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EFFECTS OF VANADATE IN CULTURED RAT HEART MUSCLE CELLS

VANADATE TRANSPORT, INTRACELLULAR BINDING AND VANADATE-INDUCED CHANGES IN BEATING AND IN ACTIVE CATION FLUX

K. WERDAN, G. BAURIEDEL, M. BOZSIK, W. KRAWIETZ and E. ERDMANN

Medizinische Klinik I der Universität München, Klinikum Grosshadern, 8 München 70, Marchioninistr. 15 (F.R.G.)

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Summary

Cultured rat heart muscle cells have been used to study uptake and intracellular binding of $Na_3^{48}VO_4$ (vanadate), as well as the influence of vanadate on beating and $^{86}Rb^+$ uptake of these cells.

1. Vanadate is taken up into cultured rat heart muscle cells in an energy-independent manner by a saturable transport system ($K_m \approx 60$ μM, $V \approx 200$ pmol per mg protein per min at 37°C). Analysis of intracellular binding of vanadate reveals a curved Scatchard plot indicating more than one binding site. Maximal binding amounts to $3 \cdot 10^9$ molecules of vanadate per cell.

2. Vanadate exerts a positive chronotropic and inotropic effect and increases automaticity. First effects can be seen at $1 \cdot 10^{-7}$ M Na_3VO_4. Concentrations higher than $1 \cdot 10^{-3}$ M induce toxic effects (arrhythmias, fibrillation and standstill of the cell).

3. Vanadate-induced alterations of beating is paralleled by a vanadate-induced stimulation of ($^{86}Rb^+ + K^+$) uptake into the cells of up to 75%. Maximal stimulation is obtained at concentrations of $1 \cdot 10^{-4}$—$1 \cdot 10^{-3}$ M vanadate. The stimulation is thought to be due to an increased activity of ($Na^+ + K^+$)-ATPase, since it can be inhibited by ouabain. This result is in contrast to in vitro experiments with purified membrane preparations of ($Na^+ + K^+$)-ATPase of different organs, where an inhibition of ($Na^+ + K^+$)-ATPase by vanadate has been found.

4. The results indicate a possible role of vanadate as an endogenous regulator of active cation flux in heart tissue.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

Introduction

The discovery of vanadate as an inhibitor of $(Na^+ + K^+)$-ATPase [1,2] has stimulated anew the search for endogenous regulators of this enzyme. In contrast to cardiac glycosides, which also bind to and thereby modulate the activity of $(Na^+ + K^+)$-ATPase [3—6], compounds of the trace element, vanadium, can be found in considerable ($1 \cdot 10^{-6}$—$1 \cdot 10^{-7}$ M, see Ref. 1) amounts in sera and tissues of vertebrates [1,7—14]. For chicken and rat [15,16] and probably also for man [8], it represents an essential nutritional factor. At least two pharmacological effects of vanadium compounds may possibly be attributed to a direct interaction of vanadium with $(Na^+ + K^+)$-ATPase of heart and kidney respectively: (1) in feline papillary muscle, vanadate exerts a positive inotropic effect [17] analogous to cardiac glycosides; (2) vanadate administered to rats dramatically stimulates diuresis [18].

This simple model, however, is complicated by the fact that vanadium not only inhibits $(Na^+ + K^+)$-ATPase activity, but also stimulates adenylate cyclase isolated from fat tissue [19] and heart [20,21]. Therefore, some problems have to be clarified, concerning a possible role of vanadium as an endogenous regulator of $(Na^+ + K^+)$-ATPase in the intact cell:

(a) Can vanadium compounds penetrate or bind to the cell membrane?

(b) Are the vanadium effects on $(Na^+ + K^+)$-ATPase, obtained with membrane preparations of this enzyme, transferable to the intact cell?, i.e., do vanadium compounds alter $(Na^+ + K^+)$-ATPase activity in vivo?

(c) If vanadium compounds alter $(Na^+ + K^+)$-ATPase activity in vivo, is this effect correlated with modifications of cellular metabolism and function?

(d) Are the physiological concentrations of vanadium sufficient to account for these effects?

As a suitable model for answering these questions simultaneously, cultured rat myocardial cells are qualified for several reasons:

(1) Transport processes across the cell membrane and cellular binding of radioactively-labelled compounds such as $Na_3^{48}VO_4$ can be investigated without the disturbing diffusion effects one has to be aware of when working with whole tissue.

(2) The activity of $(Na^+ + K^+)$-ATPase, located in the cell membrane, can be easily monitored by measuring the active flux of sodium or potassium across the cell membrane.

(3) Physiological consequences of an altered activity of $(Na^+ + K^+)$-ATPase, the enzymatic basis of active cation transport [22], are mirrored by changes in beating frequency and contraction velocity of the cell. This may be deduced from the fact that cardiac glycosides inhibit $(Na^+ + K^+)$-ATPase [23] and increase beating frequency and contraction velocity [24] in cultured rat heart muscle cells.

To elucidate the role of vanadium as a possible regulator of $(Na^+ + K^+)$-ATPase in heart tissue in vivo, we have studied the uptake and binding of radioactively-labelled vanadate in cultured rat heart muscle cells. Further, the influence of various vanadium compounds on the transport of $^{86}Rb^+$ ion, a potassium analogue, across the cell membrane, as well as on the beating of the cells has been investigated. Part of these results has been presented at the Spring Meeting of the German Society for Biological Chemistry, Berlin (1979) [25].

Materials and Methods

Cultivation of rat heart muscle cells

Primary cultures of rat myocardial cells were prepared under sterile conditions from hearts of 1—5-day-old rats according to the method of Harary et al. [26]: 10—50 hearts were cut into pieces (approx. 1 mm^3) and then disaggregated into single cells by repeated trypsinization (duration, 15 min each) at 37 °C with Serva trypsin 1 : 250, 0.12% (Serva Biochemica, Heidelberg, F.R.G.) in phosphate-buffered saline A [26] supplemented with 5 mM D-glucose. The products of the first three trypsinization steps were discarded because they contain debris and mainly mesenchymal cells [27]. Subsequent supernatants (10—20 ml each) were poured into centrifuge tubes containing 1.0 ml pre-cooled (4 °C) growth medium (CMRL 1415 ATM, Seromed, München, F.R.G., according to Healy and Parker [28], supplemented with 10% fetal calf serum (Seromed), 10% horse serum (Seromed) and 0.05 mg/ml gentamycin). The cell suspensions were centrifuged (10 min, $300 \times g$), the supernatants discarded and the cell pellets resuspended in fresh growth medium. The resulting cell suspension was then distributed in 75 cm^2 plastic culture flasks (Nunclon Plastics, Denmark) and incubated at 37 °C in a water-saturated atmosphere for 2 h. During this period, the majority of fibroblastoid cells, accounting for 30—40% of total heart cells [27], become attached to and spread out on the surface of the flask [27]. At the end of the incubation period, the supernatants containing the myocardial muscle cells were pooled in a beaker. Cell counts were carried out with a haemocytometer and the volume was adjusted with additional growth medium to give the final cell concentration desired. The cell suspension was then distributed into 25 cm^2 plastic culture flasks (Nunclon Plastics, Denmark), 5 ml suspension per flask, with cell densities of $1 \cdot 10^4$—$1 \cdot 10^5$ cells/cm^2. Further incubation of the cells was carried out at 37 °C in a water-saturated atmosphere. Since CMRL 1415 ATM has a high buffer capacity, incubation of the cells in a 5% CO_2 atmosphere is unnecessary.

All studies with heart muscle cells were carried out after cultivation of the cells for 3—5 days. During this time, either a synchronously-contracting monolayer of heart muscle cells had formed, or the cells were lying isolated or in small clusters of 2—5 cells, depending on the initially-chosen cell density. Cultures with single lying cells were preferentially used in the experiments where beating frequency and percentage of beating cells were measured.

