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Effects of perturbation of the Na^+ electrochemical gradient on influx and efflux of alanine in isolated rat hepatocytes

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Transmembrane alanine transport was studied in hepatocytes isolated from 48-h fasted rats. Aminooxyacetate was used to render alanine nonmetabolizable. Gramicidin D eliminated the transmembrane Na^+ electrochemical gradient. At 135 mM Na^+ and 0.1 mM alanine gramicidin D decreased the steady-state intracellular-to-extracellular alanine distribution ratio from 20.2 to 0.9. The underlying kinetic changes appeared to be a decrease in alanine influx to one-third of the control value and an increase in the rate constant of alanine efflux by a factor of 9. Analogous changes were observed when the Na^+ gradient was decreased by ouabain. The inhibitory effect of gramicidin D on alanine influx was confined to the Na^+-dependent, saturable component which showed a prominent increase in the apparent K_m for alanine and a small decrease in the apparent V_{max}. The effect of gramicidin D on alanine efflux was related to the increased cytosolic Na^+ concentration: the rate constant of alanine efflux was increased by cytosolic Na^+ with half-maximal stimulation at 30 mM; voltage-sensitive alanine efflux could not be demonstrated.

Introduction

The liver occupies a central position in the metabolism of amino acids in the body and alanine accounts for a large fraction of the amino acid carbon and nitrogen carried from peripheral tissues to the liver [1,2]. The uptake of alanine in rat hepatocytes is concentrative and occurs by an electrogenic 1 Na^+/1 alanine cotransport system [3,4], where the transmembrane Na^+ electrochemical gradient seems to represent the immediate energy source for the uphill movement of alanine [3,5–7]. From an energetic point of view, this situation implies that the steady-state transmembrane alanine gradient should be a function of, and never exceed, the Na^+ electrochemical gradient. From a kinetic point of view, the influence of the Na^+ gradient on the cellular accumulation of alanine should result from effects on the influx and/or the efflux of alanine. However, as discussed in general terms by Aronson [8], the effect of the Na^+ gradient on each of these fluxes depends on the molecular details of the transport mechanism(s) and cannot be predicted without knowledge of such details. Moreover, as a cosubstrate for the transport systems, sodium ions appear to influence the transport kinetics even in the absence of a transmembrane Na^+ electrochemical gradient [9].

In the case of Na^+/alanine cotransport across the rat liver cell membrane, previous studies have shown that extracellular Na^+ and cell membrane hyperpolarization stimulate the influx of alanine [3,6]. Furthermore, indirect evidence suggests that part of the alanine efflux occurs via the transport system mediating Na^+-coupled alanine influx [5], and recent data in hepatoma cells [10] indicate that the efflux is sensitive to Na^+. In an attempt to

further delineate the role of the Na^+ electrochemical gradient, we have studied the influx and efflux of alanine in isolated rat hepatocytes under conditions where perturbations of the Na^+ gradient were induced by the cation-selective ionophore, gramicidin D, and by ouabain.

Materials and Methods

Hepatocyte isolation and incubation conditions. Hepatocytes were isolated from 48-h fasted female Wistar rats (weight about 150 g) by liver perfusion with collagenase as previously described [5]. The cells were incubated in a modified Krebs-Ringer bicarbonate buffer containing 2% (w/v) bovine albumin and of the following composition (mM): Na^+ 135, K^+ 4.8, Ca^{2+} 2.4, Mg^{2+} 1.2, Cl^- 117.2, HCO_3^- 25, $H_2PO_4^-$ 2.4, and SO_4^{2-} 1.2. Choline was substituted for Na^+ when lower Na^+ concentrations were desired. In a few experiments 110 mM NaCl in the buffer was substituted by the Na^+ salts of SCN^- or isethionate. All experiments were performed at 37°C and pH 7.4 (continuous equilibrium with 95% O_2 and 5% CO_2).

Initial experiments showed that in the presence of 2% (w/v) albumin, a concentration of 10–25 μg gramicidin D per ml of cell suspension was necessary to obtain maximal effects after a few min of exposure. Consequently, pretreatment of the cells with gramicidin D comprised 5 min of exposure to a concentration of 25 μg/ml suspension, added as a solution of gramicidin D in ethanol (2.5 mg/ml). Suspensions of control cells received ethanol in a final concentration of 1% (v/v).

