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STIMULATORY (INSULIN-MIMETIC) AND INHIBITORY (OUABAIN-LIKE) ACTION OF VANADATE ON POTASSIUM UPTAKE AND CELLULAR SODIUM AND POTASSIUM IN HEART CELLS IN CULTURE *

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(1) The influence of vanadate (Na_3VO_4) on sodium and potassium uptake as well as on cellular ion contents of sodium and potassium has been studied in heart muscle and non-muscle cells obtained from various species. An ouabain-like inhibition of potassium uptake (up to 50%), combined with a decrease of cellular potassium (up to 20%) has been observed by vanadate (10^{-4}–10^{-3} M) in heart non-muscle cells obtained from neonatal guinea pigs and chick embryos. In heart muscle and non-muscle cells prepared from neonatal rats, as well as in Girardi human heart cells, a vanadate-induced stimulation of potassium uptake (up to 100%), combined with a rise in cellular potassium (up to 20%) and without significant alteration of cellular sodium, has been found. A slight increase of $^{22}Na^+$ influx can be measured in rat heart muscle cells and in Girardi human heart cells in the presence of vanadate (10^{-4}–10^{-3} M). (2) In beating rat heart muscle cells in culture, detrimental effects of serum deprivation—concerning beating properties, potassium uptake and cellular potassium—can at least in part be overcome by addition of vanadate. Furthermore, this compound prevents ouabain-induced signs of toxicity (contractures) in these cells. (3) The stimulatory effects of vanadate on potassium can be mimicked by insulin (1–10 mU/ml). Furthermore, vanadate produces an insulin-like stimulation of 2-deoxy-D-glucose uptake in rat heart muscle and non-muscle cells as well as in Girardi human heart cells. (4) The experimental data demonstrate an ouabain-like inhibition as well as an insulin-mimetic stimulation of potassium-uptake in various heart cells. The reason for this antagonistic mode of action may be due to the different capabilities of the heart cell types to reduce vanadium in the V-valence state to vanadium in the IV-valence state, thereby favouring either ouabain-like inhibition (vanadium V) or insulin-mimetic stimulation (vanadium IV) of potassium transport.

Introduction

Compounds of the trace element vanadium (e.g. vanadate, VO_4^{3-} /VO_3^-) exert a positive inotropic effect in a variety of ventricular muscle preparations [2,3], and increase contraction velocity, beating frequency and automaticity in rat heart muscle cells in culture [4]. These effects have attained much interest, since vanadium compounds (analogous to cardiac glycosides) inhibit ($Na^+ + K^+$)-ATPase enriched from different organs and species

* Part of these results has been presented at the International Congress of Cell Biology, Berlin, 1980 [1]

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[5–7]. Though the molecular mechanism of positive inotropy of cardiac glycosides is still a matter of controversy, it is generally accepted that interaction of glycosides with $(Na^+ + K^+)$-ATPase is the primary step involved in this process [8].

In contrast to the effect on enzyme in vitro, the action of vanadium compounds on active Na^+/K^+-flux across the cell membrane of intact cells (representing the physiological function of $(Na^+ + K^+)$-ATPase) is complex: inhibition is observed in human erythrocytes [9]; active potassium influx is unaltered by vanadate in isolated rat adipocytes [10] and in guinea pig and rat heart muscle [11]; even a stimulation of active potassium influx has been observed in cultured rat heart muscle cells and non muscle cells as well as in Girardi cells, a human heart cell line [4].

Looking for a possible vanadate-induced alteration of active cation-flux in the intact cell (according to the inhibition of $(Na^+ + K^+)$-ATPase in vitro) we have investigated the effects of Na_3VO_4 and of ouabain on sodium and potassium fluxes across the cell membrane of heart cells obtained from different species (muscle and non-muscle cells from hearts of neonatal rats, Girardi human heart cells, non-muscle cells from hearts of chick embryos and neonatal guinea pigs). Furthermore, the consequences of altered cation-fluxes on intracellular sodium and potassium contents are characterized.

Materials and Methods

Experiments have been carried out with the following cell types: muscle cells and non-muscle cells obtained from hearts of neonatal (1–5-day-old) rats; non-muscle cells prepared from chick embryos (9–11-day-old) and from neonatal guinea pigs; Girardi human heart cells, an epitheloid-like growing cell line [12] purchased from Flow Labs., D-5300 Bonn, F.R.G.. The term 'non-muscle cells' refers to heart cells lacking sarcomeres, mainly consisting of fibroblasts and endothelial cells [13].

Preparation and cultivation of rat heart muscle cells according to the method of Harary et al. [14] have been described previously [4]. For disaggregation of heart tissue, a collagenase (0.03%)-trypsin (0.12%) solution has been used. Heart non-muscle cells from rats, guinea pigs and chickens have been obtained from the same tissue as heart muscle cells, separated from the latter by differential attachment technique and cultivated in growth medium [4] without horse serum; experiments have been carried out after 1–8 subcultivations (0.15% trypsin + 0.1 mM EDTA) at confluency. Measurements with Girardi human heart cells have been done after culturing the cells in growth medium [4] without horse serum, after passage 500–550 at confluency.

Measurement of $^{42}K^+$, $^{22}Na^+$, $^{86}Rb^+$ and 2-deoxy-D-[^3H]glucose uptake into heart cells. Standard measurements have been carried out at 37°C with cells in 25 cm^2 plastic culture flasks (Nunclon Plastics, DK-Roskilde, Denmark) in a Hepes-buffered salt solution. Unless stated otherwise, this solution consisted of 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 11 mM D-glucose, 20 mM Hepes; salt solution has been finally adjusted with NaOH to pH 7.25. For measurement of $^{86}Rb^+$ uptake see [4], experiments with $^{42}K^+$ and $^{22}Na^+$ have been carried out under identical conditions. In experiments measuring uptake of 2-deoxy-D-[^{3}H]-glucose, cells have been preincubated in a Hepes-buffered salt solution containing 11 mM D-glucose (see above). In the assay medium, D-glucose has been omitted and substituted by 100 μM 2-deoxy-D-glucose.

In each series of experiments, a 'zero-time' assay has been carried out [4]. This 'zero-time' value closely correlates with the extracellular space of the cell monolayer after thorough washing, determined with inulin-[^{14}C]carboxylic acid, which does not penetrate or bind to heart muscle cells in culture [15].

Cell protein was determined according to Lowry et al. [16]. The variation in protein contents per flask within one experiment was $\leq \pm 7\%$.

Determination of cellular contents of potassium and sodium ions has been obtained by two independent methods [17]: (a) determination of potassium ions by flame photometry: in these experiments, uptake measurements of $^{86}Rb^+$, $^{22}Na^+$ and $^{42}K^+$, respectively, have been carried out as described above, with subsequent washing of the cells in a Ca^{2+} (1.8 mM)-sorbitol (280 mM) washing-solution instead of the Hepes-buffered salt solution, with 4 mM Hepes, final pH 7.25. After dissolving the cells, 500 μl were taken for radioac-

tivity measurement [4], and K^+ has been determined in two 100 µl aliquots by flame photometry; (b) the exchangeable cellular potassium pool has been measured by incubation of the cells with $^{86}Rb^+$ and $^{42}K^+$, respectively, with subsequent determination of radioactive tracer under equilibrium conditions.

In a similar manner, the exchangeable pool of intracellular sodium ions has been obtained by measurement of cellular $^{22}Na^+$ tracer under equilibrium conditions. In all heart cells studied, equilibrium is obtained within 3 h in the case of $^{42}K^+/^{86}Rb^+$ and $^{22}Na^+$. In all measurements, values have been corrected for contamination by extracellular radioactive tracer by inulin-[^{14}C]carboxylic acid and 'zero-time' value, respectively.

Insulin experiments. In all experiments comparing the influence of Na_3VO_4 and insulin on ($^{86}Rb^+ + K^+$)-uptake, cellular potassium contents and 2-deoxy-D-[^{3}H]-glucose uptake, 5 µg bovine albumin have been added per ml Hepes-buffered salt solution. Insulin-Hepes buffered salt solution has been prepared in the following manner: bovine insulin (23.6 U/mg) has been dissolved in 0.01 M HCl (about 100 µl 0.01 M HCl per 1 mg insulin), followed by desired dilution in Hepes-buffered salt solution containing 5 µg bovine albumin per ml, and final adjustment with NaOH to pH 7.25.