To ensure that cell cultures mainly consisted of muscle cells, the percentage of beating cells was determined in each preparation on the day the experiment was carried out. All measurements described in the text were carried out with cell preparations whose percentage of beating cells was 75% or more of the total cell population. Since every culture also contains quiescent muscle cells (see Fig. 5), the total percentage of muscle cells is even higher than the percentage of beating cells.

Two further cell types were cultivated under identical conditions as described for rat heart muscle cells: fibroblastoid rat heart cells, derived initially from the same tissue as heart muscle cells; and Girardi human heart cells, an epithelial-like growing cell line [29], purchased from Flow Lab.

(Bonn, F.R.G.). The cells were used for the experiments after having formed a confluent monolayer.

Monitoring of beating frequency and contraction velocity of cultivated rat heart muscle cells

The system for monitoring the beating frequency of cultured heart muscle cells that we used for our measurements has been described in detail by Kaumann et al. [30]. Briefly, the picture of the beating cell observed through the phase contrast microscope is recorded by a TV camera, displayed on the screen of a monitor, and simultaneously fed into a Grundig video recorder. In this way, up to 25 independently-beating cells were observed under identical conditions in one experiment. The phase contrast microscope and TV camera are located in a thermostatically-maintained incubation chamber, the temperature within the chamber is kept at 37°C. Contractions of the cell cause changes in light intensity on the monitor within the cell area. These light changes are recorded by means of a photocell (BPY 61 Siemens, F.R.G.) stuck to the monitor. The output signal of the photocell is amplified, electronically filtered and monitored on a conventional recorder. The velocity of cell motion (dm/dt) can be determined electronically by means of a differentiating preamplifier [31]. The electronic unit of the monitoring set was purchased from EL MED, Augsburg, F.R.G.

Measurements of $Na_3^{48}VO_4$ and $^{86}Rb^+$ uptake into cultured rat heart muscle cells

Standard measurements of $Na_3^{48}VO_4$ and $^{86}Rb^+$ uptake were carried out at 37°C with cells in 25 cm^2 plastic culture flasks in a Hepes-buffered salt solution. Unless stated otherwise, Hepes-buffered salt solution consisted of: NaCl, 135 mM; KCl, 5.4 mM; $CaCl_2$, 1.8 mM; $MgCl_2$, 1.05 mM; NaH_2PO_4, 0.36 mM; D-glucose, 5.0 mM; Hepes, 20 mM, adjusted with NaOH to pH 7.25. Before each assay, the culture medium was removed by suction, the cells were washed with Hepes-buffered salt solution containing only 3 mM Hepes and then equilibrated with 3.5—5.0 ml Hepes-buffered salt solution for 60 min. Under certain circumstances this time of incubation was longer, as indicated in the figure legends. The uptake experiment was started by addition of assay medium (Hepes-buffered salt solution) containing $Na_3^{48}VO_4$ plus unlabelled Na_3VO_4 or trace amounts of $^{86}RbCl$ (see also legends to the figures and tables). Uptake was determined by removing the radioactive assay medium and washing the cells three times with 5 ml of cold (4°C) Hepes-buffered salt solution containing only 3 mM Hepes. The washing procedure lasted less than 20 s, a time sufficient to remove the extracellular radioactive ions without loss of the intracellular ions. Washed cells were then dissolved in 1 ml NaOH (0.1 M)/EDTA (4.5 mM) solution. 700 μl were taken for radioactivity measurement in 10 ml Unisolve (Zinsser, München, F.R.G.) after neutralization with 1.0 M HCl, using a Berthold-Frieseke liquid scintillation counter (Berthold-Frieseke, München, F.R.G.). Two 50-μl portions were taken for protein determination according to Lowry et al. [32]. The variation in protein content per flask within one experiment was $\leqslant \pm 7\%$.

In each series of experiments, a 'zero-time' assay was carried out by adding radioactive assay medium to the cultures and immediately (in less than 10 s) washing the cells by the standard procedure. The zero-time value was sub-

tracted from every uptake measurement. The data presented in this report are mean values from closely correlating duplicates. All experiments presented were carried out at least three times.

In transport processes across the cell membrane, rubidium ions behave in nearly the same way as potassium ions [33—35], which has been shown for heart tissue [36] and cultured heart muscle cells [37], as well as for a variety of cultured cells [38—41]. Thus, in our experiments, because of its more convenient half-life, trace amounts of $^{86}Rb^+$ were used instead of $^{42}K^+$ to measure the uptake of potassium in cultured heart cells. To test the reliability of this method, the following experiment has been carried out: uptake of $^{86}Rb^+$ (0.5 μM) into cultured rat heart muscle cells was measured in the presence of 0.1 or 5.0 mM K^+ and Rb^+, respectively (measuring time, 10 min). Calculation of the rates of uptake, expressed as nmol($^{86}Rb^+ + Rb^+$) per mg protein per min and nmol($^{86}Rb^+ + K^+$) per mg protein per min, respectively, yields the following: $[K^+]$ = 0.1 mM, 1.01 ± 0.01; $[Rb^+]$ = 0.1 mM, 1.14 ± 0.05; $[K^+]$ = 5.0 mM, 10.11 ± 0.36; $[Rb^+]$ = 5.0 mM, 10.49 ± 1.16. Because of this fairly good agreement, results of $^{86}Rb^+$ uptake are expressed as nmol-($^{86}Rb^+ + K^+$) per mg protein per min in the following. This generally accepted [38] form of presentation has been preferred to expressing uptake as cpm $^{86}Rb^+$ (the actually measured value) for better comparison of the results of different experiments under different conditions.

Materials

$Na_3^{48}VO_4$ was purchased from Amersham-Buchler, Braunschweig, F.R.G. and $^{86}RbCl$ from NEN, Dreieich, F.R.G. Na_3VO_4 was obtained from BDH Chemicals, Poole, U.K. and V_2O_5, VCl_3, $NaVO_3$ and $C_{10}H_{14}O_5V$ from Riedel-de Haen, Seelze-Hannover, F.R.G. All other chemicals were of analytical grade and purchased from Merck, Darmstadt, F.R.G. and Boehringer, Mannheim, F.R.G.

Results

Uptake of $Na_3^{48}VO_4$ into cultured rat heart muscle cells

Fig. 1a shows the kinetics of $Na_3^{48}VO_4$ uptake ($1 \cdot 10^{-8}$ and $1 \cdot 10^{-6}$ M, respectively) into cultured rat heart muscle cells. During an initial phase lasting about 1—3 min, uptake is rapid, followed by a much slower increase in intracellular vanadate accumulation. The single components of this double-exponential process are further characterized in Fig. 1b: linearity of uptake is only maintained during the first 60 s after addition of $Na_3^{48}VO_4$ (inset of Fig. 1b). Washing the cells after 60 s with 2.5 mM noradrenaline (which forms a complex with vanadate [41]) does not remove cellularly-fixed ^{48}V: control, 147 ± 10 fmol per mg protein per min; noradrenaline washing, 141 ± 3 fmol per mg protein per min ($[Na_3VO_4] = 1 \cdot 10^{-8}$ M, mean ± S.D., n = 3). In human erythrocytes, this result has been taken as evidence that vanadate is not complexed with lipids or proteins on the extracellular side of the plasma membrane, but taken up into the cells [41]. Therefore, one may assume that during this initial phase of vanadate uptake, transport across the cell membrane is the rate-limiting step in vanadate uptake.

