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Diffusion characteristics dissociate ouabain binding from inotropic effect in guinea-pig myocardium

F. Ebner & H. Siegl

Institut für Pharmakologie und Toxikologie der Technischen Universität München, Biedersteiner Str. 29, D-8000 München 40, Federal Republic of Germany

1 [^{3}H]-ouabain uptake in resting guinea-pig papillary muscles depended directly on incubation time and inversely with muscle radius. The equivalence of both parameters support the relevance of diffusion. A particular mechanism of receptor-controlled diffusion was implicated by the saturation of initial rates of uptake with increasing ouabain concentrations. Saturation of initial uptake indicates an inhomogeneity of receptor occupancy with ouabain-equilibrated receptors in the superficial areas of the preparation and free receptors in the muscle core.

2 For comparison [^{3}H]-ouabain diffusion was evaluated in a non-cellular preparation i.e. glass fibre filters soaked with modified Krebs-Henseleit solution. Diffusion was approximately 3 orders of magnitude faster when compared with the papillary muscles.

3 The time course of the inotropic effect of ouabain on rested-state contractions dissociated from the tissue content of ouabain with an increased steroid concentration. This dissociation appears to be associated with the inhomogeneity of receptor occupancy, since equal amounts of the steroid correspond to different concentration profiles in the tissue when the bath concentration is changed. Functional coupling between different muscle areas may have modulated the influence of inhomogeneous receptor occupancy.

Introduction

Receptor-controlled diffusion limits the onset of the inotropic effect of cardioactive steroids in guinea-pig papillary muscle (Ebner *et al.*, 1985; Ebner, 1987); that is, receptor occupation reduces the drug concentration in the extracellular space and retards diffusion. Provided that the interaction of ouabain with the receptors does not completely halt diffusion, both processes could develop simultaneously throughout the tissue. In this case the concentration gradient of ouabain would be less steep than with a sequential mechanism where local receptor occupation binds all the available ouabain until a local equilibrium is reached and diffusion is resumed. For the degree of local and, relative to the entire time course, early equilibration of the receptors with the steroid, the saturation behaviour of initial ouabain uptake seems to be important, since initial rates of receptor occupation depend on ouabain concentration linearly while local equilibria would induce saturation of initial uptake.

Whereas with homogeneous distribution of the drug in the tissue a causal relationship between different experimental parameters may be straightforward, this may not be the case with concentration inhomogeneity. The magnitude of the inhomogeneity of receptor occupancy therefore determines the effects of the entire tissue, depending on the extent of electrical and mechanical cell-to-cell coupling (for review cf. Loewenstein, 1981) which tends to equilibrate the effects of different areas of the tissue. In consequence, during diffusion, apparently equal changes may not produce equivalent effects.

At present, studies on the relationship between receptor occupation and the inotropic effect of cardioactive steroids still require multi-cellular preparations. Therefore the properties of concentration gradients and their influence on experimental parameters need to be considered when causal relationships are to be established. Saturation of initial ouabain uptake as a measure of the inhomogeneity of receptor occupancy, in particular, allows us to judge the role of diffusion in apparent isolation from otherwise closely related parameters.

Methods

Cylindrical papillary muscles of largely different diameters from the right ventricle of guinea-pigs (250–350 g) of either sex were connected to a force transducer and incubated in modified Krebs-

Henseleit solution of the following composition, if not stated otherwise (mmol l^{-1}): NaCl 115, $NaHCO_3$ 24.9, KH_2PO_4 1.2, $MgSO_4$ 1.2, KCl 1.2, $CaCl_2$ 3.2, glucose 10. The incubation medium was constantly gassed with 95% O_2 plus 5% CO_2; the temperature was 35°C and the pH 7.5. Resting force of the muscles was maintained at 3.96 mN. Muscle diameters were determined with a microscope before the experiments at constant resting force (for details see Ebner & Waud, 1978).