Preparation and measurement of enzyme activity of ($Na^+ + K^+$)-ATPase from hearts of neonatal rats. The hearts of 200 young Wistar rats (1–3 days after birth) were quickly excised after decapitation. Then the preparation of ($Na^+ + K^+$)-ATPase-containing cardiac cell membranes was carried out as described previously [18]. ($Na^+ + K^+$)-ATPase activity was measured with the coupled optical assay [19]. The reaction was continuously recorded. One enzyme unit (U) is defined as the amount of enzyme hydrolizing 1 µmol ATP per min at 37°C. Protein was measured according to Lowry et al. [16].

Materials. Chemicals used were purchased from (see also text): Seromed, D-8000 München, F.R.G. (Collagenase 'Worthington', 168 U/mg, CLS II); Serva Biochemica, D-6900 Heidelberg, F.R.G. (bovine serum albumin, No. 11920; Trypsin 1:250, No. 37920); Sigma Chemie, D-8028 Taufkirchen, F.R.G. (2-deoxy-D-glucose, grade III, No. D6134; bovine insulin, 24 I.U./mg, No. 15500); Boehringer Mannheim, D-6800 Mannheim, F.R.G. (tetrodotoxin, No. 161560); BDH Chemicals, Poole, U.K. (Na_3VO_4); Amersham Buchler, D-3300 Braunschweig, F.R.G. (Inulin [^{14}C]carboxylic acid, 2.02 µCi/mg; ^{42}KCl, 22 µCi/mg; 2-deoxy-D-[1-^{3}H]glucose, 15 Ci/mmol); NEN Chemicals, D-6072 Dreieich, F.R.G. (^{22}NaCl, carrier free; ^{86}RbCl, 3.9 mCi/mg).

D-600-HCl was a gift from Knoll AG, D-6700 Ludwigshafen, F.R.G.. All other chemicals were of analytical grade and purchased from Merck, D-6100 Darmstadt, F.R.G. and Boehringer Mannheim, D-6800 Mannheim, F.R.G.. Na_3VO_4 has been prepared as stock solution (10 M) in the Hepes-buffered salt solution adjusted to pH 7.25 with HCl.

The data presented in this report are mean values from closely correlating duplicates or triplicates. All experiments described have been carried out at least three times.

Results

Inhibitory action of vanadate on ($Na^+ + K^+$)-ATPase enriched from hearts of neonatal rats

The inhibitory effect of vanadate on ($Na^+ + K^+$)-ATPase enriched from hearts of neonatal rats is shown in Fig. 1. As it has been demon-

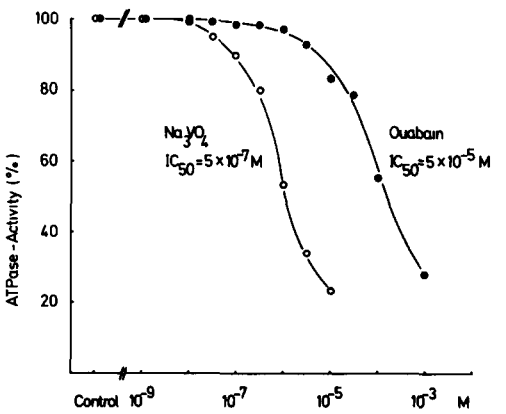


Fig 1 Inhibition of rat heart ATPase activity by Na_3VO_4 and ouabain. Cell membranes (0 6–1 0 mg protein, ($Na^+ + K^+$)-ATPase activity 0 22 U/mg protein) were incubated at 37°C in the coupled optical assay mixture [19] with the indicated ouabain or Na_3VO_4 concentrations for 60 min For further experimental details see Ref 18

strated for $(Na^+ + K^+)$-ATPase preparations from a variety of organs and tissues, vanadate inhibits in a concentration dependent manner enzyme activity of $(Na^+ + K^+)$-ATPase from hearts of neonatal rats, the same tissue, from which heart muscle and non-muscle cells have been isolated (see Materials and Methods). Half maximal inhibition occurs at $5 \cdot 10^{-7}$ M vanadate, this compound being therefore even more potent than ouabain ($IC_{50} = 5 \cdot 10^{-5}$ M), a well-known specific inhibitor of this enzyme.

Vanadate-induced stimulation and inhibition of active potassium-influx in heart cells of different species

Fig. 2A demonstrates the influence of vanadate and ouabain, respectively on $^{42}K^+$ influx in beating rat heart muscle cells in culture. Uptake is linear for at least 10 min. According to the inhibition of enriched $(Na^+ + K^+)$-ATPase (Fig. 1), ouabain at high concentrations (10^{-3} M) reduces uptake rate to 39%. In the presence of vanadate (10^{-3} M), however, the rate of $^{42}K^+$ uptake is not inhibited but stimulated, thereby yielding 129% of control value. Doing the identical experiment with the potassium analogue $^{86}Rb^+$ instead of $^{42}K^+$ as radioactive tracer (Fig. 2B), nearly the same result is obtained. Thus, in the following experiments (because of its more convenient radioactive half life) trace amounts of $^{86}Rb^+$ were used instead of $^{42}K^+$ to measure the uptake of potassium ions in cultured heart cells [4,20,21]. To study the discrepancy of vanadate action on isolated $(Na^+ + K^+)$-ATPase (Fig. 1) and on potassium uptake of beating rat heart muscle cells (Fig. 2) in more detail, the influence of increasing concentrations of vanadate on $(^{86}Rb^+ + K^+)$-uptake has been studied in a variety of heart cells (muscle and non-muscle cells) from different species (Fig. 3). In muscle and non-muscle cells obtained from hearts of neonatal rats as well as in Girardi human heart cells (an epitheloid-like growing transformed cell line [12]) vanadate enhances $(^{86}Rb^+ + K^+)$-uptake up to 204, 162 and 165%, respectively, the vanadate concentration necessary for half maximal stimulation being 22, 140 and 34 μM, respectively. This vanadate-induced stimulation can be completely abolished by ouabain, which has been demonstrated for rat heart muscle cells and Girardi human cells [4,22].

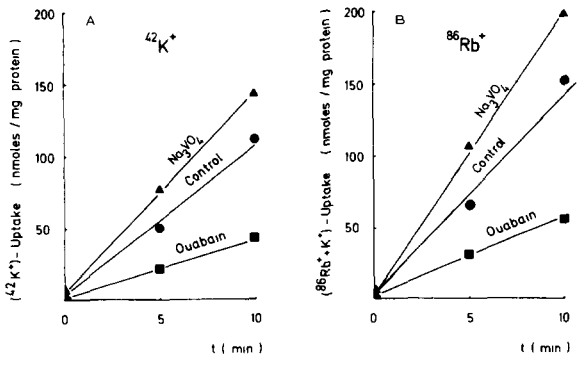


Fig. 2 Kinetics of $^{42}K^+$- and $^{86}Rb^+$-uptake into cultured rat myocardial cells. Influence of Na_3VO_4 (10^{-3} M) and ouabain (10^{-3} M). Preincubation time 180 min, 3.8 10^6 cpm $^{42}K^+$ and 1.8 $\cdot 10^6$ cpm $^{86}Rb^+$, respectively, per flask, 0.54 mg protein per flask

In cardiac non-muscle cells obtained from neonatal guinea pigs and chick embryos, however, a concentration-dependent inhibition of $(^{86}Rb^+ + K^+)$-influx occurs. Half-maximal inhibition is found at 40 and 100 μM, respectively, the amount of maximal inhibition (50 and 34%, respectively) being comparable with the action of ouabain (see legend to Fig. 3, and also Table I).