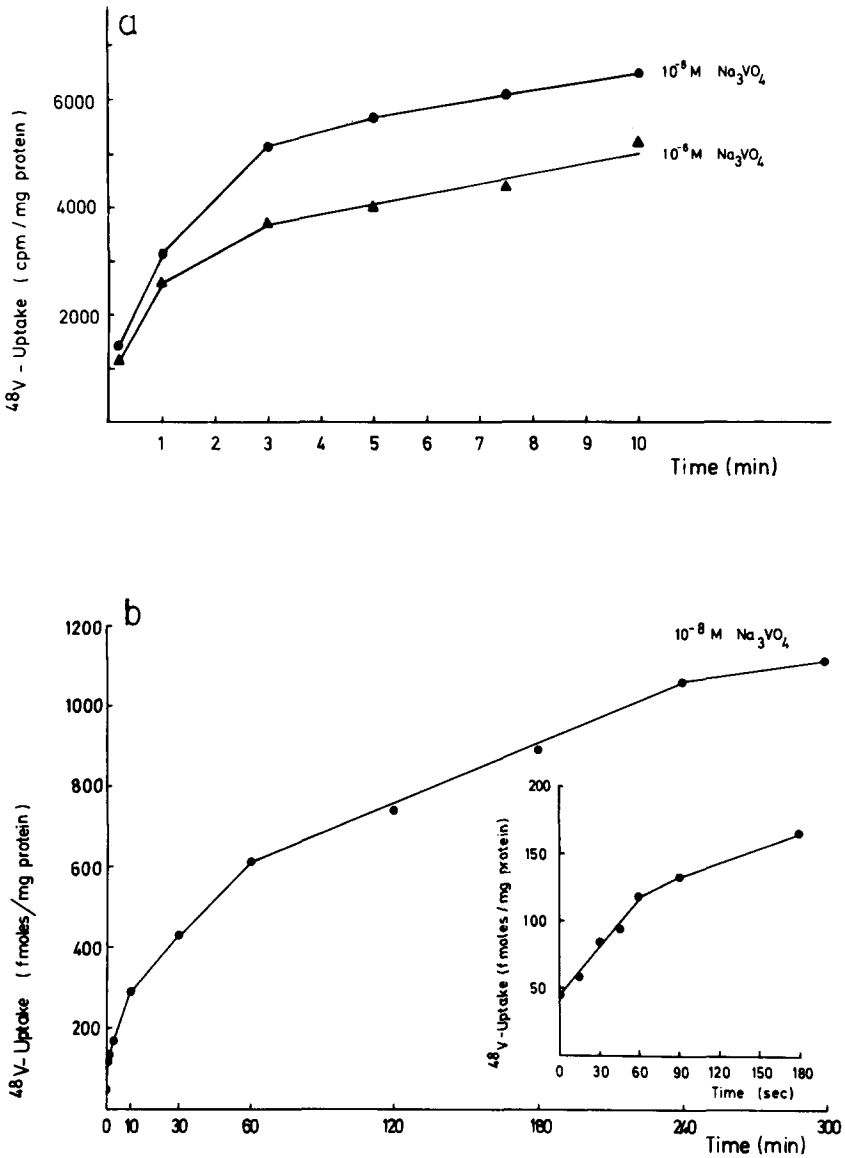

Fig. 1. Kinetics of $Na_3^{48}VO_4$ uptake into culture rat heart muscle cells. (a) Temperature 37°C; 439 000 cpm $Na_3^{48}VO_4$ per flask; 0.23 mg protein per flask. (b) Temperature 37°C; 83 000 cpm $Na_3^{48}VO_4$ per flask; 0.66 mg protein per flask; $1.03 \cdot 10^6$ cells per flask. For determination of cell number per flask, see legend to Table II. Inset of Fig. 1b: assuming a cell volume of 2.6 μl/10^6 cells [23], the intracellular concentration of $Na_3^{48}VO_4$ can be calculated: $0.5 \cdot 10^{-8}$ M (15 s); $1.2 \cdot 10^{-8}$ M (30 s); $1.4 \cdot 10^{-8}$ M (45 s); $2.6 \cdot 10^{-8}$ M (60 s); $3.4 \cdot 10^{-8}$ M (90 s); $4.1 \cdot 10^{-8}$ M (180 s). For further experimental details see Materials and Methods.

Plotting these initial uptake rates against the Na_3VO_4 concentration in the incubation solution yields a curve characteristic of a saturable transport mechanism for this compound (Fig. 2a). The data fit a Hanes plot (Fig. 2b) with a K_m value of 59 μM and a maximal transport capacity (V) of 190 pmol per mg cell protein per min at 37°C. Two similar experiments revealed K_m

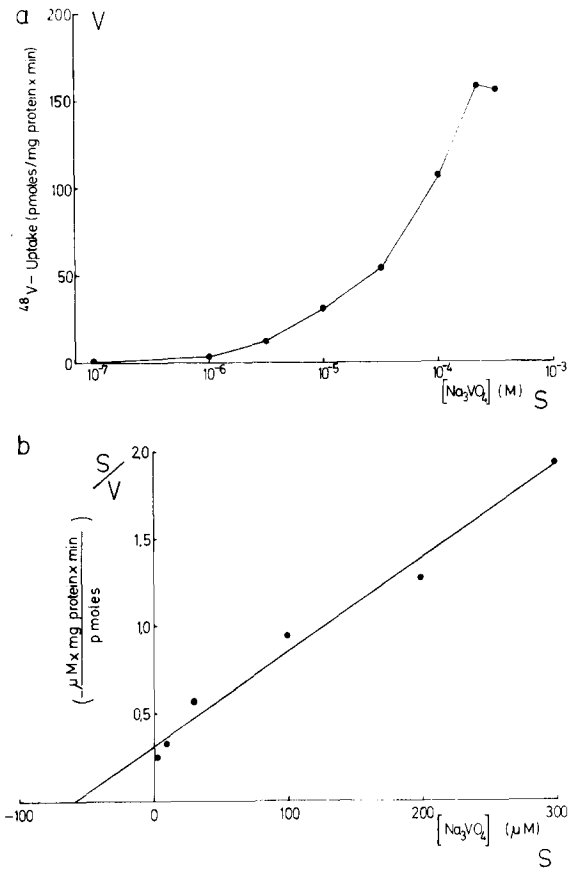

Fig. 2. Concentration-dependence of $Na_3^{48}VO_4$ uptake into cultured rat heart muscle cells. Measurements were carried out within the time range of linear $Na_3^{48}VO_4$ uptake. Temperature 37°C; abscissa, concentration of $Na_3^{48}VO_4$ in incubation solution; 338 000 cpm $Na_3^{48}VO_4$ per flask; 0.97 mg protein per flask. In Fig. 2b, the data of Fig. 1a (without the values for $1 \cdot 10^{-7}$ and $1 \cdot 10^{-6}$ M Na_3VO_4 in incubation solution) were plotted as a Hanes plot, yielding a K_m value of 59 μM and a V value of 190 pmol per mg protein per min for vanadate uptake ($r = 0.99$). For further experimental details see Materials and Methods.

vanlues of 83 and 100 μM and V values of 110 and 421 pmol per mg protein per min, respectively. Transport rates for $Na_3^{48}VO_4$ of cultured rat heart muscle cells are comparable to those of cultured fibroblastoid rat heart cells (rat heart muscle cells, 88.9 ± 21.6 fmol per mg protein per min; fibroblastoid rat heart cells, 67.1 ± 9.1 fmol per mg protein per min; concentration of $Na_3^{48}VO_4$ in incubation solution, $1 \cdot 10^{-8}$ M, $n = 4$, 37°C).

This initial, linear phase of vanadate uptake into rat heart muscle cells does not require energy, since incubation of the cells with 0.5 μM *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone (uncoupling of oxidative phosphorylation) and 0.5 mM sodium iodoacetate (inhibition of glycolysis), which completely abolishes beating of the cells within 20—60 s, does not lower the uptake rate of vanadate: control, 158 ± 10 fmol per mg protein per min; *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone + sodium iodoacetate, 172 ± 20

fmol per mg protein per min ($[Na_3VO_4] = 1 \cdot 10^{-8}$ M, mean ± S.D., $n = 3$). Though vanadate accumulates in considerable amounts within the cells at low concentrations of Na_3VO_4 in the incubation solution (see below), this seems not to be mediated by an active transport process, but by an intracellular binding of vanadate: linearity of uptake is lost when the intracellular vanadate concentration exceeds that in the incubation solution by a factor of 2—3 (see legend to Fig. 1b).