Measurements of alanine and Na^+ uptake. Aminooxyacetate in a final concentration of 2.5 mM was added to the cell suspensions at least 5 min before initiation of the transport measurements in order to block the first step in the intracellular metabolism of alanine [3,11], i.e. to render alanine nonmetabolizable during the measurements. The uptake of alanine and Na^+ was measured with L-[^{14}C]alanine (1 μCi/ml suspension) and $^{22}Na^+$ (10 μCi/ml suspension). At appropriate times after additions of tracer(s) and desired amounts of unlabelled alanine, the cellular uptake was terminated by centrifugation of 20-μl samples from the cell suspension in a manner designed to minimize the trapping of extracellular radioactivity as described previously [4]. Briefly, the samples were flushed into 300 μl isotope-free buffer and centrifuged immediately (within 1–2 s) through silicone oil into perchloric acid. In cases where the tracer equilibrates betwen intra- and extracellular water, the trapped extracellular radioactivity by this separation procedure amounts to about 3% of the intracellular radioactivity. In order to permit sampling at short intervals, a bank of four Beckman Microfuges B was used. The centrifuges were connected to electronic timers and their starting times were printed out and taken as the time of termination of the cellular uptake. The cell precipitates and samples of the supernatants were counted for radioactivity [3]. The cellular uptake of $^{22}Na^+$ and L-[^{14}C]alanine was expressed as the ratio between the concentrations in intra- and extracellular water (distribution ratio) or as the initial rate of uptake (influx) in $mmol \cdot min^{-1} \cdot (l\ cell\ water)^{-1}$. In order to correct for trapping of radioactivity in extracellular water and to obtain the cellular water content as a reference unit, these parameters were determined in separate experiments in each cell batch with ^3H_2O and hydroxy[^{14}C]methylinulin as previously described [3].

Measurements of alanine efflux. One ml of cell suspension preloaded with L-[^{14}C]alanine and unlabelled alanine to the desired concentration was sedimented by centrifugation. After removal of the supernatant, the efflux was initiated by resuspending the cell pellet in 10 ml of isotope-free buffer containing 2.5 mM aminooxyacetate and the desired concentration of test substances. At appropriate times the cells were isolated from 300-μl samples of the suspension by centrifugation through silicone oil into perchloric acid and the cell precipitate was counted for radioactivity. The efflux curves were analysed as described previously [5] and in the legend of Fig. 4.

Liver perfusion and measurements of cell membrane potential. Isolated rat livers were perfused in a non-recirculating system [12] with the modified Krebs-Ringer bicarbonate buffer described above. The flow rate was 40 ml per min. Gramicidin D dissolved in ethanol (or ethanol alone in control periods) was supplied to the medium close to the portal vein by a Holter® pump (900 Series, Extracorporeal Medical Specialities, Inc., PA). Cell

membrane potentials were recorded with standard microelectrode techniques and evaluated according to criteria previously described [12].

Chemicals. Collagenase (CLS II) was supplied from Worthington Biochemicals, Freehold, NJ. Silicone oil AR 200 (density 1.04) was from Wacker Chemie, Munich, F.R.G. Hydroxy[^{14}C]methylinulin and $^{22}Na^+$ was obtained from the Radiochemical Centre, Amersham, U.K.; and [^{14}C] alanine (about 200 mCi/mmol) dissolved in 0.01 M HCl, was from New England Nuclear, Boston, MA. Ouabain was a product of Fluka A.G., Buchs, Switzerland. Gramicidin D and unlabelled alanine were purchased from Sigma Chemical, St. Louis, MO.

Results

The effect of gramicidin D on the transmembrane Na^+ electrochemical gradient in liver cells was studied in two types of experiments. (1) The cell membrane potential was evaluated by microelectrode measurements in isolated, perfused rat livers (Fig. 1). During five min of perfusion with gramicidin D, the cell membrane potential changed from about −30 mV to a value close to 0 mV. (2)

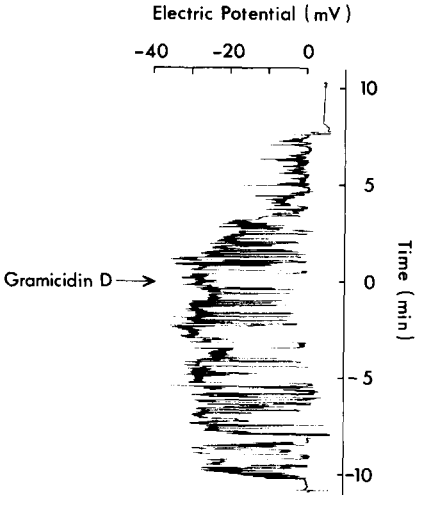

Fig. 1. Effect of gramicidin D on the cell membrane potential in isolated, perfused rat liver. Gramicidin D was present in the perfusate from zero time (final concentration 0.5 μg per ml). The microelectrode was slowly advanced through the liver, successively penetrating new cells with intervening extracellular recordings (0 mV in the figure).

