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Differential effect of extracellular calcium on the Na^+-K^+ pump activity in intact polymorphonuclear leucocytes and erythrocytes

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The effect of extracellular calcium on the Na^+-K^+ pump activity in human polymorphonuclear leucocytes and erythrocytes was studied and compared with the activity in mixed peritoneal leucocytes from rats. While there was maximal decrease in the pump activity (25–30%) of leucocytes from both rat and human by calcium 0.6 mM, a concentration of 0.1 mM caused a substantial decrease indicating a high sensitivity for extracellular calcium. In contrast, calcium had no effect on the pump activity in erythrocytes. The effect of calcium on the pump activity in leucocytes may be due to regulation of the influx of sodium across the plasma membrane, since in human leucocytes calcium had no effect on the pump activity if the cells were loaded with sodium.

Na^+-K^+ pump; Na^+,K^+-ATPase; Calcium sodium; Potassium; Leucocyte; Erythrocyte

1. INTRODUCTION

Previously, evidence has been provided that extracellular calcium inhibits the activity of the Na^+-K^+ pump in intact rat peritoneal mast cells [1,2]. The aim of this investigation was to study if regulation of the Na^+-K^+ pump activity by extracellular calcium may be a more general phenomenon. The effect of extracellular calcium on the pump activity was determined in human polymorphonuclear leucocytes and erythrocytes and mixed peritoneal leucocytes.

2. MATERIALS AND METHODS

2.1. *Isolation of rat peritoneal leucocytes*

Two male Sprague-Dawley rats, 330–380 g, were used for each experiment. The rats were killed by decapitation under light ether anaesthesia. Peritoneal leucocytes were isolated by differential centrifugation of mixed peritoneal cells in a selfgenerating gradient of Percoll as described previously [1]. The leucocytes were concentrated in the upper part of the gradient. The cells were harvested by aspiration into a syringe and washed twice by centrifugation (160 × *g* for 10 min) and resuspension in order to remove the remaining Percoll. Then the leucocytes were suspended in a calcium-free Krebs-Ringer solution for the experiments. The whole isolation procedure was performed at 4°C. The purity of the leucocyte suspensions was determined by inspection or smears stained with Toluidine blue. The leucocytes constituted 97–99% (98.5 ± 0.5%, mean and SE, $n = 6$) of the cell population.

2.2. *Isolation of human erytrocytes*

Whole venous blood (10 ml) from 5 healthy males, age 23–32 anticoagulated with heparin (50 IE/ml) was diluted with 10 ml of a calcium-free Krebs-Ringer solution. After centrifugation, 160 × *g* for 10 min, at 4°C the supernatant was discarded and the cells were washed 3 times by centrifugation (300 × *g*, 15 min, 4°C) and resuspension. The cells were then suspended in a calcium-free Krebs-Ringer solution for the experiments. The erythrocytes constitute 99.3–100% (99.8 ± 0.3%, mean and SE, $n = 5$) of the cell suspension.

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2.3. *Isolation of human polymorphonuclear leucocytes*

Suspensions of intact polymorphonuclear leucocytes were prepared from whole venous blood by centrifugation on a discontinuous density gradient of Percoll. Whole venous blood (30 ml) from 5 healthy males, age 23–32 anticoagulated by heparin (50 IE/ml) was diluted with an equal volume of calcium-free Krebs-Ringer solution, and mixed with dextran (M_w 500 000 Da, final concentration 1.2% w/v). This solution was left for 30 min at room temperature to allow sedimentation of the erytrocytes.