The competitive effect of various vanadium compounds on uptake of radioactively-labelled Na_3VO_4 (orthovanadate) is presented in Table I. Raising the orthovanadate concentration from $1 \cdot 10^{-8}$ to $1 \cdot 10^{-4}$ M increases vanadate uptake from 101 fmol per mg protein per min to $3.07 \cdot 10^5$ fmol per mg protein per min. Addition of unlabelled metavanadate, vanadium(IV) oxobis-(2,4-pentanedionate), vanadium pentoxide and vanadium(III) chloride at final concentrations of $1 \cdot 10^{-4}$ M decreases uptake of radioactively-labelled orthovanadate ($1 \cdot 10^{-8}$ M). This indicates competition of these compounds with orthovanadate for binding to the same carrier. The data also indicate that the affinity of all vanadium compounds tested is similar to that of Na_3VO_4. In contrast, phosphate ions at concentrations of $1 \cdot 10^{-4}$ and even $1 \cdot 10^{-3}$ M (not shown) do not lower uptake of radioactively-labelled orthovanadate ($1 \cdot 10^{-8}$ M) into cultured rat myocardial cells (Table I).

Though experiments to characterize further transport of vanadate into cultured rat heart muscle cells are still in progress, one may predict from our data that vanadate uptake does not occur by simple diffusion, but by an energy-independent carrier system, most probably as facilitated diffusion. This carrier would, therefore, only facilitate equilibration of vanadate across the cell membrane, with equal concentrations of vanadate in the incubation solution and the water phase of the cells, as is postulated for human erythrocytes [41]. The nature of this transport system is not yet clear. In human erythrocytes, vanadate transport is catalyzed by the anion-exchange system [41]. No decision can yet be made, as to whether this also holds true for rat myocardial

TABLE I

UPTAKE OF [^{48}V]VANADATE INTO CULTURED RAT HEART MUSCLE CELLS AND COMPETITION BY VARIOUS VANADIUM COMPOUNDS

Measurements were carried out within the time range of linear [^{48}V]vanadate uptake between 10 and 60 s. Temperature, 37°C; 463 480 cpm $Na_3^{48}VO_4$ per flask; 1.69 mg protein per flask. $Na_3^{48}VO_4$ and competitive compounds ($1 \cdot 10^{-4}$ M) were added simultaneously after incubation of the cells for 60 min in Hepes-buffered salt solution without phosphate. For further experimental details see Materials and Methods. ($C_{10}H_{14}O_5V$ = vanadium(IV) oxobis(2,4-pentanedionate)

Competitive compound	Concentration of $Na_3^{48}VO_4$ (M)	Uptake of $Na_3^{48}VO_4$	
		cpm per assay	fmol/mg protein per min
—	$1 \cdot 10^{-8}$	918	101
—	$1 \cdot 10^{-4}$	128	307 200
V_2O_5	$1 \cdot 10^{-8}$	82	9
$NaVO_3$	$1 \cdot 10^{-8}$	80	9
$C_{10}H_{14}O_5V$	$1 \cdot 10^{-8}$	141	25
VCl_3	$1 \cdot 10^{-8}$	106	12
Na_2HPO_4	$1 \cdot 10^{-8}$	968	105

TABLE II

INTRACELLULAR ACCUMULATION OF $Na_3^{48}VO_4$ IN CULTURED RAT HEART MUSCLE CELLS

Cultured rat heart muscle cells were incubated with Hepes-buffered salt solution plus $Na_3^{48}VO_4$ at the concentrations indicated for 7 h, until equilibrium of uptake was obtained. Further incubation of the cells with $Na_3^{48}VO_4$ ($2.5 \cdot 10^{-8}$ M, $1 \cdot 10^{-4}$ M) for an additional 60 min did not increase intracellular radioactivity of $Na_3^{48}VO_4$. 218 700 cpm $Na_3^{48}VO_4$ per flask; 0.49 mg protein per flask; 766 000 cells per flask (determination of cell number per flask: trypsinization of the cells per flask (n = 3), subsequent washing with Hepes-buffered salt solution, counting of the cells by the aid of a haemocytometer, determination of the ratio mg protein per 10^6 cells).

Accumulation factor

$$= \frac{\text{total intracellular } [^{48}V] \text{ (pmol/}10^6 \text{ cells)}}{\text{cell volume (92.6 } \mu\text{l/}10^6 \text{ cells)} \times \text{concentration of } Na_3^{48}VO_4 \text{ in incubation solution } (\mu M)}$$

Calculation of free intracellular [^{48}V] (pmol/10^6 cells): concentration of $Na_3^{48}VO_4$ in incubation solution (μM) × volume of cell water (1.93 μl/10^6 cells). The values for cell volume and volume of cell water were taken from the data of McCall [23]. For further experimental details see Materials and Methods. In two similar experiments, the maximal number of intracellular-bound [^{48}V]was 8800 and 3500 pmol/10^6 cells.

Concentration of $Na_3^{48}VO_4$ in incubation solution (M)	^{48}V accumulation factor intra-/extracellular	Intracellular [^{48}V] (pmol per 10^6 cells)		
		Total	Free	Bound
$2.5 \cdot 10^{-8}$	93	6.05	0.05	6.0
$1 \cdot 10^{-7}$	54	14.0	0.2	13.8
$1 \cdot 10^{-6}$	33	84.5	1.9	82.6
$1 \cdot 10^{-5}$	11	272	19	253
$1 \cdot 10^{-4}$	6.0	1 564	193	1371
$5 \cdot 10^{-4}$	3.1	4 050	965	3085
$1 \cdot 10^{-3}$	1.9	4 964	1 930	3034
$5 \cdot 10^{-3}$	1.2	14 952	9 650	5302
$1 \cdot 10^{-2}$	0.9	24 632	19 300	5332

cells (see Discussion). Though the experimental data presented in this chapter are consistent with an energy-independent carrier system for vanadate uptake into rat heart muscle cells, this initial linear uptake of $Na_3^{48}VO_4$ might also be interpreted in terms of binding to the cell membrane (see Discussion).

The rapid phase of vanadate uptake across the cell membrane is followed by a less rapid increase in intracellular vanadate, represented by the second part of the biphasic uptake kinetics (Fig. 1a, b). At low vanadate concentrations in the incubation solution ($1 \cdot 10^{-8}$ M), steady-state conditions are obtained only after an incubation period of 6—7 h (see legend to Table II), whilst at higher concentrations ($1 \cdot 10^{-4}$ M) equilibrium is reached after about 60 min (not shown).