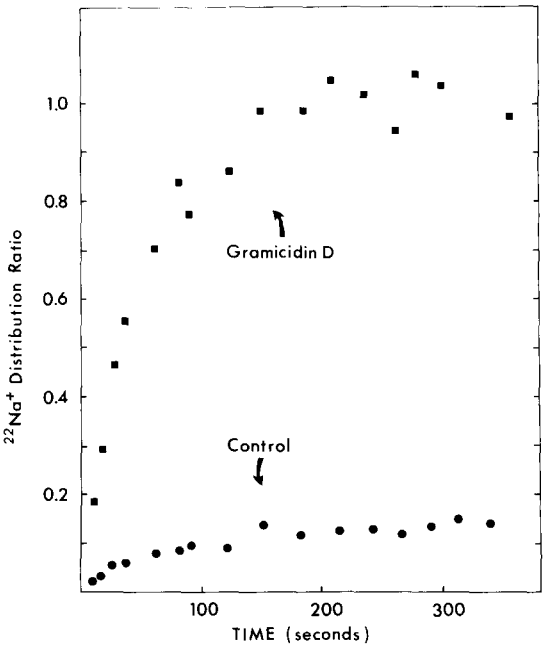

Fig. 2. Effect of gramicidin D on the time course of $^{22}Na^+$ uptake in isolated rat hepatocytes. The cells were incubated in a buffer containing 135 mM Na^+. Gramicidin D (25 μg per ml) or the solvent (ethanol 1% v/v) was present 5 min before the measurements were initiated at zero time by addition of $^{22}Na^+$. Sampling was performed at the times indicated. The $^{22}Na^+$ content of the isolated cell pellets is expressed as the intracellular-to-extracellular distribution ratio. Each point represents the mean of two measurements in different cell preparations.

After addition of $^{22}Na^+$ to suspensions of isolated rat hepatocytes at an extracellular Na^+ concentration of 135 mM, a steady-state intracellular-to-extracellular distribution ratio of $^{22}Na^+$ was attained within 3 min (Fig. 2). Prior exposure of the cells to gramicidin D for 5 min induced a marked increase in the influx of $^{22}Na^+$ and increased the distribution ratio in steady state from about 0.15 in control cells to about 1.0. The cellular water volume after exposure to gramicidin D showed only a slight increase to 112 ± 4% (S.E., $n = 6$) of the control value. The results mentioned above served to support the assumption that gramicidin D can be used as a tool in short term experiments to eliminate (or strongly reduce) the transmembrane Na^+ electrochemical gradient in liver cells. Our previous finding [3] that a 5-min exposure to gramicidin D is accompanied by only a small decrease in the cellular ATP content was con-

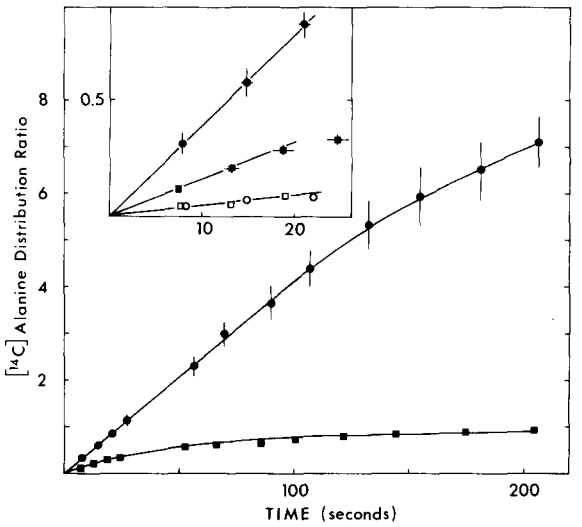

Fig. 3. Effect of gramicidin D on the time course of alanine uptake in isolated rat hepatocytes. The cells were incubated in a buffer containing 135 mM Na^+. Gramicidin D, 25 μg per ml, (■), or the solvent, ethanol 1% v/v, (●), was present 5 min before [^{14}C]alanine was added at zero time in a final concentration of 0.1 mM. Sampling was performed at the times indicated. The [^{14}C]alanine content of the isolated cell pellets is expressed as the intracellular-to-extracellular distribution ratio. Each point represents the mean of four experiments in different cell preparations, with bars indicating ± S.E. when greater than extension of the point. Inset: magnified presentation of the initial time courses, and (with open symbols) the results from a single parallel experiment in Na^+-free buffer in the presence and absence of gramicidin D.

firmed (data not shown).