The supernatant was removed and centrifuged at 140 × *g* for 10 min at 4°C. Then the cell pellet was resuspended in a small volume of Krebs-Ringer solution, layered onto a discontinuous density gradient of Percoll and centrifuged at 300 × *g* for 30 min at 4°C. The gradient containing two layers of Percoll (1.06 and 1.08 g/ml) retained thrombocytes and mononuclear leucocytes and allowed polymorphonuclear leucocytes and the remaining erythrocytes to pass. These were removed by a mild hypotonic lysis, i.e. 10 s exposure to distilled water followed by adjustment for isotonicity to reappear. The polymorphonuclear leucocytes were washed once (centrifugation at 140 × *g*, 10 min, 4°C), layered onto a second gradient of Percoll (1.08 g/ml) and centrifuged (300 × *g*, 15 min, 4°C) in order to remove the residual cell debris. Then the cells were washed twice by centrifugation (140 × *g*, 10 min, 4°C) and resuspension. The isolation procedure was performed with a calcium-free Krebs-Ringer solution in the dose-response experiment (Fig. 2) and a calcium-free, potassium-free Krebs-Ringer solution in the Na^+-loading experiment (Fig. 4). The purity of the final cell suspension was tested by inspecting smears stained with Hemacolor. The polymorphonuclear leucocytes constituted 96.8%–98.5% (97.5 ± 0.3%, mean and SE, $n = 5$) in the dose-response experiment and 97.5–98.3% (97.8 ± 0.3%, mean and SE, $n = 5$) in the Na^+-loading experiment.

2.4. *Incubation procedure*

Samples of cells having the same cell density in a final volume of 0.5 ml were preincubated at 37°C for 5–60 min with various combinations

of calcium, potassium and ouabain. A coulter counter (Model 134, Analys Instrument AB, Sweden) was used to count the number of cells. The number of rat leucocytes varied between 6.75×10^5 and 8.26×10^6, human polymorphonuclear leucocytes between 8.90×10^5 and 4.08×10^6 and human erytrocytes between 2.65×10^8 and 3.55×10^8. For further details concerning preincubation and incubation conditions, see legends to figures.

2.5. *Measurement of Na^+-K^+ pump activity*

For determination of cellular potassium-uptake (K^+ ($^{86}Rb^+$)-uptake), 100 μl of a solution, containing, in addition to potassium, trace amounts of $^{86}Rb^+$, were added to the samples. The radioactive concentration during the incubation was mean 1.5 μCi/ml (1.2–2.2 μCi/ml). The incubation with the K^+ ($^{86}Rb^+$)-solution lasted for 2–10 min and was terminated by the addition of 9.5 ml ice-chilled Krebs-Ringer solution. The cells were washed twice by centrifugation at 300 × g for 15 min at 4°C followed by the addition of 0.5 ml of an aqueous solution of NaOH (47.6 mM) to the samples. The whole content of each sample was then transferred to a scintillation vial and mixed with Ecoscint. Cellular K^+($^{86}Rb^+$)-uptake was measured in a Mark III Liquid Scintillation Spectrometer (Nuclear Chicago) using the preset window for ^{32}P. The specific activity of potassium in the extracellular medium was used to calculate the cellular uptake of K^+ ($^{86}Rb^+$).

2.6. *Presentation of data*

Statistical analysis was performed by use of Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant. Data are presented as mean ± standard error of the mean.

2.7. *Solutions*

The calcium-free Krebs-Ringer solution had the following composition (mM): NaCl 136.8, KCl 4.75, $MgSO_4$ 1.2, Tris-HCl 12.5. When the concentrations of calcium and potassium varied the concentration of sodium was varied accordingly in order to maintain isotonicity. All solutions contained bovine serum albumin, 1 mg/ml, and glucose, 1 mg/ml. The pH was 7.4 (room temperature).

2.8. *Materials*

Bovine serum albumin was supplied by Sigma (St. Louis, USA), Hemacolor and glucose by E. Merck (Darmstadt, W.Germany), Dextran and Percoll by Pharmacia Fine Chemicals (Sweden), Ecoscint by BN Plastics (Helsinge, Denmark) and $^{86}Rb^+$ by Amersham (Buckinghamshire, UK). The radioactive rubidium was always used within 3 months of manufacture in order to minimize complications resulting from the progressive increase in $^{134}Cs^+$ relative to $^{86}Rb^+$. Ouabain (Mecobenzon, Denmark) and all other chemicals were of analytical grade.