Influence of vanadate on intracellular sodium and potassium levels in heart cells

Due to the different action of vanadate and ouabain on $(^{86}Rb^+ + K^+)$-uptake in cultured heart cells, these compounds should alter steady state levels of intracellular sodium and potassium ions in an opposite manner. In the experiment of Table I the influence of vanadate and ouabain on steady-state levels of intracellular sodium and potassium ions have been compared in heart cells of different species. Potassium contents have been determined by flame photometry and $^{86}Rb^+$ under steady-state conditions, while exchangeable intracellular sodium ions have been measured with radioactively labeled $^{22}Na^+$ under steady-state conditions (for further experimental details see Materials and Methods). As one would predict from the inhibition of isolated $(Na^+ + K^+)$-ATPase (Fig. 1) as well as of $(^{86}Rb^+ + K^+)$-uptake (Fig. 2), ouabain decreases cellular potassium and increases cellular sodium ions in all cell types tested. Also the observed vanadate effects can be deduced from the different action of this compound on $(^{86}Rb^+$

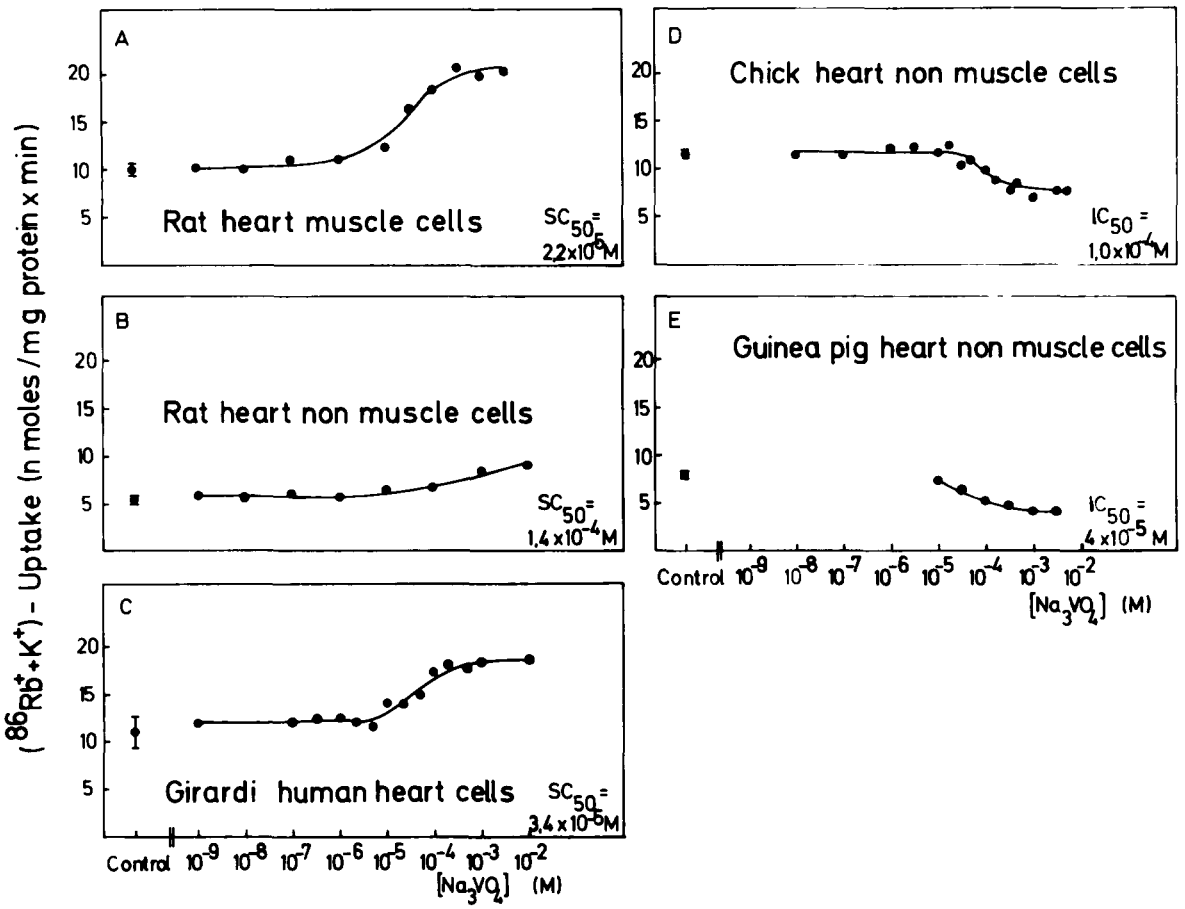


Fig 3 Concentration-dependent stimulation and inhibition of ($^{86}Rb^+ + K^+$)-uptake by Na_3VO_4 into various types of cultured heart cells Preincubation time, $^{86}Rb^+$ radioactivity and cell protein per flask (A) 240 min, 1 01 10^6 cpm, 0 71 mg, (B) 60 min, 0 95 10^6 cpm, 0.89 mg, (C) 150 min, 3 23·10^6 cpm, 0 60 mg, (D) 240 min, 3 68 10^6 cpm, 0 18 mg; (E) 240 min, 1 80 10^6 cpm, 0 34 mg $[K^+] \approx 2$ 7 mM (B, C) and 5 4 mM (A, D, E), respectively Inhibition of ($^{86}Rb^+ + K^+$)-uptake by ouabain (10^{-3} M) (B) 40% , (D) 55%, (A, C and E) not determined

$+K^+$)-uptake as described in Fig. 3: stimulation of ($^{86}Rb^+ + K^+$)-uptake by vanadate in rat heart muscle and non-muscle cells as well as in Girardi human heart cells increases intracellular potassium contents (17, 19 and 12%, respectively). Cellular pool of sodium ions is either slightly lowered (as shown in the experiments of Table I) or remains unchanged (experiments not shown) in the presence of vanadate. In guinea pig and chick heart non-muscle cells, cellular potassium is reduced by vanadate to 84 and 81% of control value. Though vanadate and ouabain inhibit ($^{86}Rb^+ + K^+$)-uptake in both cell types of fairly the same degree, ouabain-caused reduction of cellular potassium exceeds by far the one induced by vanadate.

Comparison of morphological effects of ouabain and vanadate in rat heart muscle cells in culture

In beating rat heart muscle cells in culture, toxic concentrations of ouabain (10^{-3} M and 10^{-2} M) produce drastic morphological alterations (Fig. 4): the cells stop beating, they are rounded and attach to the ground with thin contracted branches. These contractures are not seen in the presence of toxic concentrations of vanadate (10^{-3} M and 10^{-2} M, for signs of toxicity see Ref. 4). Even more, in the presence of vanadate, ouabain-induced contractures of the cells are prevented (Fig. 4).

TABLE I

CELLULAR CONTENTS OF POTASSIUM AND SODIUM, AND ($^{86}Rb^+ + K^+$)-UPTAKE IN VARIOUS TYPES OF CULTURED HEART CELLS INFLUENCE OF Na_3VO_4 AND OUABAIN

Cell type. I, rat heart muscle cells, II, Girardi human heart cells, III, Guinea pig heart non-muscle cells, IV, chick heart non-muscle cells, V, rat heart non-muscle cells Cellular potassium has been determined by flame photometry (I, II, IV, V) and by $^{86}Rb^+$ measurement under equilibrium conditions (3 h) in experiment III, cellular sodium has been determined by $^{22}Na^+$ measurement under equilibrium conditions (260 min in experiment I, 250 min in experiment II) $[Na_3VO_4] = 3\ 10^{-4}$ M (I–III) and 10^{-3} M (IV, V), [ouabain] $= 10^{-3}$ M (I–V) Values are given as mean ± S D for cellular contents of K^+ and Na^+ ($n = 4$–10), the values of ($^{86}Rb^+ + K^+$)-uptake are means of closely correlating duplicates (I–III) and mean ± S D ($n = 3$, IV, V), respectively Preincubation time, $^{86}Rb^+$- and $^{22}Na^+$-radioactivity and cell protein per flask (I) 260 min, 3 12 10^6 cpm, 18 52 10^6 cpm, 0 67 mg, (II) 250 min, 1 63 10^6 cpm, 17 57 10^6 cpm, 1 03 mg, (III) 240 min, 1 32 10^6 cpm $^{86}Rb^+$, 0 78 mg, (IV) 240 min, 2 38 10^6 cpm $^{86}Rb^+$, 0 26 mg, (V) 240 min, 1 41 10^6 cpm $^{86}Rb^+$, 1 30 mg For further experimental details see methods