Fig. 3 and Table I show the major effects of gramicidin D on alanine uptake with the extracellular concentrations of alanine and Na^+ at 0.1 mM and 135 mM. In control cells the uptake of alanine was linear with time for about 120 s (Fig. 3), and within 20–40 min a steady state of the intracellular-to-extracellular alanine distribution ratio close to 20 was attained (Table I). This is in agreement with previous reports [3,5]. With gramicidin D present, the uptake of alanine was linear with time for only about 20 s (Fig. 3, inset), and the steady-state distribution ratio, which was reached within 2 or 3 min, did not exceed 1 (i.e. one-twentieth of the control value). As shown in Table I, gramicidin D reduced the influx of alanine to about one-third of the control value.

The observation that the steady-state distribution ratio of alanine was attained more rapidly in the presence of gramicidin D and was decreased more strongly than the alanine influx suggested that the kinetics of alanine efflux was also affected by gramicidin D. As shown in Fig. 4 and Table I, the rate constant of the efflux was increased about 9-fold in the presence of gramicidin D. It should be noted that the influxes of alanine and the rate constants of alanine efflux were the same under steady-state conditions as under net flux conditions. Thus, in the case of alanine efflux from gramicidin D-treated cells, rate constants (min^{-1}) were (i) 1.27 ± 0.10 (S.E., $n = 4$) when measured under net flux conditions with an initial intracellular alanine concentration of 2 mM, (ii) 1.14 ± 0.18 (S.E., $n = 3$) under equilibrium exchange conditions with 2 mM alanine, and (iii) 1.27 ± 0.10 (S.E., $n = 3$) under equilibrium exchange condi-

TABLE I

EFFECTS OF GRAMICIDIN D AND OUABAIN ON INTRACELLULAR-TO-EXTRACELLULAR $^{22}Na^+$ DISTRIBUTION RATIO AND ALANINE TRANSPORT ACROSS THE CELL MEMBRANE IN ISOLATED RAT HEPATOCYTES AT EXTRACELLULAR CONCENTRATIONS OF 0.1 mM ALANINE AND 135 mM Na^+

Isolated rat hepatocytes were incubated in the absence or presence of either gramicidin D (25 μg per ml for 5 min) or ouabain (1 mM, 2 h). The steady state distribution ratios of $^{22}Na^+$ and [^{14}C]alanine were measured as described under Materials and Methods. Influxes of alanine were calculated from the initial linear slopes in experiments similar to those shown in Fig. 3. Rate constants of alanine efflux were obtained from experiments similar to those in Fig. 4. Values are given as means ± S.E. of 3–5 measurements in different cell preparations.

	Steady-state $^{22}Na_i^+/^{22}Na_o^+$	Steady-state $[Ala]_i/[Ala]_o$	Alanine influx (mmol·min^{-1}·(l cell water)$^{-1}$)	Rate constant of alanine efflux
Control	0.14 ± 0.02	20.2 ± 0.9	0.25 ± 0.03	0.14 ± 0.05
Gramicidin D	0.94 ± 0.05	0.9 ± 0.1	0.09 ± 0.01	1.27 ± 0.10
Ouabain	0.63 ± 0.05	2.5 ± 0.4	0.17 ± 0.04	0.82 ± 0.10