3. RESULTS

The Na^+-K^+ pump activity of intact peritoneal leucocytes from rats was inhibited by extracellular calcium. Maximal inhibition (30%) was observed with calcium 0.6 mM ($P < 0.05$), and there was an inhibition of 25% by calcium 0.1 mM ($P < 0.05$). (Fig. 1). A similar dose-dependent inhibition in the pump activity by calcium was found in intact human polymorphonuclear leucocytes (Fig. 2). Calcium 0.6 mM caused maximal inhibition (25%, $P < 0.05$). However, a substantial inhibition (20%) could be observed in the presence of 0.1 mM calcium although the level of significance was not attained ($P < 0.1$). The ouabain-resistant uptake of K^+ ($^{86}Rb^+$) in the absence of calcium was 203 ± 9 and 29 ± 7 pmol/10^6 cells/min in

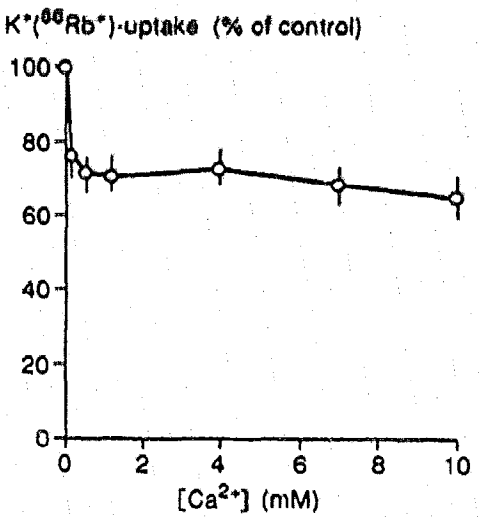

Fig. 1. Dose-response effect of extracellular calcium on the Na^+-K^+ pump activity in intact peritoneal leucocytes from rats. The cells were preincubated in a calcium-free medium for 60 min at 37°C in the presence of potassium, 4.75 mM. The preincubation continued without or with ouabain 1 mM for 15 min. Then calcium, 0.1–10 mM was added (abscissa) for another 15 min before determination of the Na^+-K^+ pump activity by incubation with K^+ ($^{86}Rb^+$) for 5 min. Ordinate scale: the activity of the Na^+-K^+ pump in percent of control value from cells incubated without calcium: 451 ± 49 pmol/10^6 cells/min. The pump activity was calculated as the total uptake of K^+ ($^{86}Rb^+$) into the cells corrected for the ouabain-resistant uptake. Mean value from 6 experiments; vertical lines show SE.

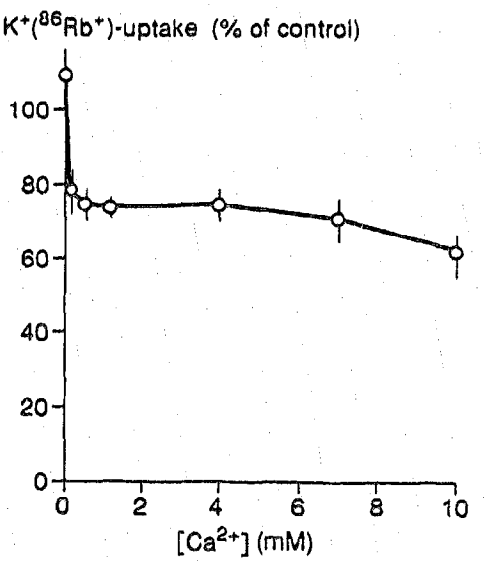

Fig. 2. Dose-response effect of extracellular calcium on the Na^+-K^+ pump activity in intact human polymorphonuclear leucocytes. The cells were preincubated in a calcium-free medium for 60 min at 37°C in the presence of potassium, 4.75 mM. The preincubation continued without or with ouabain 10 μM for 15 min. Then calcium, 0.1–10 mM was added (abscissa) for another 15 min before determination of the Na^+-K^+ pump activity by incubation with K^+ ($^{86}Rb^+$) for 10 min. Ordinate scale: the activity of the Na^+-K^+ pump in percent of control value from cells incubated without calcium: 109 ± 8 pmol/10^6 cells/min. The pump activity was calculated as the total uptake of K^+ ($^{86}Rb^+$) into the cells corrected for the ouabain-resistant uptake. Mean value from 5 experiments; vertical lines show SE.