The binding of [^3H]-ouabain

After preincubation of the non-stimulated papillary muscles for 30–45 min, [^{14}C]-sorbitol (96 nmol l^{-1} equal to 18.5 KBq per 15 ml bath volume) was added. After an additional 30 min, [^{3}H]-ouabain (18.5 nmol l^{-1} equal to 185 KBq per 15 ml bath volume) and unlabelled ouabain of variable amounts were applied. At the end of the experiment the muscles were rinsed, gently blotted and weighed. After digestion in 1 ml tissue solubilizer (BTS-450) overnight at room temperature, and addition of 100 μl glacial acetic acid and 10 ml NA scintillation cocktail, the radioactivity was determined in a Beckman LS-7500 liquid scintillation counter. Samples of medium (500 μl) were counted in 10 ml scintillation cocktail HP. Tissue: medium ratios were calculated by $(\text{d.p.m.}_{\text{tissue}}\ \mu l_{\text{medium}})\ (\text{d.p.m.}_{\text{medium}}\ \text{mg}_{\text{wet weight}})^{-1}$; where d.p.m. is disintegrations per min. Tissue contents were calculated from tissue: medium ratio × ouabain concentration in the medium (in nmol l^{-1}) which gives tissue contents in fmol ouabain mg^{-1} wet weight.

The inotropic effect of ouabain

The muscles were preincubated for 1 h in modified Krebs-Henseleit solution (KCl, 4.7 mmol l^{-1}) and stimulated at a frequency of 1 Hz with electrical square-wave pulses of 3 ms duration at an intensity slightly above stimulation threshold. KCl concentration was lowered to 1.2 mmol l^{-1} and rested-state contractions elicited every 1000 s. Thereafter the appropriate ouabain concentration was added and the development of its effect was followed. Peak force of contraction was evaluated from the tracings of a pen recorder. The data were normalized as percentage of the value at the end of the initial equilibration period at 1 Hz.

The diffusion of [^3H]-ouabain in glass fibre filters

The diffusion of [^{3}H]-ouabain was estimated using strips of glass fibre filters (Whatman GF/C), soaked initially in Krebs-Henseleit solution containing [^{14}C]-sorbitol (63 nmol l^{-1}) in the absence and presence of unlabelled ouabain (1 μmol l^{-1} ouabain). The strips were mounted vertically, their lower ends dipping in the Krebs-Henseleit solution which covered the bottom of a closed vial maintained at 35°C. Thirty or 60 min after the addition of [^{3}H]-ouabain (55.5 nmol l^{-1}), the strips were cut into sections of approximately 3 mm in length, eluted in scintillation cocktail HP for 24 h and radioactivity then determined. The length of each individual section was determined accurately by measuring the amount of [^{14}C]-sorbitol in it relative to the total [^{14}C]-sorbitol content of the entire strip. Equilibrium values of [^{3}H]-ouabain of those sections which had not yet reached their final values of [^{3}H]-ouabain were calculated on the basis of the equilibrated sections. According to Crank (1975), in a plane sheet sorption initially obeys

$$M_t M_\infty^{-1} = 4\pi^{-0.5}(D \times t \times l^{-2})^{0.5} \qquad (1)$$

where M_t and M_∞ are the amounts of [^{3}H]-ouabain at time t and at equilibrium, respectively, D = diffusion constant, l = length. From the slope of $M_t M_\infty^{-1}$ vs $(t \times l^{-2})^{0.5}$ the diffusion constant is obtained.

Statistics

The data are presented as individual values or as arithmetic means ± s.e.mean. Statistical significance was assumed at 5% probability of error. Regressions were calculated by the method of least squares. Goodness of fit was estimated with the F-test.

Drugs and materials

Unlabelled ouabain was from Serva, Heidelberg, Germany. [^{3}H]-ouabain (specific activity 666 GBq mmol $^{-1}$) in ethanol: benzene (9:1 v/v), [^{14}C]-sorbitol (specific activity 12.802 GBq mmol $^{-1}$) in ethanol: water (9:1 v/v) were purchased from New England Nuclear, Dreieich, Germany. Tissue solubilizer BTS-450 (0.5 N quaternary ammonium hydroxide in toluene), scintillation cocktails NA and HP were obtained from Beckman Instruments, München, Germany.

Results

[^{3}-H]-ouabain diffusion in non-stimulated guinea-pig papillary muscle

The linear relationship between [^{14}C]-sorbitol tissue content and muscle wet weight after 30 min incubation with 96 nmol l^{-1} [^{14}C]-sorbitol suggests a homogeneous distribution of [^{14}C]-sorbitol in the muscle (Figure 1a). By contrast, with 18.5 nmol l^{-1}