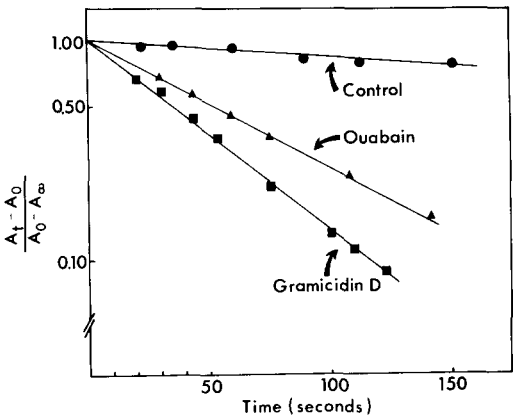

Fig. 4. Effects of gramicidin D and ouabain on alanine efflux from rat hepatocytes. The cells were incubated in a buffer containing 135 mM Na^+ and were used for efflux determination, either as control cells, or after exposure to gramicidin D (25 μg per ml) for five min, or exposure to ouabain (1 mM) for 2 h. After preloading with [^{14}C]alanine to a steady state at an extracellular concentration of 0.1 mM, the efflux was initiated at zero time by resuspension of the cells in radioisotope-free buffer. At the times indicated, the cellular ^{14}C radioactivity (A_t) was determined. Data are plotted according to the equation $A_t = (A_0 - A_\infty)\exp(-kt) + A_\infty$, which describes an exponential decrease of the radioactivity from its value (A_0) at time 0 to a new lower equilibrium value (A_∞) at infinite time. A_∞ was measured as cellular ^{14}C radioactivity after at least six half-times. The results are from single representative experiments.

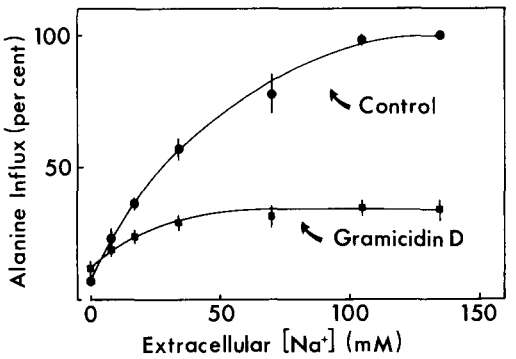

Fig. 5. Relationship between extracellular Na^+ concentration and alanine influx in the absence or presence of gramicidin D. Na^+ concentrations from 0 to 135 mM were obtained by substitution of Na^+ with choline. The influx of alanine was measured with [^{14}C]alanine at an extracellular concentration of 0.1 mM, and the estimate was made from three or four samples obtained during the initial 20 s of incubation. Alanine influx was expressed as per cent of the result in control cells at 135 mM Na^+. Each point represents the mean of three experiments in different cell preparations, with bars indicating ±S.E. when greater than extension of the point.

tions with 0.1 mM alanine. Besides confirming that alanine does not exert appreciable trans-effects [5], these results show that the rate constant of the efflux could be considered independent of the intracellular alanine concentration within the range employed in the study. From the results so far one may conclude that elimination of the Na^+ gradient by gramicidin D also eliminates the ability of hepatocytes to concentrate alanine above the extracellular level. Moreover, the gramicidin D-induced decrease in the steady state distribution ratio of alanine from 20.2 to 0.9 can be kinetically accounted for by a decrease in the influx to about one third of the control value and an increase in the rate constant of the efflux by a factor of about 9.

Fig. 5 shows that the alanine influx was dependent on Na^+ both in the absence and presence of gramicidin D. With 0.1 mM alanine in the medium the apparent K_m for Na^+ was about 38 mM in control cells and about 16 mM in gramicidin D-treated cells. Thus, the inhibition of alanine influx by gramicidin D is not due to a decrease in the affinity of the alanine transport system(s) for Na^+. The effect of gramicidin D on the kinetic parameters of alanine influx was studied at 135 mM Na^+ in three experiments where extracellular alanine concentrations from 0.05 to 50 mM were employed (Fig. 6). Computerized analysis of these data by a method described previously [5] showed that the non-saturable component of alanine influx was uninfluenced by gramicidin D. In contrast, the apparent K_m of the saturable component, which is Na^+-dependent [5], was increased from 2.20 ± 0.59 mM (S.D.) in control cells to 3.78 ± 0.59 mM (S.D.) in gramicidin D-treated cells ($P < 0.02$, t-test). There was also a decrease in the apparent V_{max} from 5.43 ± 1.34 to 4.18 ± 0.68 mmol/l cell water per min, but this difference did not reach statistical significance ($0.3 > P > 0.2$, t-test). In the present type of experiments there was a decrease in the electrical potential difference as well as an increase in cellular Na^+ concentration. Therefore, it was not possible to separate the effects of the two perturbations on the kinetic parameters of alanine influx.