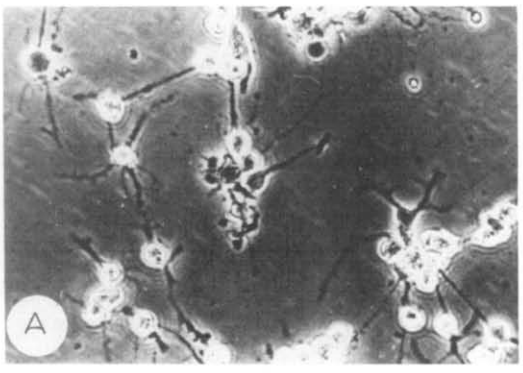
Cell type	Cellular contents of K^+ (nmol/mg protein)		
	Control	Na_3VO_4	Ouabain
I	547 ±30	639 ±26	130 ± 8
II	599 ±35	673 ±51	69 ±20
III	660 ±53	556 ±67	198 ±23
IV	617 ±28	500 ±39	47 ±14
V	491 ±39	586 ±31	264 ±17
	Cellular contents of Na^+		
	Control	Na_3VO_4	Ouabain
I	27 ± 7	23 ± 3	144 ±11
II	54 ± 3	40 ± 4	436 ±19
	($^{86}Rb^+ + K^+$)-uptake (nmol/mg protein per min)		
	Control	Na_3VO_4	Ouabain
I	90	150	36
II	79	106	14
III	84	59	67
IV	149± 03	97± 15	90± 10
V	65± 01	78± 01	51± 01

Long term effects of vanadate in beating rat heart muscle cells in culture

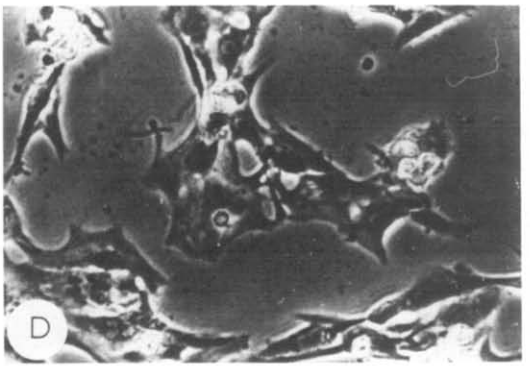
To study long term effects of vanadate in beating rat heart muscle cells in culture on contractility, ($^{86}Rb^+ + K^+$)-uptake and steady-state levels of cellular potassium ions, the cells have been incubated for up to 24 h under different conditions: (a) cultivation of the cells in complete medium containing 10% horse serum and 10% fetal calf serum; (b) incubation of the cells in buffered salt solution (see Methods) in the presence and absence of 10^{-4} M Na_3VO_4.

Contractility has been characterized by beating frequency and automaticity by the number of beating cells per cm^2; potassium uptake rates have been determined by $^{86}Rb^+$ flux measurements; steady-state levels of cellular potassium ions have been obtained by flame photometry. In the presence of complete culture medium, these parameters remain fairly constant over 24 h (Fig. 5). However, incubation of the cells in buffered salt solution leads to a drastic decline in beating frequency and number of beating cells/cm^2 within this time period; ($^{86}Rb^+ + K^+$)-uptake is time-dependent lowered up to about 50% of its original value; cellular potassium contents declines to 64% of control. These detrimental effects are at least in part prevented by adding Na_3VO_4 (10^{-4} M) to the buffered salt solution (Fig. 5).

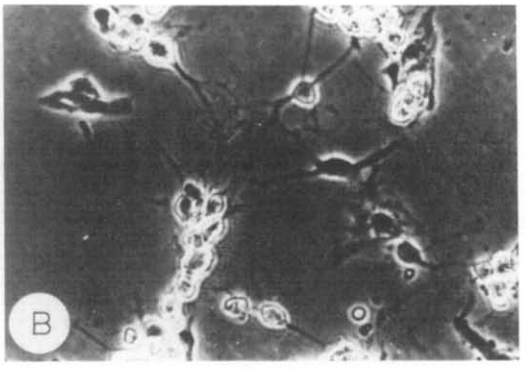
The time-dependent detrimental effects of serum deprivation in the experiment of Fig. 5 are abnormally strong. In most cases, the decline in beating frequency, beating cells/cm^2, ($^{86}Rb^+ + K^+$)-uptake and cellular potassium concentration is not as drastic as in the above shown experiment; however, always a beneficial effect of vanadate concerning these parameters is found. Additionally, with prolonged incubation of rat heart muscle cells in buffered salt solution up to 24 h, an increasing percentage of the cells develops arrhythmias, which (at least in part) can be prevented by addition of 10^{-4} M Na_3VO_4. However, the described beneficial effects of vanadate are only seen, when rat heart muscle cells are incubated for prolonged intervals in buffered salt solution. In complete medium substituted with horse serum and fetal calf serum, neither stimulation of ($^{86}Rb^+ + K^+$)-uptake nor marked alterations of cellular potassium content can be ob-



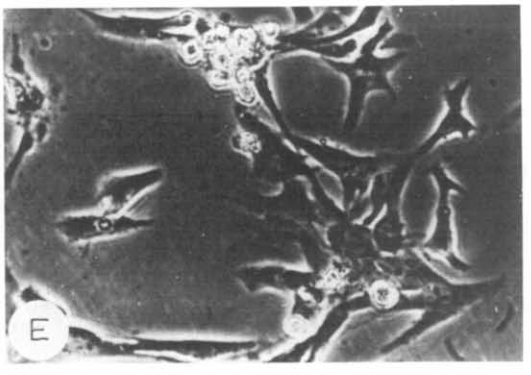
Ouabain (10^{-2}M)



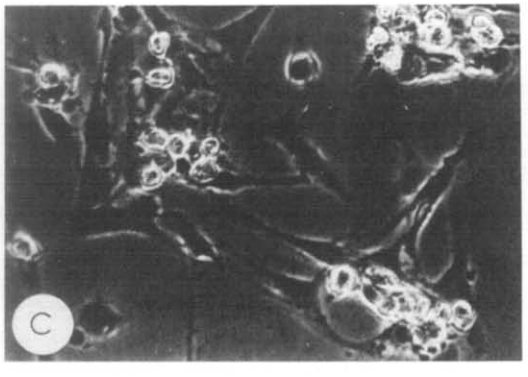
Na_3VO_4 (10^{-2}M)



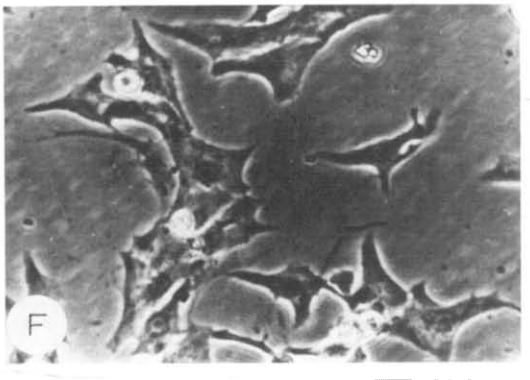
Ouabain (10^{-3}M)



Na_3VO_4 (10^{-3} M)



Ouabain (10^{-3}M) + Na_3VO_4 (10^{-4}M)



Control

Fig 4 Effects of ouabain and Na_3VO_4 in cultured rat heart muscle cells morphological aspects Preincubation time 180 min, beating frequency (control) 127 ± 12 min^{-1} ($M \pm SD$, $n=4$) A, 10^{-2} M ouabain, B, 10^{-3} M ouabain, C, 10^{-3} M ouabain + 10^{-4} M Na_3VO_4, D, 10^{-2} M Na_3VO_4; E, 10^{-3} M Na_3VO_4; F, control Cells in ouabain, Na_3VO_4 and ouabain + Na_3VO_4 have stopped beating.