In the experiment described in Table II, the intracellular amount of ^{48}V after an incubation period of 7 h has been measured at increasing Na_3VO_4 concentrations in the incubation solution. The intracellular accumulation factor for vanadate ranges from 93 ($2.5 \cdot 10^{-8}$ M) to 0.9 ($1 \cdot 10^{-2}$ M), as it can be calculated by dividing the intracellular amount of vanadate by the cell space (see legend to Table II). Cantley et al. [41] have shown that in erythrocytes, most of the cellular vanadate taken up is bound to molecules of high molecular weight, whereas the concentration of free vanadate inside the cell equals that

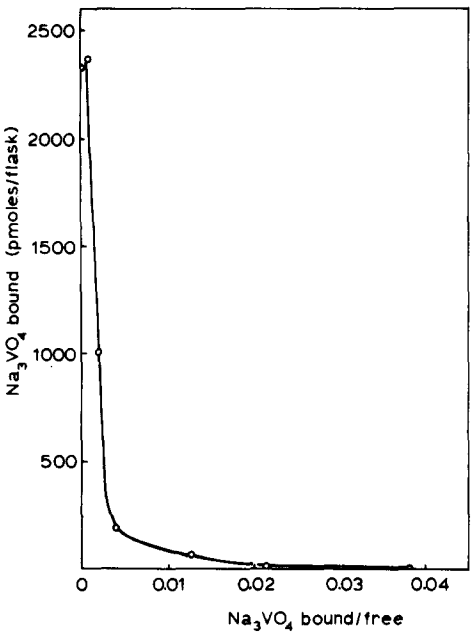

Fig. 3. Intracellular binding of $Na_3^{48}VO_4$ to cultured rat heart muscle cells after treatment of the cells with 2.5 mM noradrenaline. The amount of cell-bound Na_3VO_4 per flask was obtained by multiplication of cell-bound Na_3VO_4 per 10^6 cells (see Table II) by the amount of cells per flask (in millions, 0.766). The amount of free Na_3VO_4 per flask was calculated from the Na_3VO_4 concentration in the incubation solution, incubation volume (5 ml) and cell-bound Na_3VO_4 per flask. For further details see Table II.

in the incubation solution. Assuming that in cultured rat heart muscle cells the vanadate transport system also facilitates an equilibrium of similar concentrations of vanadate in the incubation solution and in the cellular water space, the amount of bound or complexed vanadate can be calculated (see legend to Table II). It reaches a maximum level of 5300 pmol per 10^6 cells, which equals $3.2 \cdot 10^9$ vanadate molecules bound per cell.

A Scatchard plot [42] of these data (Fig. 3) reveals a curved line. The consequence of this result will be discussed later (see Discussion).

Vanadate-induced changes in beating of cultured rat heart muscle cells

In Fig. 4, contractions of a single rat myocardial cell are monitored by an electro-optical system described in Materials and Methods. Adding Na_3VO_4 to give a final concentration of $1 \cdot 10^{-2}$ M induces some characteristic alterations of beating. After a short lag phase of 1—2 min, an increase in beating frequency and velocity of contraction is seen, lasting about 4 min. Thereafter, toxic effects (due to the high concentration of Na_3VO_4 used) become visible, resulting in arrhythmias and decrease of contraction velocity. Finally, only uncoordinated, weak oscillations in some areas of the cell are observable. If this experiment is carried out with lower concentrations of Na_3VO_4, e.g., $1 \cdot 10^{-4}$ M, only the increases in beating frequency and contraction velocity are found. In cultured heart cells, an increase in contraction velocity, within limitations, is representative of a positive inotropic effect [24]. Therefore,

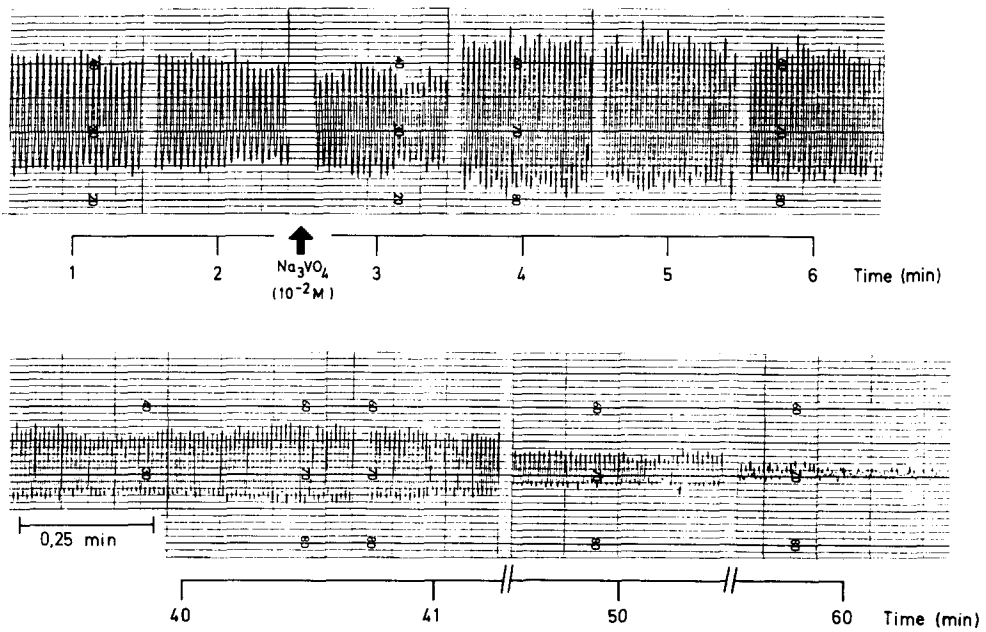

Fig. 4. Influence of Na_3VO_4 on contractility behaviour of a cultured rat heart muscle cell. Contractility of the cell was monitored by the electro-optical system described in Materials and Methods. Ordinate: arbitrary units of contraction velocity (see Materials and Methods). For further details see text.

Fig. 4 demonstrates the positive chronotropic and inotropic actions of Na_3VO_4 in cultured rat heart muscle cells. Using quiescent heart muscle cells, a further effect of vanadate can be demonstrated: muscle cell cultures obtained from

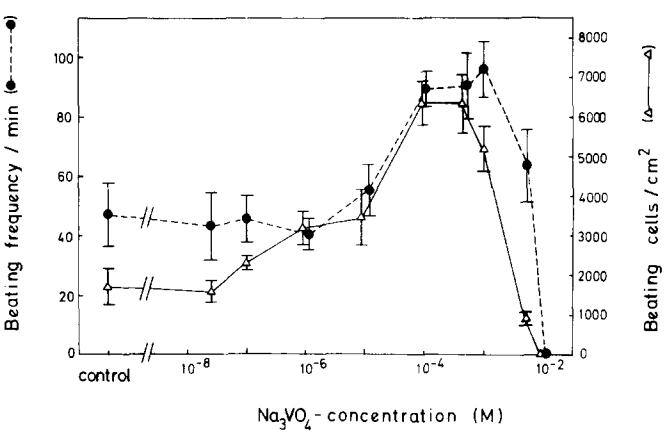

Fig. 5. Influence of Na_3VO_4 on contractility behaviour of cultured rat heart muscle cells. Abscissa: concentration of Na_3VO_4 in incubation solution (Hepes-buffered salt solution). Rat heart muscle cells had been incubated for 7 h in Hepes-buffered salt solution plus Na_3VO_4, thereafter beating frequency and number of beating cells per cm^2 were determined (at microscope magnification, ×300). Values are the mean ± S.E. (n = 8). For determination of beating cells per cm^2, a total of 200 cells per value was counted. Calculation of total cells per culture area yielded 8000 cells per cm^2. Same experiment as described in Table II. For further explanation see text.

hearts of 4—6-day-old rats have a lower beating frequency and show a higher percentage of quiescent muscle cells than in heart cells obtained from 1—2-day-old rats [43]. Addition of Na_3VO_4 to these cells often results in inducing spontaneous regular beating (experiments not shown). This increase in automaticity has been observed in quiescent muscle cells, i.e., cells where no beating was observed during a time period of 3 min, as well as in muscle cells showing, only occasionally, some irregular beating.

The positive chronotropic effect of vanadate as well as the increase in automaticity induced by this compound have been quantified in a representative experiment shown in Fig. 5 (same experiment as described in Table II). As stated above, heart cell cultures from 4—5-day-old rats were used to obtain a high percentage of quiescent cells. In this experiment, cultured rat heart muscle cells were incubated for 7 h in Hepes-buffered salt solution with different concentrations of Na_3VO_4. Na_3VO_4 increases, in a concentration-dependent manner, beating frequency and the number of beating cells per cm^2. First effects were seen at $1 \cdot 10^{-7}$ M (number of beating cells per cm^2) and $1 \cdot 10^{-5}$ M (beating frequency). Half-maximal stimulation was obtained for both effects at a concentration of approx. $3 \cdot 10^{-5}$ M Na_3VO_4. At concentrations $\geqslant 1 \cdot 10^{-3}$ M Na_3VO_4, signs of toxicity were registered as described for the experiment of Fig. 4. Similar effects to those described in Fig. 5 can be observed, even after 27 h of incubation of the cells in Na_3VO_4 (not shown).