To ensure that the effects of gramicidin D on

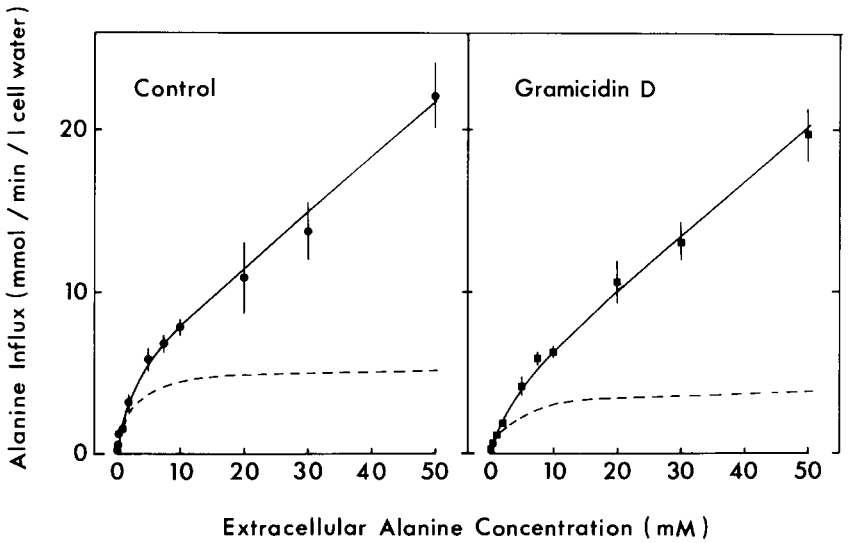

Fig. 6. Effect of gramicidin D on the relationship between alanine influx and extracellular alanine concentration in the presence of 135 mM Na^+. In the three cell preparations the influxes of alanine were measured in control cells (left panel) and in gramicidin D-treated cells (right panel) at extracellular alanine concentrations from 0.05 to 50 mM. Each influx measurement was based on three or four samples obtained during the initial 20 s of incubation. The results are given as means ± S.E.. The weighted data were analyzed by direct computerized fitting as described previously [5]. The influx of alanine was assumed to consist of a passive component and a single Michaelis-Menten component, and the best fit of the inferred equation to the data is shown as a solid line. The broken line gives the Michaelis-Menten component. Equations of the curves are: ●——●, Influx = 0.331 [Ala]+5.43 [Ala]/(2.20+[Ala]). ■——■, Influx = 0.326 [Ala]+4.18 [Ala]/(3.78+[Ala]).

the transport of alanine reflected changes induced in the Na^+ electrochemical gradient (rather than non-specific effects of the ionophore) additional experiments were performed with ouabain as a tool to change the gradient. Treatment of hepatocytes with ouabain for 1–2 h is accompanied by changes in both components of the Na^+ electrochemical gradient similar to those observed with gramicidin D, albeit not of the same magnitude (Table I and Ref. 12). The effects of ouabain on the transport of alanine were also somewhat smaller than, but analogous to those of gramicidin D (Table I and Fig. 4). Thus, ouabain decreased the steady-state alanine distribution ratio with a factor of 8 and this appeared to represent the net effect of a decrease in the influx to about two thirds of the control value and a 6-fold increase in the rate constant of alanine efflux.

The conspicious effect of gramicidin D (and of ouabain) on alanine efflux indicated that this process might depend on the intracellular Na^+ concentration and/or the cell membrane potential. In order to relate the rate constant of alanine efflux to the intracellular Na^+ concentration, rate con-

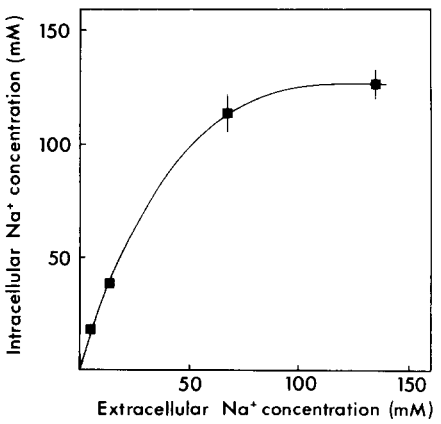

Fig. 7. Relationship between intracellular and extracellular Na^+ concentrations in gramicidin D-treated cells. Different extracellular Na^+ concentrations were obtained by substitution of Na^+ with choline. The intracellular Na^+ concentration was calculated from the intra- to extracellular $^{22}Na^+$ distribution ratio measured at steady state after about 6 min of incubation with the isotope. Each point represents the mean of 3–5 experiments in different cell preparations, with bars indicating ± S.E. when greater than extension of the point.