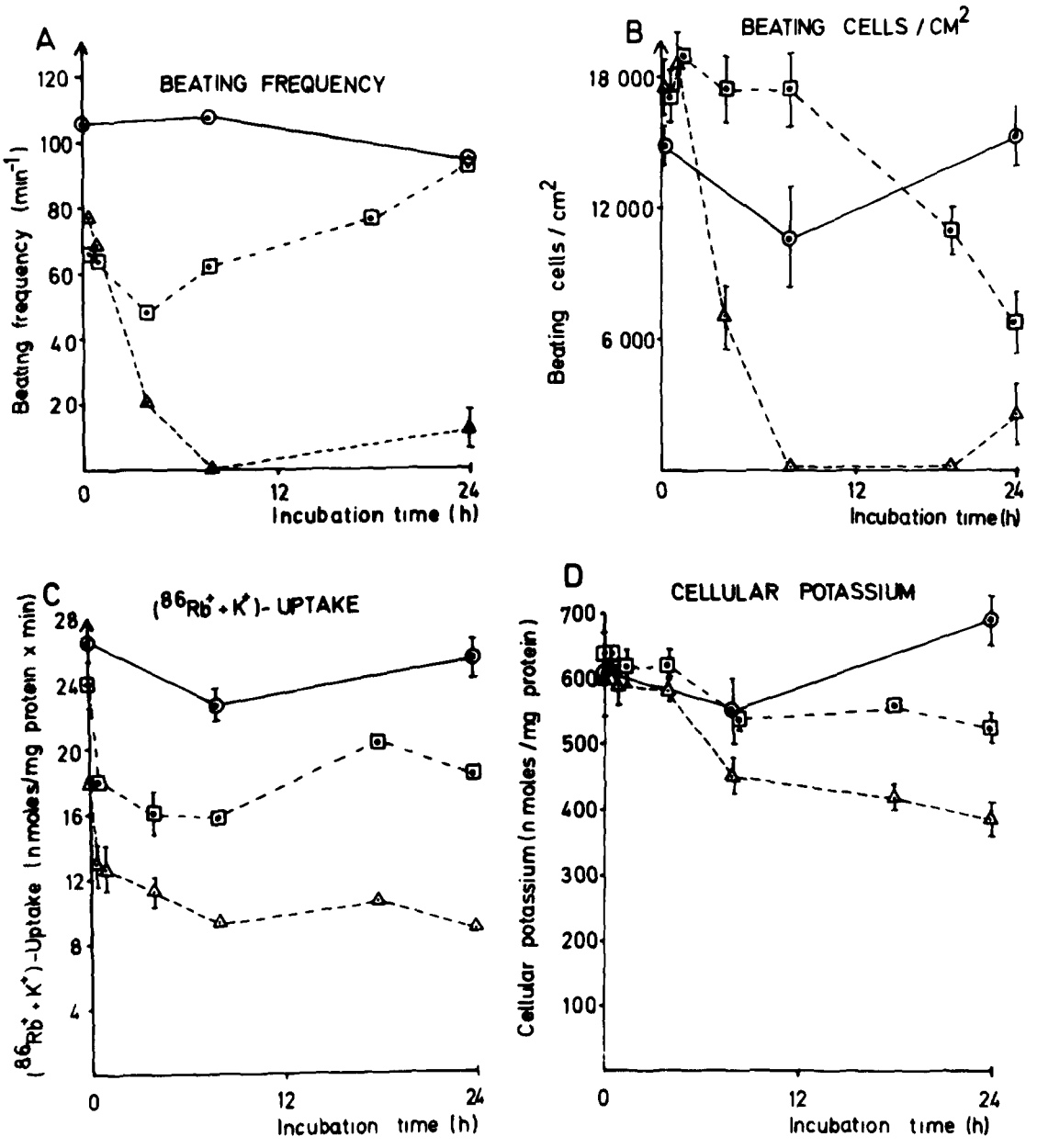


Fig 5 Long term effects of Na_3VO_4 on beating frequency, automaticity, ($^{86}Rb^+ + K^+$)-uptake and cellular potassium in beating rat heart muscle cells in culture Cells have been incubated for the time indicated in complete culture medium supplemented with 10% horse serum and fetal calf serum (○), Hepes-buffered salt solution (△), and Hepes-buffered salt solution + 10^{-4} M Na_3VO_4 (□), respectively Values are mean ± S E (beating frequency and beating cells per cm^2, $n = 8$) and mean ± S D (($^{86}Rb^+ + K^+$)-uptake and cellular potassium, $n = 3$), the standard deviations lying within the symbols used, if arrows are not indicated Cellular potassium has been measured by flame photometry, 1 39 10^6 cpm ($^{86}Rb^+$)/flask, 0 70 mg protein/flask Beating frequency, ($^{86}Rb^+ + K^+$)-uptake and cellular potassium contents of beating rat heart muscle cells cultured in complete medium + 10% horse serum + 10% fetal calf serum (values in the presence of 10^{-4} M Na_3VO_4 are given in brackets) Preincubation time 0 h 106 ± 1 min^{-1}, 26 5 ± 0 7 (27 6 ± 1 4) nmol/mg protein per min, 609 ± 63 (605 ± 40) nmol/mg protein, preincubation time 8 h 107 ± 2 (0) min, 22 7 ± 1 0 (18 6 ± 0 5) nmol/mg protein per min, 548 ± 47 (602 ± 18) nmol/mg protein, preincubation time 24 h 94 ± 1 (0) min^{-1}, 25 3 ± 1 1 (19 1 ± 0 4) nmol/mg protein per min, 691 ± 35 (557 ± 8) nmol/mg protein

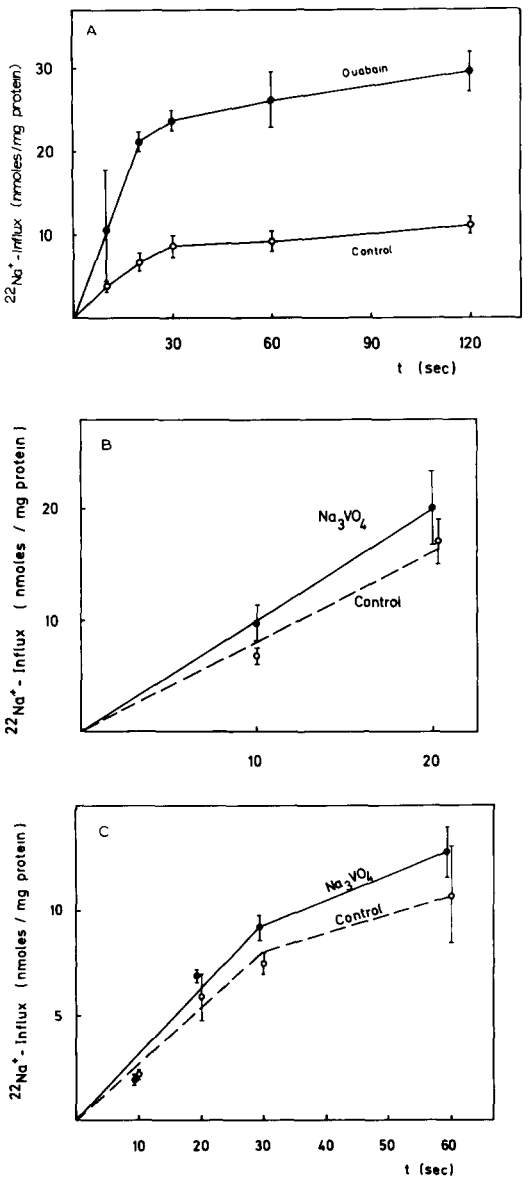


Fig 6 (A) Kinetics of $^{22}Na^+$ influx in beating rat heart muscle cells Influence of ouabain (10^{-3} M) Preincubation time 15 min, 6 20 10^6 cpm $^{22}Na^+$ per flask; 1 46 mg protein per flask Values are given as mean ± S E ($n=3$), zero-time values (see Materials and Methods) have been subtracted (control 1 7 ± 0.6 nmol/mg protein, ouabain 5 1 ± 0 9 nmol/mg protein) (B) Kinetics of $^{22}Na^+$ influx in beating rat heart muscle cells in the presence of ouabain (10^{-3} M) Influence of Na_3VO_4 (3 10^{-4} M) Preincubation time (ouabain ± Na_3VO_4) 45 min; 7 21 10^6 cpm $^{22}Na^+$ per flask, 1 28 mg protein per flask Values are given as mean ± S E ($n=3$); zero-time value (see Materials and Methods have been subtracted (control 5 4 ± 0 8 nmol/mg protein, Na_3VO_4 7 5 ± 1 2 nmol/mg protein) (C) Kinetics of $^{22}Na^+$ influx in beating rat heart muscle cells Influence of Na_3VO_4 (10^{-3} M) Preincubation time 15 min; 9 55 10^6 cpm $^{22}Na^+$ per flask, 2 25 mg protein per flask Values are given as mean ± S E ($n=3$), zero-time values (see Materials and Methods) have subtracted (control 3 1 ± 1 5 nmol/mg protein, Na_3VO_4 2 6 ± 1 1 nmol/mg protein)

served; in contrast, even a concentration-dependent stop of automaticity of these cells occurs within minutes to hours at vanadate concentrations $\geq 10^{-4}$ M (at least in part of the experiments (see legend to Fig. 5)).