As in the case of vanadate transport across the cell membrane of cultured rat heart cells, all vanadium compounds mentioned in Table I behave similarly to orthovanadate in modifying contractility behaviour of heart muscle cells (not shown). In contrast to vanadium compounds, none of the additionally tested anions such as molybdate, sulphate and phosphate had any stimulatory effect on automaticity when applied at a final concentration of $1 \cdot 10^{-4}$ M (not shown). Therefore, the observed alterations in beating of the cells seem to be rather specifically related to vanadium-containing molecules.

Effect of vanadate on $^{86}Rb^+$ uptake in cultured rat heart cells

Vanadate has been described as a modulator of $(Na^+ + K^+)$-ATPase [1,2]. This enzyme regulates sodium and potassium fluxes across the cell membrane [22,44—46] and is thought to be the receptor for cardiac glycosides [3—6]. The question arises as to whether vanadate, which modifies beating of heart muscle cells in a similar manner to that shown for cardiac glycosides [24], also exerts its effect by interacting with $(Na^+ + K^+)$-ATPase. To test this hypothesis, the influence of Na_3VO_4 on influx of radioactively-labelled rubidium, a potassium analogue, was determined. Assuming that $^{86}Rb^+$ is taken up into the cells in a similar manner to potassium (see Materials and Methods), transport rates can be calculated as nmol of $(^{86}Rb^+ + K^+)$ from the intracellular radioactivity of $^{86}Rb^+$ and the concentrations of rubidium ions and potassium ions in the medium (see Materials and Methods).

Fig. 6 presents kinetics of $(^{86}Rb^+ + K^+)$ uptake into cultured rat heart muscle cells. Uptake is linear within the time range tested (15 min). Therefore, all further experiments to measure uptake rates of $(Rb^+ + K^+)$ were carried out by incubating the cells with $^{86}Rb^+$ for 10 min. It may be noted that these kinetics represent equilibrium conditions concerning uptake of potassium (see Materials

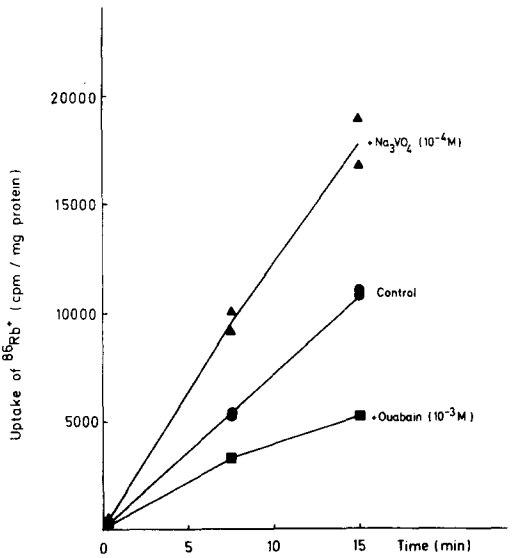

Fig. 6. Kinetics of $^{86}Rb^+$ uptake into cultured rat myocardial cells. Influence of Na_3VO_4 and ouabain. Before measuring uptake of $^{86}Rb^+$, cells were incubated for 60 min in Hepes-buffered salt solution ($[K^+]$ = 2.7 mM), or Hepes-buffered salt solution ($[K^+]$ = 2.7 mM) supplemented with Na_3VO_4 or ouabain, at final concentrations of $1 \cdot 10^{-4}$ and $1 \cdot 10^{-3}$ M, respectively. Temperature, 37°C; 714 000 cpm $^{86}Rb^+$ per flask; 1.09 mg protein per flask. For further explanation see Materials and Methods.

and Methods). Addition of the cardiac glycoside, ouabain ($1 \cdot 10^{-3}$ M), reduces ($^{86}Rb^+ + K^+$) uptake by 46%, due to inhibition of ($Na^+ + K^+$)-ATPase. This is in agreement with the data of McCall [23] who showed that potassium influx into cultured rat myocardial cells is inhibited by ouabain in a concentration-dependent manner, maximal inhibition occurring at $3 \cdot 10^{-3}$ M ouabain. In contrast to ouabain, Na_3VO_4 ($1 \cdot 10^{-4}$ M) stimulates ($^{86}Rb^+ + K^+$) uptake by 74% (Fig. 6).

As Cantley et al. [41] have described an inhibition of Rb^+ uptake in human erythrocytes by Na_3VO_4, which has been reproduced in our experiments, the question arises as to whether the vanadate-induced stimulation of ($^{86}Rb^+ + K^+$) uptake we have found in our experiments with cultured rat heart muscle cells is restricted to this cell type, perhaps connected with the vanadate-induced alteration of beating. We therefore used two further cell types to study the effect of Na_3VO_4 on ($^{86}Rb^+ + K^+$) uptake across the cell membrane: fibroblastoid rat heart cells obtained from the same origin as rat heart muscle cells (see Materials and Methods), and an epithelial-like cell line derived from human heart [29]. ($^{86}Rb^+ + K^+$) uptake into these cell types was stimulated, as well, to a similar extent by Na_3VO_4 as described in Fig. 6 for cultured rat heart muscle cells (experiments not shown). We may therefore conclude that vanadate-induced stimulation of ($^{86}Rb^+ + K^+$) influx is not restricted to cultured muscle cells from rat heart, but seems to be a more general phenomenon.

The concentration-dependence of this effect is shown in Fig. 7a–c for these three cell types. ($^{86}Rb^+ + K^+$) uptake can be increased by vanadate by 74% (rat heart muscle cells), 63% (fibroblastoid rat heart cells) and 65% (epithelial-like

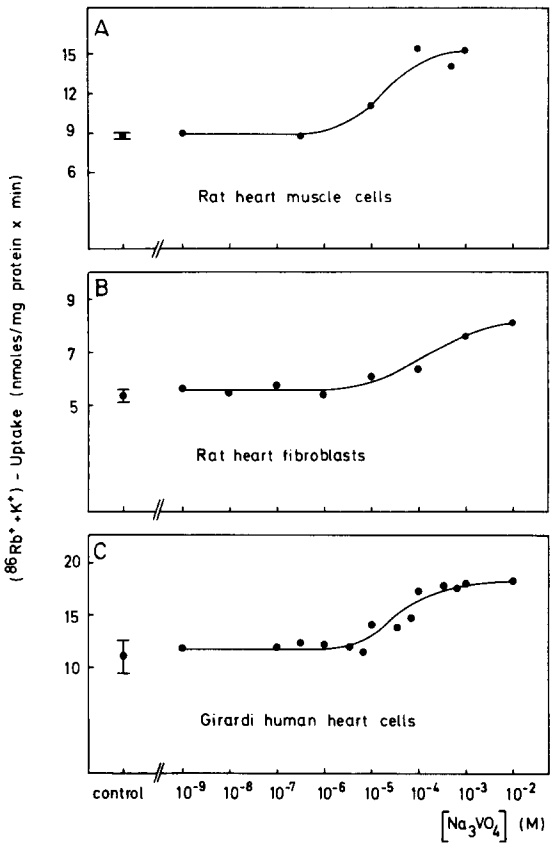

Fig. 7. Concentration-dependence of vanadate-induced stimulation of ($^{86}Rb^+ + K^+$) uptake into cultured rat heart muscle cells, fibroblastoid rat heart cells and Girardi human heart cells. Before measuring uptake of $^{86}Rb^+$, cells were incubated for 60 min in Hepes-buffered salt solution supplemented with Na_3VO_4 at final concentration indicated. Temperature, 37°C. Rat heart muscle cells: $1.61 \cdot 10^6$ cpm $^{86}Rb^+$ per flask; 1.04 mg protein per flask. Fibroblastoid rat heart cells: $0.95 \cdot 10^6$ cpm $^{86}Rb^+$ per flask; 0.89 mg protein per flask. Girardi human heart cells: $3.23 \cdot 10^6$ cpm $^{86}Rb^+$ per flask; 0.60 mg protein per flask. For further experimental details see Materials and Methods.