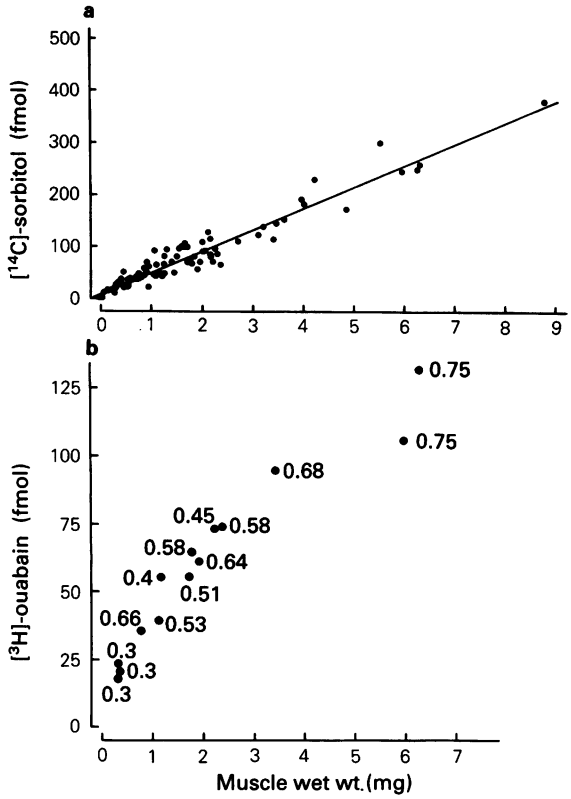

Figure 1 Dependence of the uptake of [^{14}C]-sorbitol (a) and [^{3}H]-ouabain (b) on muscle weight: guinea-pig papillary muscles were incubated for 30 min with 96 nmol l^{-1} [^{14}C]-sorbitol (data, in part, from the experiments of Figure 3) and with 18.5 nmol l^{-1} [^{3}H]-ouabain. Total tissue content was plotted on the ordinate scales in fmol and related to muscle wet weight (in mg) on abscissa scales. Numbers next to (●) in (b) show muscle radii in mm.

[^{3}H]-ouabain the corresponding relationship was curvilinear (Figure 1b) as evidenced by the improved goodness of fit of a quadratic polynomial forced through the coordinate origin ($y = bx + cx^2$; $b = 40.9 \pm 2.4$, $c = -3.5 \pm 0.5$) in comparison with a linear regression ($y = bx$; $P < 0.01$). According to the parallel of deviation from linearity to muscle radius (see numbers beside symbols in Figure 1b) the reduced effectiveness of the thicker preparations in retaining [^{3}H]-ouabain became particularly evident when the amounts of [^{3}H]-ouabain mg^{-1} wet weight were related inversely to muscle radius after 10 or 30 min incubation at the same ouabain concentration (Figure 2).

For diffusion, incubation time (t) and inverse radius (r) are equivalent; i.e., the square root of

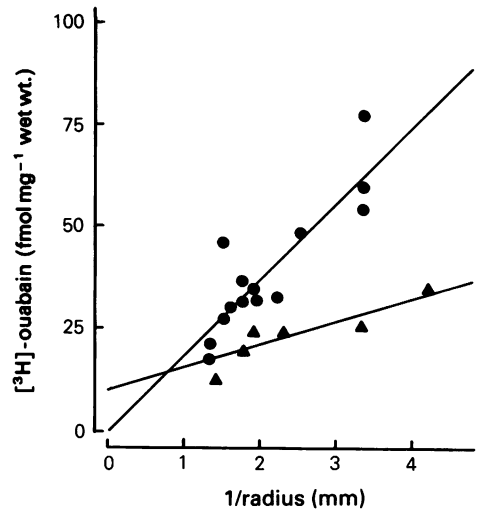

Figure 2 [^{3}H]-ouabain uptake depends on muscle radius. After 10 (▲) or 30 (●) min incubation with 18.5 nmol l^{-1} [^{3}H]-ouabain, tissue content of [^{3}H]-ouabain (in fmol mg^{-1} wet weight, ordinate scale) was inversely related to the muscle radius (mm^{-1}, abscissa scale). Regressions: (10 min) $y = (0.556 \pm 0.055) + (0.307 \pm 0.056)x$, $r = 0.939$, $P < 0.01$; (30 min) $y = (-0.005 \pm 0.12) + (1.027 \pm 0.167)x$, $r = 0.872$, $P < 0.001$.