stants were measured in gramicidin D-treated cells at different extracellular Na^+ concentrations (135, 65, 14, 4.5, and 0 mM). The corresponding intracellular Na^+ concentrations (Fig. 7) were used to plot the efflux rate constants as shown in Fig. 8. The results seem to indicate saturation kinetics of alanine efflux with an apparent K_m for Na^+ at about 30 mM. It is questionable, however, whether the cell membrane potential remained unchanged near zero in these experiments, because the intra-to-extracellular distribution ratio of Na^+ rose above unity and approached a value of 4 when the extracellular Na^+ concentration was lowered towards 4.5 mM (Fig. 7). Assuming that sodium ions are distributed close to electrochemical equilibrium in gramicidin D-treated cells, the intracellular electrical potential should be approaching −40 mV in the above-mentioned situation. Therefore, if intracellular negativity does inhibit alanine efflux, the stimulatory effect of intracellular Na^+ shown in Fig. 8 may be underestimated.

Attempts were made to clarify whether the rate constant of alanine efflux was dependent on the cell membrane potential. It was recognized that

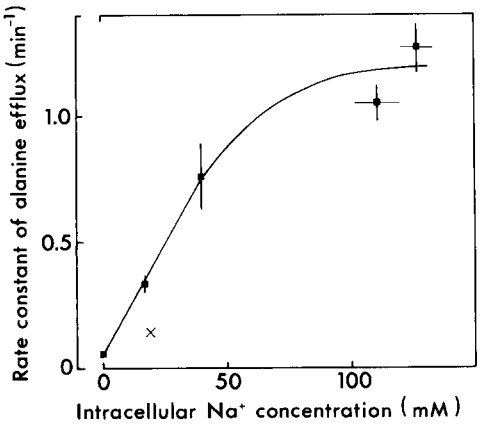

Fig. 8. Relationship between the rate constant of alanine efflux and intracellular Na^+ concentration in gramicidin D-treated cells. Rate constants of alanine efflux were measured under net flux conditions with an initial intracellular alanine concentration of about 2 mM in experiments similar to those in Fig. 4. Intracellular Na^+ concentrations were those obtained in the experiments of Fig. 7. Each point represents the mean of 3–5 experiments in different cell preparations, with bars indicating ±S.E. when greater than extension of the point. The point, ×, at (19 mM, 0.14 min^{-1}) indicates the situation in control cells at an extracellular Na^+ concentration of 135 mM (Table I).

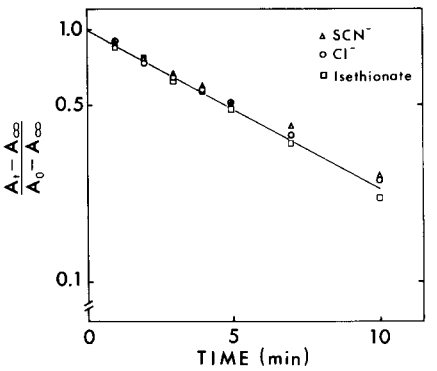

Fig. 9. Alanine efflux from rat hepatocytes in the presence of different extracellular anions. The cells were preloaded with [^{14}C]alanine in the normal Krebs-Ringer bicarbonate buffer. The efflux was initiated by resuspension in amino acid-free buffers of the following ion-compositions: (○) unchanged Krebs-Ringer biocarbonate buffer (117.2 mM Cl^-), (△) thiocyanate buffer (110 mM SCN^-, 7.2 mM Cl^-), and (□) isethionate buffer (110 mM isethionate, 7.2 mM Cl^-). The results are expressed as described in the legend of Fig. 4. The data are from single representative experiments.

use of a valinomycin-induced K^+ diffusion potential (hyperpolarization) was unsuitable to settle this point since the cellular ATP concentration decreased to near zero [3] implying shortage of substrate for the Na^+/K^+ pump and therefore an increase in the cytosolic Na^+ concentration. This sequence of events may explain that valinomycin slightly increased the rate constant of alanine efflux (data not shown) – a result that obviously also speaks against a marked inhibitory effect of the intracellular negativity. In other experiments the hepatocytes were preloaded with alanine, and the efflux was initiated by resuspending the cells in buffers where 110 mM Cl^- in the Krebs-Ringer bicarbonate buffer had been substituted by either SCN^- or isethionate with the purpose to induce different anion diffusion potentials. However, no differences in the rate constant of alanine efflux were detectable between the different media (Fig. 9).

Discussion

In the present study we have attempted to specify the importance of the transmembrane Na^+ electrochemical gradient for the energetics and kinetics of alanine transport in rat hepatocytes.

