. Studies in rat allowed a clear dissociation between the high and the low affinity component of ouabain inotropic effect showing that only the high affinity was suppressed by EIPA treatment (Finet & Godfraind, 1986). Such a species difference could be related to an effect of EIPA not associated with its Na-H inhibition.

As already discussed above, the high sensitivity to extracellular acidification of the inotropic response to low doses of ouabain may be related to the time required to reach the peak inotropic effect. In purified (Na-K)-ATPase preparations, there is no evidence that the rate of association of ouabain is different at low and high affinity binding sites. They differ only in their dissociation constants (De Pover & Godfraind, 1979). Furthermore, there is no indication that these two sites have different cellular localizations (Wibo *et al.*, 1990). Therefore, the slow onset of action of the low doses interacting with high affinity sites could be related to the time required to reach binding steady state rather than to other mechanisms.

In summary, the results presented in this paper do confirm that activation of Na-H exchange is involved in the action of ouabain on the ionic composition of cardiac cell. They show that inhibition of this process by extracellular acidification selectively blocked the action of the low doses of ouabain. This indicates that the coupling process between the interaction of ouabain with high and low affinity sites may be different in guinea-pig heart as has already been shown in rat heart (Finet & Godfraind, 1986).

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