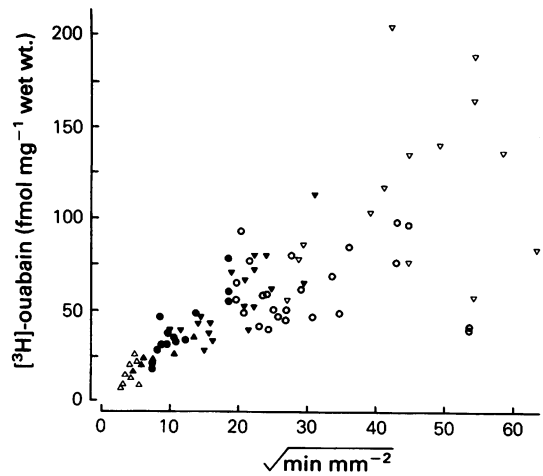

Figure 3 Incubation time and the inverse of the muscle radius are equivalent in the time course of [^{3}H]-ouabain uptake. Total tissue content of [^{3}H]-ouabain (in fmol mg^{-1} wet weight, ordinate scale) was determined in papillary muscles after different incubation periods with 18.5 nmol l^{-1} [^{3}H]-ouabain. Abscissa scale: square root of incubation time × radius^{-2} in min mm^{-2}. The data from individual preparations are shown. Incubation time (min): △ (5), ▲ (10), ● (30), ▼ (60), ○ (180), ▽ (360).

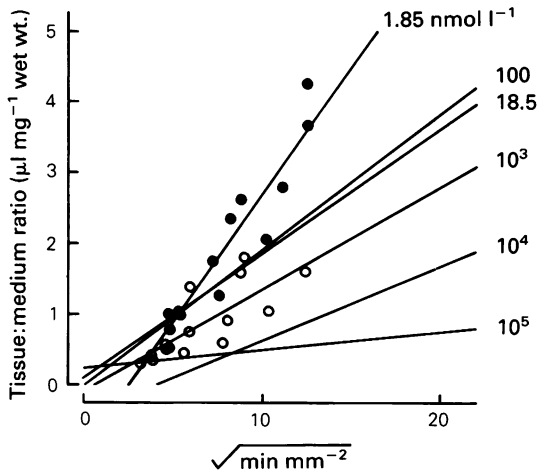

Figure 4 Initial rates of [^{3}H]-ouabain uptake in papillary muscles saturate with increasing ouabain concentrations. After incubation with 1.85 (lowest concentration) or 18.5 nmol l^{-1} [^{3}H]-ouabain and various amounts of unlabelled ouabain for 5 to 30 min tissue-medium ratios in μl mg^{-1} wet weight (ordinate scale) were related to the square root of incubation time × radius^{-2} (min mm^{-2}; abscissa scale). For the sake of clarity only the data with 1.85 and 10^3 nmol l^{-1} ouabain (● and ○, respectively) are shown. With the exception of 100 and 10 μmol l^{-1} ouabain, all correlation coefficients were significantly different from zero ($P < 0.001$). Regression coefficients (ouabain concentration in nmol l^{-1} in parentheses); 0.358 ± 0.048 (1.85), 0.175 ± 0.016 (18.5), 0.189 ± 0.027 (100), 0.146 ± 0.032 (1000), 0.105 ± 0.042 (10^4), 0.024 ± 0.023 (10^5). The number of experiments ranged from 6 to 28.

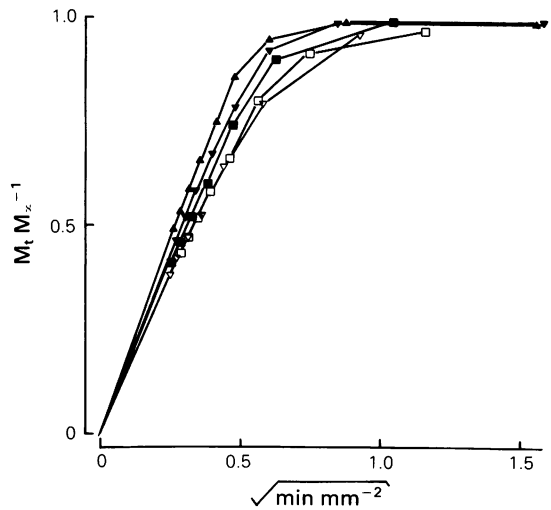

Figure 5 The diffusion of [^{3}H]-ouabain in modified Krebs-Henseleit solution. The uptake of [^{3}H]-ouabain in glass fibre filters is shown with relative values $M_t M_\infty^{-1}$ on the ordinate scale, where M_t is the amount of [^{3}H]-ouabain in filter of length l at time t, M_∞ is the corresponding equilibrium value, extrapolated from equilibrated sections. Abscissa scale: square root of incubation time × length^{-2} (min mm^{-2}). The symbols represent the following conditions (incubation time in min): 55 nmol l^{-1} [^{3}H]-ouabain, ▽ (30), ▲ (60), ▼ (60); 1 μmol l^{-1} ouabain + 55 nmol l^{-1} [^{3}H]-ouabain ■ (30), □ (60).