human heart cell line), the Na_3VO_4 concentration for half-maximal stimulation of ($^{86}Rb^+ + K^+$) uptake being 10, 100 and 30 μM, respectively. For rat heart muscle cells, a mean value for stimulation of ($^{86}Rb^+ + K^+$) uptake of 47 ± 27 (±S.D.) has been found ($n = 12$) at a concentration of $1 \cdot 10^{-4}$ M Na_3VO_4. It may be emphasized that neither in the experiments of Fig. 7 nor in other experiments where ($^{86}Rb^+ + K^+$) uptake into cultured rat heart muscle cells was studied, could a vanadate-induced inhibition of uptake be observed within the carefully-tested concentration range of $1 \cdot 10^{-9}$—$1 \cdot 10^{-3}$ M Na_3VO_4.

As in the case of vanadate uptake and vanadate-induced modification of contractility behaviour of the cells, all vanadium compounds mentioned in Table I increase ($^{86}Rb^+ + K^+$) uptake into cultured rat heart muscle cell (not shown). In contrast, no stimulation could be seen with $1 \cdot 10^{-4}$ M molybdate, sulphate and phosphate (not shown).

Potassium influx into cultured rat heart muscle cells is composed of an

TABLE III

VANADATE-INDUCED STIMULATION OF ($^{86}Rb^+ + K^+$) UPTAKE INTO CULTURED RAT HEART MUSCLE CELLS AND GIRARDI HUMAN HEART CELLS AND THE INFLUENCE OF OUABAIN

Before measuring uptake of $^{86}Rb^+$ for 10 min, cells were incubated for 60 min in Hepes-buffered salt solution (control), or Hepes-buffered salt solution supplemented with Na_3VO_4, ouabain and Na_3VO_4 plus ouabain, respectively, at final concentrations of $1 \cdot 10^{-4}$ M (Na_3VO_4) and $1 \cdot 10^{-3}$ M (ouabain). Temperature, 37°C; Hepes-buffered salt solution contained 2.5 mM K^+.
Heart muscle cells: 611 000 cpm $^{86}Rb^+$ per flask; 1.09 mg protein per flask.
Girardi human heart cells: 611 000 cpm $^{86}Rb^+$ per flask; 1.13 mg protein per flask.
For further experimental details see Materials and Methods.

Incubation conditions	Uptake of ($^{86}Rb^+ + K^+$)			
	Rat heart muscle cells		Girardi human heart cells	
	nmol/mg protein per min	%	nmol/mg protein per min	%
Control	14.4	100	7.8	100
Na_3VO_4	17.1	119	10.3	132
Ouabain	8.9	62	4.5	58
Na_3VO_4 + ouabain	8.8	61	4.6	59

ouabain-sensitive transport, mediated by ($Na^+ + K^+$)-ATPase, and an ouabain-insensitive uptake [23]. Therefore, the observed vanadate-induced stimulation of ($^{86}Rb^+ + K^+$) uptake could be due to both of these mechanisms of influx. To discriminate between these two possibilities, vanadate-induced stimulation of ($^{86}Rb^+ + K^+$) uptake has been studied in cultured rat heart muscle cells and epithelial-like human heart cells in the presence and absence of ouabain (Table III). In contrast to $Na_3^{48}VO_4$ transport into cultured rat heart muscle cells, which is not inhibited at all by $1 \cdot 10^{-3}$ M ouabain (not shown) ouabain completely abolishes vanadate stimulation of ($Rb^+ + K^+$) uptake in both cell types, suggesting that the increased ($^{86}Rb^+ + K^+$) influx is due to vanadate-induced stimulation of ($Na^+ + K^+$)-ATPase.

Discussion

Interaction of vanadate with isolated ($Na^+ + K^+$)-ATPase is well-established [1,2]. As this enzyme spans the plasma membrane [47], vanadate can exert its effects either from the extracellular or the cytoplasmic side. Cardiac glycosides, for instance, have binding sites on ($Na^+ + K^+$)-ATPase facing the extracellular side of the enzyme [48]. For vanadate, binding from the cytoplasmic side is assumed [41]. This implies transport of vanadate across the plasma membrane prior to interaction of this substance with ($Na^+ + K^+$)-ATPase.

The results described in Fig. 1 suggest transport of vanadate across the cell membrane of cultured rat heart muscle cells, facilitated by a saturable transport system (Fig. 2) in an energy-independent manner. In human erythrocytes, vanadate uptake is facilitated by the anion-exchange system [41]. The affinity of this carrier for vanadate is relatively low ($K_m \approx 40$ mM) and of the same order as that for phosphate ($K_m \approx 80$ mM) [41]. In contrast, the Michaelis constant for vanadate transport in cultured rat myocardial cells amounts to

60—100 μM. At present, the nature of this transport system is not clear. If it is identical with an anion-exchange system, then the affinity of this carrier for vanadate is much higher in rat heart muscle cells than in human erythrocytes. Addition of excess phosphate does not lower uptake of $Na_3^{48}VO_4$ (Table I), indicating a high specificity of this transport system for vanadate compared with phosphate. In contrast, various vanadium compounds tested compete with $Na_3^{48}VO_4$ for binding to the carrier system. This may, at least in part, be explained by the complex chemistry of vanadium (see below). However, as stated in Results, although our experimental data obtained so far are consistent with the existence of a carrier-mediated uptake of vanadate into rat heart muscle cells, no direct proof exists up to now that vanadate appears inside the cells and that this involves a carrier. The initial linear uptake phase could also represent simple binding of vanadate to the extracellular side of the plasma membrane. Passive penetration through nonspecific pores followed by binding to interior sides should also be discussed. These two possibilities, however, seem unlikely for several reasons. (a) Noradrenaline washing of the cells does not remove $Na_3^{48}VO_4$ from the cells. (b) Kinetics of vanadate uptake do not fit a curve of second-order kinetics which one would expect if uptake kinetics represent merely binding of vanadate to a binding site according to the following scheme: vanadate + binding site ↔ vanadate-binding-site complex. (c) Linearity of uptake is lost when the intracellular concentration of vanadate equals that of the extracellular concentration. This can be well-explained by a carrier-mediated, energy-independent transport system. (d) The glycocalyx of the cell membrane contains mainly negatively-charged molecules which would not bind the also negatively-charged vanadate. However, if vanadate is indeed bound to the plasma membrane, these binding sites would be very specific for vanadium-containing molecules (see Table I).

The plasma membrane of cultured rat heart muscle cells contains $1.6 \cdot 10^6$ molecules of $(Na^+ + K^+)$-ATPase per cell [23], assuming a stoicheiometry of one molecule ouabain bound per molecule $(Na^+ + K^+)$-ATPase. The number of maximally-bound molecules of vanadate per rat heart muscle cell, however, can be calculated to be $3.2 \cdot 10^9$ (Table II), which is 2000-times the number of $(Na^+ + K^+)$-ATPase molecules per cell. Na_3VO_4, the vanadium compound used most in our experiments, contains vanadium in the V-oxidation state. At pH 7, $H_2VO_4^-$ is the predominating form at concentrations $<1 \cdot 10^{-4}$ M. At higher concentrations, condesation reactions occur leading to polyvanadates such as $V_3O_9^{3-}$ and $V_4O_{12}^{4-}$ [49]. Furthermore, vanadium(III) compounds are easily oxidized by oxygen and vanadium(V) compounds reduced by mild reducing agents. Additionally, plasma membrane preparations from heart tissue have been found to contain a high activity of NADH-vanadate reductase [60]. Human erythrocytes contain vanadium predominantly in the IV-oxidation state, though administered in the V-oxidation state as vanadate [50]. This altogether makes it difficult to determine the actual state of vanadium in biological material. But it may explain why all vanadium compounds tested in our experiments (a) inhibit uptake of $Na_3^{48}VO_4$ (Table I) and (b) behave similarly to Na_3VO_4 in stimulating $(^{86}Rb^+ + K^+)$ influx and in inducing a positive chronotropic effect and in increasing automaticity in cultured rat heart muscle cells.