Effect of vanadate on sodium-influx in cultured heart cells

For investigation of the mechanism of action of vanadate on monovalent cation flux across the cell membrane of cultured heart cells, $^{22}Na^+$ influx has been studied. $^{22}Na^+$ influx in beating rat heart muscle cells is shown in Fig. 6A: as it has been described recently [17], $^{22}Na^+$ uptake occurs rapidly, having a half-time of 15–30 s (Fig. 6A, control). Inhibition of $(Na^+ + K^+)$-ATPase by high doses of ouabain produces increased intracellular sodium levels, but also a marked stimulation of sodium-uptake rate [23] (Fig. 6A, ouabain). Figs. 6B and 6C demonstrate the effect of vanadate on $^{22}Na^+$ uptake rates in the presence (Fig. 6B) as well as in the absence (Fig. 6C) of ouabain. In both cases, a reproducible, slight increase of $^{22}Na^+$-uptake velocity by vanadate of about 20% can be demonstrated. A similar (5–30%) increase of $^{22}Na^+$ influx has been also observed in Girardi human heart cells, incubated in $3 \cdot 10^{-4}$ M Na_3VO_4 (experiments not shown).

Cultured heart muscle cells possess two different channels for uptake of sodium ions: the 'fast channel' can be blocked by tetrodotoxin, the entrance of sodium ions by the 'slow channel' is abolished by D 600 (for references see Ref. 24).

Increased uptake of sodium ions in the presence of vanadate (mediated by the fast and slow sodium channel) would explain the vanadate-induced stimulation of the sodium pump by increasing intracellular sodium concentration, the rate limiting step of sodium pump activity [25]. To test this possibility, we therefore studied vanadate-induced stimulation of $(^{86}Rb^+ + K^+)$-uptake in the presence of inhibitors of fast and slow medium channels (Table II): increasing concentrations of D 600

TABLE II

INFLUENCE OF D 600 AND TETRODOTOXIN ON VANADATE-INDUCED STIMULATION OF ($^{86}Rb^+ + K^+$)-UPTAKE IN RAT HEART MUSCLE CELLS IN CULTURE

$[Na_3VO_4] = 10^{-4}$ M, Values are given as mean ± S D ($n = 3$)
Experiment with D 600 A stock solution of D 600 has been prepared in ethanol and subsequently diluted in Hepes-buffered salt solution In the control measurement without D 600, an appropriate amount of ethanol has been added to the buffered salt solution Preincubation in the presence of D 600 ± Na_3VO_4 60 min, 1 06 10^6 cpm $^{86}Rb^+$ per flask, 1 72 mg protein per flask Beating frequency Control in the absence of D 600 and Na_3VO_4 67 ± 2 min^{-1}, in the presence of Na_3VO_4 72 ± 2 min^{-1} (mean ± S E, $n = 5$) At D 600 = 10^{-7} M in the absence of Na_3VO_4, beating ceases; in the presence of Na_3VO_4 and D 600 (10^{-7} M) only some of the cells are beating (0–25 min^{-1}), at higher concentrations of D 600, beating is blocked
Experiment with Tetrodotoxin Preincubation in the presence of tetrodotoxin ± Na_3VO_4 150 min, 1 41 10^6 cpm $^{86}Rb^+$ per flask, 0 99 mg protein per flask Beating frequency Control in the absence of tetrodotoxin and Na_3VO_4 42 ± 1 min^{-1}, in the presence of Na_3VO_4 50 ± 2 min^{-1} Tetrodotoxin (10^{-4} M) without Na_3VO_4 32 ± 2 min^{-1}, in the presence of Na_3VO_4 37 ± 2 min^{-1} (mean ± S E, $n = 5$)

Concentration of D 600/tetrodotoxin (M)	($^{86}Rb^+ + K^+$)-uptake (nmol/mg protein per min) in the presence of			
	D 600		Tetrodotoxin	
	Control	Na_3VO_4	Control	Na_3VO_4
–	12 9 ± 0 4	19 4 ± 1 5	12 6 ± 1 0	14 4 ± 0 6
10^{-7}	9 2 ± 0 3	13 0 ± 0 5	12 4 ± 0 9	15 9 ± 0 6
10^{-5}	9 5 ± 0 2	12 5 ± 0 9	11 7 ± 0 3	14 0 ± 0 9
10^{-4}			11 7 ± 0 9	14 1 ± 0 1

progressively reduce beating frequency, beating ceases at 10^{-7} M (see legend to Table II). Concomitantly (due to the frequency-dependent ($^{86}Rb^+ + K^+$)-uptake in heart muscle [25]) potassium-uptake decreases (Table II). Vanadate-induced stimulation of ($^{86}Rb^+ + K^+$)-uptake, however, cannot be abolished even by the highest concentration of D 600 used, demonstrating that: (a) this vanadate effect is independent from beating, and (b) this vanadate effect is independent from sodium entry by D 600-sensitive uptake mechanism.

According to the postulation that 'fast sodium channels' may be latent in cultured heart muscle cells (for references see Ref. 24), beating frequency in cultured rat heart muscle cells is only slightly decreased by tetrodotoxin up to 10^{-4} M (Table II). Also the increase in ($^{86}Rb^+ + K^+$)-uptake by vanadate is not diminished, even at high concentrations (10^{-4} M) of tetrodotoxin (Table II).

Comparison of vanadate- and insulin-effects in cultured heart cells

In rat adipocytes, vanadate produces an insulin-like stimulation of glucose uptake and glucose metabolism [10]. As insulin is known to alter cellular monovalent cation contents of heart tissue by either enhancement of ($Na^+ + K^+$)-ATPase activity or by changing permeability of the cell membrane for sodium and potassium ions (for review, see Ref. 26), the influence of Na_3VO_4 and insulin concerning ($^{86}Rb^+ + K^+$)-uptake, cellular potassium level and uptake of 2-deoxy-D-[^{3}H]-glucose have been compared in heart cells of different species.

Fig. 7 shows the effect of increasing activities of bovine insulin on ($^{86}Rb^+ + K^+$)-uptake in rat heart muscle cells. A stimulatory action can clearly be demonstrated, being half maximal at 0.1–1.0 mU/ml insulin, and leading to a maximal stimulation of uptake rate of about 80%. A similar in-

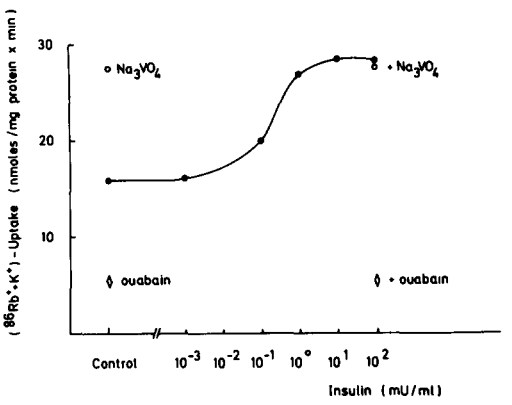


Fig 7 Concentration dependent stimulation of ($^{86}Rb^+ + K^+$)-uptake by insulin into rat heart muscle cells in culture Preincubation time 240 min, 1 02 10^6 cpm $^{86}Rb^+$ per flask; 0 52 mg protein per flask. [ouabain] = 10^{-3} M [Na_3VO_4] = 3 10^{-4} M. Values are means of closely correlating duplicates For further experimental details see methods

crease in ($^{86}Rb^+ + K^+$)-uptake has been measured in the same experiment in the presence of 10^{-3} M Na_3VO_4 instead of insulin (Fig. 7). This insulin action is ouabain-sensitive, since it can be completely abolished by high concentrations of

TABLE III

COMPARISON OF INSULIN AND VANADATE EFFECTS ON UPTAKE OF ($^{86}Rb^+ + K^+$) AND 2-DEOXY-D-[^{3}H]-GLUCOSE, AND ON CELLULAR POTASSIUM CONTENTS IN VARIOUS TYPES OF CULTURED HEART CELLS