After elimination of the Na^+ gradient with gramicidin D (and no appreciable ATP depletion), a rapid equilibration of alanine across the cell membrane occurred. In pigeon erythrocytes [13] and Ehrlich cells [14,15], gramicidin D likewise eliminated concentrative amino acid uptake (but caused marked ATP depletion). In contrast, after elimination of the Na^+ gradient in Ehrlich cells with nigericin [16], another cation ionophore, concentrative amino acid uptake remained present and could be enhanced by pyruvate and lactate, which also increased the ATP level. On the basis, coupling of Na^+-dependent amino acid transport to an unrecognized energy source related to cellular ATP ws suggested [16]. However, the present results together with previous evidence in hepatocytes [3,5] imply no need to assume other energy sources than the Na^+ electrochemical gradient to explain the asymmetric steady-state distribution of alanine.

The Na^+ gradient was shown to exert its influence as the driving force for net accumulation of alanine by affecting the kinetics of the influx as well as the efflux of alanine. At a low extracellular alanine concentration, where the distribution ratio of alanine is maximal [5], elimination of the Na^+ electrochemical gradient decreased the alanine influx by a factor of 3 (Table I and Figs. 3 and 5). The role of Na^+ as a cosubstrate for the transport was illustrated by the finding that the alanine influx could be further reduced to about 10% of the control value by decreasing the Na^+ concentration towards zero (Fig. 5). Simple formulations of the kinetics of cotransport systems for Na^+ and amino acids or sugars [17–19] consider the Na^+-coupled influx of such substrates to depend on the extracellular Na^+ concentration and the intracellular negativity. According to this model, the observed difference between alanine influxes in control cells and gramicidin D-treated cells at an extracellular Na^+ concentration of 135 mM should be ascribed exclusively to the difference in cell membrane potential. It is a question, however, whether intracellular Na^+ might contribute by inhibiting Na^+-coupled influx of substrates. This was not observed in the case of Na^+-dependent 3-*O*-methylglucose influx in isolated intestinal cells [17]. On the other hand, Aronson [20] showed in isolated renal microvillus membranes that the rate of phlorizin binding (reflecting the binding of glucose to its Na^+-dependent transporter) was decreased by intravesicular Na^+. Anyway, the sensitivity of the alanine influx to changes in the Na^+ electrochemical gradient supports the idea [21–23] that different types of substrates and different amino acids might compete, not only for common transport systems, but also (via changes in cell membrane potential or intracellular Na^+ concentration) for the common driving force.

The rate constant of alanine efflux was influenced to an even greater degree than the influx when the Na^+ gradient was eliminated. These data confirm and extend early investigations in Ehrlich cells [14,15] and in lysed and restored pigeon red cells [24] where the glycine efflux was shown to depend on the intracellular Na^+ concentration. The results of Fig. 8 show the strong dependency of the efflux rate constant on the intracellular Na^+ concentration within the physiological range. It should be noted, however, that in control cells with an intracellular Na^+ concentration of 0.14×135 mM = 19 mM (Table I), the rate constant of alanine efflux was clearly lower than in gramicidin D-treated cells with the same intracellular Na^+ concentration (Fig. 8). An explanation for this difference might be that measurements of the $^{22}Na^+$ distribution ratio in control cells overestimate the cytosolic Na^+ concentration due to nuclear sequestration of Na^+ [25,26]. Such sequestration is probably eliminated by gramicidin D resulting in a more valid estimation of cytosolic Na^+ concentrations. It is interesting that a rate constant of alanine efflux similar to that in control cells was obtained in gramicidin D-treated cells with an intracellular Na^+ concentration (6 mM, Fig. 8) close to the intracellular Na^+ activity measured with Na^+-specific microelectrodes in the perfused rat liver [27]. As noted under Results it appears reasonable to believe that, as in control cells, the cell membrane potential in gramicidin D-treated cells with an intracellular Na^+ concentration of 15–20 mM lies between -40 and -30 mV. This contention is in fair agreement with fluorescent dye measurements of the cell membrane potential in nigericin-treated Ehrlich cells at low extracellular Na^+ concentrations [16]. The above considerations lead to the conclusion that the rate constant of alanine efflux

in control cells can be accounted for predominantly by a low cytosolic Na^+ concentration, with little room left for an inhibitory effect of intracellular negativity. It is consistent with this view that voltage-sensitive alanine efflux could not be demonstrated in the present study. This observation might be important for the consideration of different models of Na^+-coupled solute transport and deserves more detailed investigation. For this purpose, the use of plasma membrane vesicles with different orientations (right-side-out and inside-out) might be advantageous [28].

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