(t × r^{-2}) determines the time course (see Smith, 1969; Crank, 1975). With the pertinent plot of ouabain uptake (Figure 3) the data from preparations with short periods of incubation but large radii actually overlapped those with inverse parameters. From the initial slope in Figure 3, disregarding saturation behaviour and the higher order terms which expand equation 1 for cylinders (cf equation 5.25 in Crank, 1975) an apparent diffusion constant for [^{3}H]-ouabain in papillary muscle of 2.56×10^{-8} cm^2 s^{-1} was estimated; equilibrium (M_∞ in equation 1) was assumed after 6 h incubation. Although the present findings support the role of diffusion in the time course of [^{3}H]-ouabain uptake, its intimate relationship to receptor binding became evident when the initial rates of uptake were considered with increased ouabain concentration. In the case of concentration-independent diffusion tissue-medium ratios should be superimposable. Their reduction (Figure 4), therefore, demonstrates that initial rates of ouabain uptake are saturable as a consequence of receptor occupation.

[^{3}H]-ouabain diffusion in modified Krebs-Henseleit solution

For comparison, the diffusion of [^{3}H]-ouabain (55 nmol l^{-1}) in glass fibre filters soaked with modified Krebs-Henseleit solution in the absence and presence of 1 μmol l^{-1} unlabelled ouabain was determined (Figure 5). From the initial rates of uptake the diffusion constants for [^{3}H]-ouabain were calculated according to equation 1 with values from 5.3 to 9.6 × 10^{-5} cm^2 s^{-1}.

Dissociation of ouabain binding from its inotropic effect

If according to the mechanism of diffusion, identical tissue contents of ouabain correspond to different concentration profiles with changing bath concentrations, the inotropic effects of papillary muscle should reflect the different receptor occupancies. Actually at 0.3 μmol l^{-1} ouabain, the inotropic effect on rested-state contractions was below the value observed at 0.5 μmol l^{-1} ouabain when the effect was related to the tissue content of ouabain (Figure 6). The result

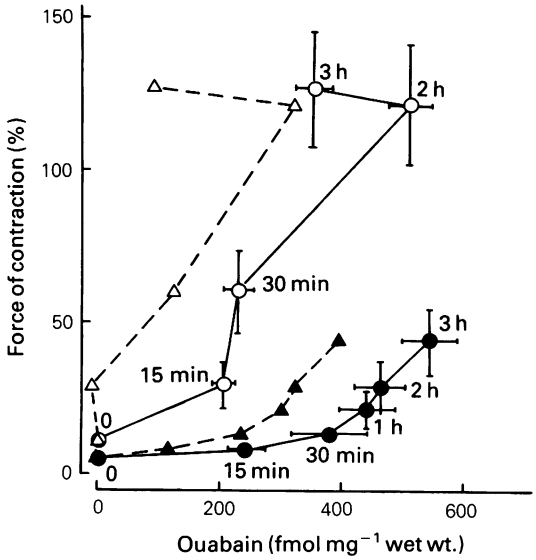

Figure 6 The relationship between tissue content of ouabain and force of contraction depends on ouabain concentration. The magnitude of rested-state contractions (0.001 Hz), as a percentage of the control value after the initial equilibration period at 1 Hz (ordinate scale, 100% = 13.6 ± 1.7 and 15.2 ± 2.5 mN in 0.5 and 0.3 μmol l^{-1} ouabain group, respectively), was related to the tissue content of ouabain in fmol mg^{-1} wet weight (abscissa scale) of different groups of muscles incubated under the same experimental conditions. The diameter of the muscles was comparable to those in the preceding figures. Broken lines indicate the data which were corrected for that amount of ouabain corresponding to the individual [^{14}C]-sorbitol space, assuming a homogeneous distribution of the steroid in the extracellular space; solid lines show uncorrected data. The numbers beside the symbols represent the incubation times. Arithmetic means with some s.e.mean of 5 or 6 preparations are shown. Ouabain concentration was 0.3 (●, ▲) or 0.5 (○, △) μmol l^{-1}.

was the same when total or 'specific' (i.e., after correction for the amount of ouabain corresponding to the individual [^{14}C]-sorbitol space) amounts of ouabain were compared (solid vs broken lines in Figure 6).