Cell type. I, rat heart muscle cells, II, Girardi human heart cells, III, guinea pig heart non-muscle cells, IV, chick heart non-muscle cells, V, rat heart non-muscle cells Cellular potassium has been determined by flame photometry Bovine insulin = 10 mU/ml (I, II, III, V) and 100 mU/ml (IV) [Na_3VO_4] for measurement of ($^{86}Rb^+ + K^+$)-uptake and cellular contents of K^+ 3 10^{-4} M (I, II, III) and 10^{-3} M (IV, V) [Na_3VO_4] for measurement of 2-deoxy-D-[^{3}H]glucose uptake. 3 10^{-5} M (I, III), 10^{-4} M (V), 10^{-3} M (II) In I, III and V, higher concentrations of Na_3VO_4 than the ones used reduce uptake of 2-deoxy-D-[^{3}H]glucose Values are given as mean ± S D (n = 3–6), [K^+] in incubation medium = 5.4 mM, concentration of 2-deoxy-D-[^{3}H]glucose = 0 1 mM. Preincubation time, $^{86}Rb^+$- and 2-deoxy-D-[^{3}H]glucose radioactivity and cell protein per flask. (I) 240 min, 1 02 · 10^6 cpm, 0 94 10^6 cpm, 0 52 mg, (II) 200 min, 2 69 · 10^6 cpm, 1 03 10^6 cpm, 0 80 mg; (III) 240 min, 1 97 10^6 cpm, 0 92 · 10^6 cpm, 0.65 mg, (IV) 240 min, 2 38 10^6 cpm $^{86}Rb^+$, 0 26 mg; (V) 240 min, 1 41 10^6 cpm, 0 94 10^6 cpm, 1 30 mg For further experimental details see methods

Cell type	($^{86}Rb^+ + K^+$)-uptake (nmol/mg protein per min)		
	Control	Insulin	Na_3VO_4
I	15 8 ± 0 4	28.4 ± 0 3	27 6 ± 1 3
II	10.2 ± 0 8	12 6 ± 0 6	13 2 ± 1 4
III	14 3 ± 0 2	17 8 ± 0 4	11 0 ± 0 1
IV	14 9 ± 0 3	11 9 ± 1 4	9 7 ± 1.5
V	6 5 ± 0.1	8 6 ± 0 1	7 8 ± 0 1
	2-Deoxy-D-[^{3}H]glucose uptake (nmol/mg protein per min)		
	Control	Insulin	Na_3VO_4
I	0 21 ± 0.01	0 53 ± 0 06	0 42 ± 0 02
II	0 21 ± 0.01	0 38 ± 0.01	0 32 ± 0 02
III	0 23 ± 0.02	0.52 ± 0 04	0 23 ± 0 02
V	0 19 ± 0.02	0 28 ± 0 02	0 28 ± 0 02
	Cellular contents of K^+ (nmol/mg protein)		
	Control	Insulin	Na_3VO_4
I	588 ±16	621 ±20	620 ±15
II	715 ±66	863 ±39	820 ±47
IV	617 ±28	625 ±39	500 ±39
V	491 ±39	565 ±39	586 ±31

ouabain (Fig. 7). Due to the stimulated potassium-uptake, cellular level of potassium ions is increased by insulin (Table III).

The similarity of Na_3VO_4 and insulin action in rat heart muscle cells is not limited to stimulation of ($^{86}Rb^+ + K^+$)-uptake, but vanadate does also mimic insulin's enhancement of uptake of 2-deoxy-D-[^{3}H]glucose, as it has been first described for isolated rat adipocytes [10]. Also in rat heart muscle cells, Na_3VO_4 increases uptake rate of 2-deoxy-D-[^{3}H]glucose in an insulin-like manner, as it is shown in the uptake kinetics of Fig. 8: in the presence of insulin (10 mU/ml), velocity of hexose uptake is enhanced. A similar stimulation is also observed in the presence of $3 \cdot 10^{-5}$ M Na_3VO_4. Ouabain, in contrast, does not stimulate 2-deoxy-D-glucose uptake into these cells (experiments not shown).

Similar comparison of the action of Na_3VO_4 and insulin concerning ($^{86}Rb^+ + K^+$)-uptake, 2-deoxy-D-[^{3}H]glucose uptake and cellular potassium concentration have also been carried out in a variety of heart cells of different species listed in Table III: in those cell types, which respond to Na_3VO_4 with an increased rate of ($^{86}Rb^+ + K^+$)-uptake and a rise of cellular potassium contents (Girardi human heart cells, rat heart non-muscle cells), Na_3VO_4 and insulin react similarly with

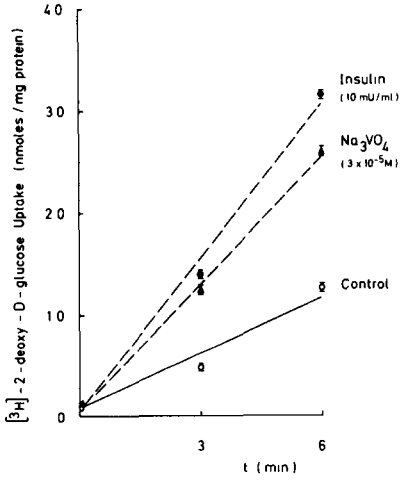


Fig. 8 Kinetics of 2-deoxy-D-[^{3}H]glucose uptake into rat heart muscle cells in culture. Influence of Na_3VO_4 and insulin Preincubation time 240 min; $0.94 \cdot 10^6$ cpm 2-deoxy-D-[^{3}H]-glucose per flask; 0.52 mg protein per flask Values are given as mean ± S.D., $n = 3$ For further experimental details see text

regard to stimulation of ($^{86}Rb^+ + K^+$)- and 2-deoxy-D-[^{3}H]-glucose-uptake, and elevation of cellular potassium concentration. In those cell types, however, which respond to Na_3VO_4 with an inhibition of ($^{86}Rb^+ + K^+$)-uptake, effects of insulin and Na_3VO_4 are different: in heart non-muscle cells from guinea pigs, Na_3VO_4 cannot mimic insulin's stimulatory action of 2-deoxy-D-[^{3}H]-glucose uptake, and ($^{86}Rb^+ + K^+$)-uptake is enhanced in the presence of insulin and inhibited in the presence of Na_3VO_4. In non-muscle cells derived from chicken heart, bovine insulin fails to stimulate ($^{86}Rb^+ + K^+$)-uptake, cellular potassium contents is unchanged; Na_3VO_4, however, inhibits ($^{86}Rb^+ + K^+$)-uptake and depresses cellular potassium (Table III).

Discussion

Summarizing the results presented in Fig. 3 and Table I one can see that ($^{86}Rb^+ + K^+$)-uptake into heart cells of different origin can either be inhibited or stimulated by vanadate. Consequently, intracellular contents of potassium ions are modified (at least in a qualitative sense) according to the primary effect of vanadate on active potassium transport.

What are the underlying mechanisms responsible for the observed heterogeneity of vanadate action?

Inhibition of ($^{86}Rb^+ + K^+$)-uptake in heart non-muscle cells obtained from fetal guinea pigs and chick embryos can easily be accounted for by inhibition of ($Na^+ + K^+$)-ATPase in the intact cell, according to the vanadate-induced inhibition of isolated ($Na^+ + K^+$)-ATPase [5–7]. A binding site of this enzyme for vanadate (located on the cytoplasmic site of ($Na^+ + K^+$)-ATPase) has been identified [9,27]. Though ouabain and Na_3VO_4 inhibit ($^{86}Rb^+ + K^+$)-uptake in both cell types to comparable degree (Fig. 3), only ouabain drastically reduces cellular potassium contents, while in the presence of vanadate cellular potassium level is only decreased to a minor extent (Table I). These differences may imply additional mechanisms than simple inhibition of the sodium pump concerning vanadate-induced alteration of monovalent cation flux.

Lack of inhibition of ($^{86}Rb^+ + K^+$)-uptake by

vanadate observed in intact rat heart cells and Girardi human heart cells does not contradict the inhibitory action of vanadate on $(Na^+ + K^+)$-ATPase in membrane preparations. Only vanadium in the V-valence state represents a potent inhibitor of the enzyme [28]. Lack of inhibition in the intact cell might therefore be explained by enzymatic [29,30] or non-enzymatic [31,32] reduction of vanadate(V) to vanadyl(IV), which has been found to occur in the intact cell [28,33]. This reduction is much more likely to explain the missing inhibition of $(^{86}Rb^+ + K^+)$-uptake than the assumption that vanadate cannot enter the interior of these cells: experimental evidence exists that vanadate does penetrate human erythrocytes [9], rat heart muscle cells in culture [4] and isolated rat adipocytes [10].