One molecule of $(Na^+ + K^+)$-ATPase binds two molecules of vanadate [51]. Though the actual number of vanadium-containing molecules per rat heart muscle cell, the calculation of which is based on the monomeric form of $Na_3^{48}VO_4$ (Table II), may be reduced by condensation reactions, it does exceed the number of $(Na^+ + K^+)$-ATPase molecules per cell by a factor of 1000. Though the curvilinear Scatchard plot of intracellular vanadate binding (Fig. 3) could represent negative co-operativity or multiple forms of specific binding sites, we favour the assumption that most of the large amount of intracellular vanadate is bound or complexed in a nonspecific manner within the cell. In human erythrocytes, a large portion of intracellular vanadate is fixed to haemoglobin [50]. Though being aware that erythrocytes represent an extremely specialized enucleated cell type, one may assume from these data that, also, in cultured rat heart muscle cells a lot of intracellularly-accumulated vanadate is nonspecifically-bound to molecules of high molecular weight. However, only approx. 0.25—1% of total binding sites has to be occupied by vanadate to increase significantly the automaticity of beating in cultured rat heart muscle cells (Table II, Fig. 5), and maximal increase in beating frequency and automaticity of beating as well as stimulation of rubidium influx is accomplished when 26% of maximal binding capacity of the cells is saturated (Table II, Figs. 5 and 7). Taking into account that, also, binding of ouabain to cultured rat heart muscle cells has to be distributed between specific binding to $(Na^+ + K^+)$-ATPase and nonspecific binding [23], whereby nonspecific binding sites exceed specific ones at concentrations $\geqslant 1 \cdot 10^{-6}$ M ouabain in the incubation solution, our results justify the conclusion that cultured rat heart cells possess a limited number of specific binding sites for vanadate. The occupation of these specific sites with vanadate may be responsible for the vanadate-induced biological effects shown in the preceding chapters. Based on work with membrane preparations, two candidates for specific binding of vanadate have to be discussed in cultured rat heart muscle cells: $(Na^+ + K^+)$-ATPase [51] and adenylate cyclase. The latter has been shown to be stimulated by vanadate [19—21]. Regarding the in vitro inhibition of $(Na^+ + K^+)$-ATPase by vanadate, influx of rubidium into human erythrocytes [41] and potassium-activated sodium efflux in squid axon [52] are inhibited. In heart tissue, a more complex action of vanadate than pure inhibition of $(Na^+ + K^+)$-ATPase seems to exist: though the isolated enzyme prepared from feline heart is also inhibited by vanadate [53], the ouabain sensitive $^{86}Rb^+$ uptake in electrically-stimulated atrial muscle of guinea-pig heart was not inhibited [54]. The results presented in this paper demonstrate a stimulation, but not an inhibition of active $(^{86}Rb^+ + K^+)$ influx in cultured rat heart muscle cells (Figs. 6 and 7, Table III). Similar results have been obtained with fibroblastoid rat heart cells and Girardi human heart cells. Therefore, this effect is not due to an increased beating frequency of the muscle cells, but seems to represent a more general phenomenon.

Closely-correlated with this stimulation of $(^{86}Rb^+ + K^+)$ influx of cultured rat heart muscle cells, a positive chronotropic effect and an increase in automaticity are found (Fig. 5). Vanadate-induced stimulation of $(^{86}Rb^+ + K^+)$ influx can be completely abolished by addition of ouabain (Table III). Therefore, the increased influx of $(^{86}Rb^+ + K^+)$ is most probably due to an increased activity of $(Na^+ + K^+)$-ATPase induced by vanadate. Several reasons can account

for this vanadate-induced stimulation of $(Na^+ + K^+)$-ATPase. (a) Nonspecific permeability of the plasma membrane for sodium and/or potassium is increased by vanadate. This would cause a rise in intracellular sodium concentration and/or extracellular potassium concentration, and thereby would induce a stimulation of $(Na^+ + K^+)$-ATPase by increased substrate levels for the enzyme. (b) Vanadate induces a direct stimulation of $(Na^+ + K^+)$-ATPase. This effect, however, does not occur in vitro. At present, this problem remains an open question. Experiments with influx and efflux measurements of $^{22}Na^+$ and $^{86}Rb^+$ will help to clarify the situation, they are still in progress. (c) Cation fluxes of sodium and potassium in avian erythrocytes are stimulated by the adenylate cyclase system [55]. But, the stimulation of potassium influx is not abolished by ouabain [55]. For this reason, it seems unlikely that the stimulation of adenylate cyclase activity by vanadate [19—21] accounts for the vanadate-induced stimulation of $(^{86}Rb^+ + K^+)$ influx in cultured rat heart cells. (d) A further, simple explanation for the increased rate of uptake of $^{86}Rb^+$ would be a co-transport of vanadate plus $^{86}Rb^+$. But, also in this case, no ouabain inhibition should occur.

It is generally believed that the positive inotropic action of cardiac glycosides is due to partial inhibition of $(Na^+ + K^+)$-ATPase and concomitant inhibition of active cation flux [3]. Vanadate, however, which alters beating of cultured rat heart muscle cells in a similar manner as cardiac glycosides, stimulates $(^{86}Rb^+ + K^+)$ influx (Figs. 6 and 7). The concentration-dependence regarding vanadate concentration in the incubation solution is similar for both phenomena. This is, of course, no conclusive evidence that the positive chronotropic effect and the increase in automaticity are dependent on the stimulation of active $(^{86}Rb^+ + K^+)$ influx and thereby on the increased activity of $(Na^+ + K^+)$-ATPase. But, it demonstrates that changes in beating of cultured rat heart cells similar to that induced by cardiac glycosides can be achieved under conditions where active influx of $(^{86}Rb^+ + K^+)$ is stimulated, but not inhibited. In view of the fact that, also, cardiac glycosides at low concentrations have been said to stimulate the sodium pump [56—58], comparison of the effects of vanadate and cardiac glycosides on contractility behaviour and active cation influx into cultured rat heart muscle cells may help to clarify the problem, whether inhibition of $(Na^+ + K^+)$-ATPase is indeed involved in the positive inotropic action of cardiac glycosides in heart tissue.

Based on our experimental data, we can conclude that the prerequisites necessary for vanadate to act as an endogenous regulator of active cation flux in heart tissue are fulfilled: vanadate can enter the cell, there exists a limited amount of intracellular binding sites and vanadate can alter active $(^{86}Rb^+ + K^+)$ influx and, concomitantly, beating of heart muscle cells. The concentrations necessary to achieve these effects are low and within, or not far above, the physiological range $(1 \cdot 10^{-6}—1 \cdot 10^{-7}\,M)$ described for various mammalian tissues [1]. Hypotheses have been discussed, concerning the mechanisms of this regulatory function [51,59], mainly based on experiments with membrane preparations of $(Na^+ + K^+)$-ATPase. Further work, however, will be necessary to clarify the discrepancy between the inhibitory action of vanadate on isolated $(Na^+ + K^+)$-ATPase and the stimulatory effect of vanadate on $(^{86}Rb^+ + K^+)$ uptake into cultured rat heart muscle cells.

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