Discussion

The properties of [^{3}H]-ouabain uptake in guinea-pig papillary muscle, in particular saturability of initial rates and dependence on muscle radius, conform to a sequential mechanism of receptor-controlled diffusion. Its extremely low rate is documented by the apparent diffusion constant of [^{3}H]-ouabain (2.56×10^{-8} cm^2 s^{-1}). Comparison with ouabain diffusion in glass fibre filters with modified Krebs-Henseleit solution ($5–9 \times 10^{-5}$ cm^2 s^{-1} corresponding to diffusion half times of 10 s in sheets of 1 mm length) clearly demonstrates the dramatic retardation, even if a barrier factor for the tissue is taken into account. For instance the diffusion of sucrose in cat myocardium is reduced to 23% of its value in water (Suenson *et al.*, 1974). The difference in rates of ouabain uptake in myocardium compared with diffusion in Krebs-Henseleit solution are even more astonishing if one considers the time course of ouabain binding to Na-K-ATPase preparations from guinea-pig hearts, where 5 to 10 min suffice for equilibration (e.g., Fricke & Klaus, 1977). Therefore, diffusion and binding are considerably faster than in a sequential chain of reactions. Thus, the fast binding rates of ouabain to membrane particles and the saturation of the initial ouabain uptake in the papillary muscle suggest that the equilibrium of receptor occupation was rapidly established in the superficial areas of the preparation. Presumably the interaction of ouabain with the receptors reduces the free drug concentration in the extracellular space to such an extent that, locally, further diffusion is suspended until the equilibrium of binding is established. Ouabain-equilibrated receptors on superficial cells consequently appear to co-exist with non-occupied receptors in the muscle core.

For linear, i.e., concentration-independent diffusion, the principle of superposition (for review see Thron, 1974) predicts superimposable concentration profiles after identical periods of incubation. During diffusion equal tissue contents obtained at different bath concentrations would, therefore, differ in concentration profile. Moreover, saturability of initial rates of uptake points to non-linear processes which must induce further distortion.

Since identical profiles of receptor occupancy elicit equivalent effects, it appears that the dissociation of ouabain binding from its effect at different bath concentrations (Figure 6) is a result of the local difference in receptor occupancy as well as the ouabain concentration in the extracellular space. The inhomogeneity of the extracellular ouabain concentration is documented by the lack of superimposition of binding and effect after the ouabain data had been corrected for [^{14}C]-sorbitol space with a homogeneous distribution of ouabain (Figure 6). In addition to diffusion, other non-specific ouabain binding could have contributed to the observed dissociation. The homogeneous dependence of [^{3}H]-ouabain binding on extracellular K concentration as steady-state binding to the value of [^{14}C]-sorbitol space is approached (Ebner *et al.*, 1986), however, makes the existence of such non-specific ouabain binding sites unlikely.

It has been shown that an electrolyte (^{42}K) can diffuse intercellularly (Weidmann, 1966). The pertinent diffusion constant of $7.9 \times 10^{-6} cm^2 s^{-1}$ corresponds to a half-time of approximately 40 s in a plane sheet of 1 mm length. Therefore, the increased intracellular Na (Na_i) produced by ouabain binding (Ebner *et al.*, 1986) may also be modulated by the diffusion of Na_i across the myocytes, regardless of an increase of longitudinal internal resistance at high ouabain concentrations (Weingart, 1977) which indicates the decoupling of cells. If intercellular diffusion of Na_i is not considerably faster than the concentration profile of the steroid changes (at 0.3 $\mu mol\,l^{-1}$ the half-time of ouabain binding to membrane preparations was 2–3 min, own unpublished results; see also Fricke & Klaus, 1977), different local Na_i activities result from local variations in the magnitude of Na-pump inhibition. Similar to the relationship of receptor occupation to effect, any local Na_i would accordingly dissociate from the effect of the entire preparation. In fact a discrepancy between Na_i and the force generated by sheep Purkinje fibres has been shown with different concentrations of strophanthidin (Boyett *et al.*, 1986). Also with intimate functional coupling of the myocytes, the mean values of Na_i should strongly depend on the respective concentration profile of ouabain at the receptors. Therefore, it seems that steroid diffusion in particular is involved in the dissociation of the inotropic effect of ouabain from its tissue content. In spite of this dissociation, the observed receptor occupancy can account for the development of the inotropic effect in view of the role of diffusion in that relationship.

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