The observed vanadate-induced stimulation of $(^{86}Rb^+ + K^+)$-uptake in rat heart cells and Girardi human heart cells implies an increased enzymatic activity of $(Na^+ + K^+)$-ATPase, since it increases cellular level of potassium ions (Table I), and it can be completely inhibited by ouabain [4,22]. This increased activity might be due to direct stimulation of the enzyme or to indirect stimulation of $(Na^+ + K^+)$-ATPase by increasing intracellular levels of enzyme substrates (e.g. Na^+):

(a) Under physiological conditions, the rate-limiting substrate of $(Na^+ + K^+)$-ATPase and thereby of active sodium-potassium transport is the intracellular concentration of sodium ions (for review, see Ref. 25). As it has been shown in the experiments of Figs. 6B and 6C, vanadate does increase $^{22}Na^+$ influx in cultured rat heart muscle cells and Girardi human heart cells. This increase (about 2–5 nmol/mg protein per min) equals the increased $(^{86}Rb^+ + K^+)$-uptake of about 2–10 nmol/mg protein per min in these cell types. If this increased $^{22}Na^+$ influx would be responsible for an indirect stimulation of $(Na^+ + K^+)$-ATPase and thereby of increased $(^{86}Rb^+ + K^+)$-uptake, one would expect an elevated level of cellular $^{22}Na^+$. This however, has not been observed (Table I). Therefore, one may assume that the increased $^{22}Na^+$ influx in the presence of vanadate reflects an additional effect, but is not the reason for stimulation of $(^{86}Rb^+ + K^+)$-uptake. This rise in $^{22}Na^+$ influx may, on the other hand, explain the fact that cellular sodium levels are not drastically lowered but remain fairly constant despite the vanadate induced stimulation of the sodium pump (Table I).

(b) Direct stimulation of $(Na^+ + K^+)$-ATPase from frog skeletal muscle is found in the presence of catechols and their orthochinone derivatives [34]. Although vanadate oxidizes catecholamines in vitro [35], no experimental evidence exists that this reaction triggers vanadate-induced stimulation of $(^{86}Rb^+ + K^+)$-uptake. Also a stimulation of adenylate cyclase by vanadate [36–38] may not be the cause of the observed stimulation: enhancement of potassium influx in turkey erythrocytes by β-adrenergic-coupled adenylate cyclase is not inhibited by ouabain [39], as it has been shown to be the case for vanadate-induced stimulation of $(^{86}Rb^+ + K^+)$-uptake in heart cells [4,22].

(c) Concerning vanadate-induced stimulation of $(^{86}Rb^+ + K^+)$-uptake, the striking similarity between Na_3VO_4 and insulin effects on $(^{86}Rb^+ + K^+)$-uptake and cellular contents of sodium and potassium should be taken into account. As it has been shown for Na_3VO_4 (Table I), also insulin elevates cellular potassium levels in chicken heart muscle cells [40], rat heart muscle and non-muscle cells and Girardi human heart cells (Table III), mediated by an increased rate of $(^{86}Rb^+ + K^+)$-uptake (Fig. 7, Table III). Cellular sodium levels, on the other hand, are not altered significantly, both in the presence of insulin [40] and vanadate (Table I). Further similarities between Na_3VO_4 and insulin have also been observed concerning glucose oxidation [41] and uptake of 2-deoxy-D-[^{3}H]-glucose in rat adipocytes [10], rat heart muscle and non-muscle cells and Girardi human heart cells (Table III, Fig. 8). However, only vanadium in the IV-valence state mimics insulin's action on glucose oxidation [41]. Further evidence for a possible insulin-mimetic action of vanadate is presented in Fig. 5: the time-dependent detrimental effect of serum depletion on potassium-uptake can at least in part be overcome by addition of vanadate. In the presence of serum, no vanadate-induced stimulation of $(^{86}Rb^+ + K^+)$-uptake occurs. Similar results concerning beneficial effects of insulin on amino acid uptake after serum depletion have been obtained in cultured chicken heart cells [40].

Based on these experimental data, the following hypothesis might explain the inhibitory as well as the stimulatory action of Na_3VO_4 on active potassium

uptake in heart cells. Depending on the different cellular capacity for reduction of vanadate(V) to vanadyl (IV) in various types of heart cells, vanadium may act either more as ouabain-like inhibitor, or more as insulin-mimetic stimulator of active potassium uptake. If vanadate can act as inhibitor of $(Na^+ + K^+)$-ATPase in the intact cell, the insulin-mimetic action is blocked, because it depends on non-suppressed enzymatic activity of $(Na^+ + K^+)$-ATPase. If vanadate is unable to act as inhibitor of $(Na^+ + K^+)$-ATPase due to reduction to vanadyl (IV), the insulin-mimetic effect clearly appears. The experimental data presented in this paper are consistent with this hypothesis: if vanadate stimulates $(^{86}Rb^+ + K^+)$-uptake (rat heart muscle and non-muscle cells, Girardi cells), it also enhances, like insulin, uptake of 2-deoxy-D-[^{3}H]-glucose (Table III, Figs. 7 and 8). If vanadate inhibits $(^{86}Rb^+ + K^+)$-uptake (guinea pig heart non-muscle cells), it also fails to stimulate hexose uptake; in contrast to insulin (Table III).

At present, two hypotheses exist to explain insulin's capability of altering the distribution of sodium and potassium ions across the plasma membrane of susceptible cells (for review, see Ref. 26): (a) modification of membrane permeability for sodium and potassium ions at different degrees by insulin, (b) direct stimulation of the sodium pump by insulin.

Concerning insulin's action in heart, experimental evidence is equivocal, supporting both hypotheses [26]. Also in the case of vanadate action in heart cells, an increased permeability for sodium ions (Figs. 6B and 6C), as well as a stimulation of the sodium pump [4,22] has been found.

Can the trace element vanadium indeed act as physiological regulator of $(Na^+ + K^+)$-ATPase by inhibiting active sodium-potassium transport [27]? At present, an inhibitory action of vanadate on active sodium-potassium transport has been demonstrated in erythrocytes [9] and heart non-muscle cells from guinea pigs and chickens (Table III). No vanadate-induced alteration of $(Na^+ + K^+)$-ATPase in intact tissue has been found in guinea pig heart muscle [11], rat aorta [42] and rat adipocytes [10]. In rat heart muscle and non muscle cells and in Girardi human heart cells [4] as well as in rat heart ventricular strips [43], even a stimulation of $(^{86}Rb^+ + K^+)$-uptake has been observed. Also the effects of vanadate in chicken heart muscle-cells [44] and in mouse skeletal muscle [45], are consistent with a stimulation of the sodium pump. Due to these experimental data, one may suppose that regulatory action of vanadate on sodium pump in vivo would be even more complex than it has been supposed, since vanadate can either inhibit or stimulate or let unaltered the sodium pump in intact cells. Our above mentioned hypothesis may help to explain this heterogeneity of vanadate action on active sodium-potassium-transport in intact cells.

Do ouabain-like inhibition and insulin-mimetic stimulation of active potassium transport contribute to the positive inotropic action of vanadate? Though vanadate influences a variety of enzymes involved in the contractility process (for review, see Ref. 46), ouabain-like inhibition of $(Na^+ + K^+)$-ATPase has been supposed as candidate for positive inotropy of vanadate [47], due to the well-known inhibition of the isolated enzyme. Experimental evidence, however, argues against that mode of action [11]. Even the opposite (a vanadate-induced stimulation of $(^{86}Rb^+ + K^+)$-uptake) has been found in rat ventricular strips, in parallel to an increase in force of contraction produced by vanadate [43]. Also in cultured rat heart muscle cells, stimulation of $^{86}Rb^+$ uptake correlates with an increase in contraction velocity, automaticity and beating frequency induced by vanadate [4]. With regard to the well-known positive inotropic effect of insulin (for review, see Ref. 48) and the striking similarities of vanadate and insulin action in rat heart muscle cells described in this paper, a similarity of positive inotropy of vandate and insulin should be taken into consideration.

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