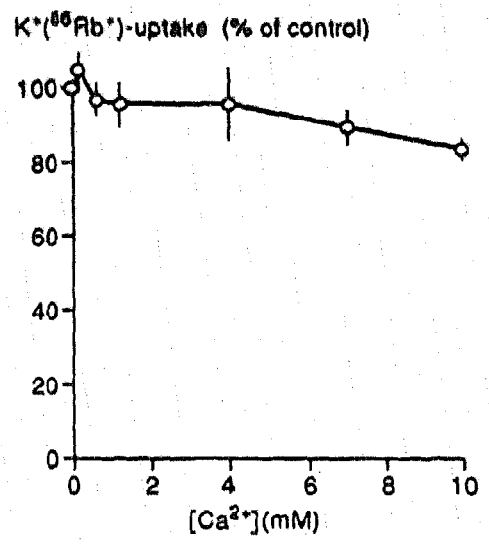

Fig. 3. Dose-response effect of extracellular calcium on the Na^+-K^+ pump activity in intact human erythrocytes. The cells were preincubated in a calcium-free medium for 60 min at 37°C in the presence of potassium, 4.75 mM. The preincubation continued without or with ouabain 10 μM for 15 min. Then calcium, 0.1–10 mM was added (abscissa) for another 15 min before determination of the Na^+-K^+ pump activity by incubation with K^+ ($^{86}Rb^+$) for 5 min. Ordinate scale: the activity of the Na^+-K^+ pump in percent of control value from cells incubated without calcium: 1335 ± 74 pmol/10^9 cells/min. The pump activity was calculated as the total uptake of K^+ ($^{86}Rb^+$) into the cells corrected for the ouabain-resistant uptake. Mean value from 5 experiments; vertical lines show SE.

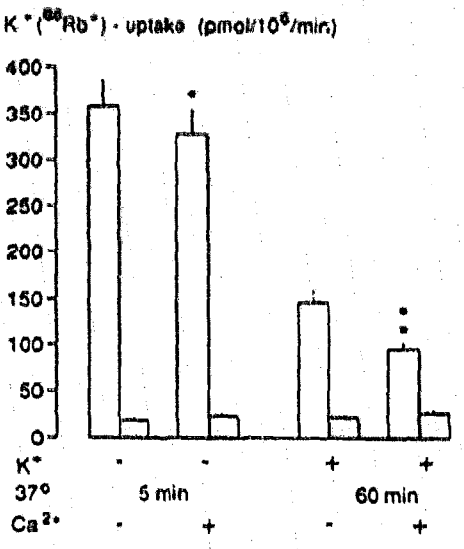

Fig. 4. Reversal of the pump inhibition due to calcium by sodium loading of intact human polymorphonuclear leucocytes. The cells were sodium loaded due to the isolation procedure at 4°C (potassium-free medium). After temperature equilibration at 37°C for 5 min in a potassium-free medium in the absence or presence of calcium, 1.2 mM (abscissa), the activity of the pump was determined by incubation of the cells for 2 min with potassium, 4.75 mM, using $^{86}Rb^+$ as a tracer. Control samples were incubated in the presence of potassium, 4.75 mM and in the absence or presence of calcium, 1.2 mM for 60 min before determination of the pump activity by incubation with K^+ ($^{86}Rb^+$) for 10 min. The ouabain-resistant K^+ ($^{86}Rb^+$)-uptake was determined in samples incubated in parallel with ouabain, 10 μM throughout the incubation at 37°C. The pump activity was calculated by correction of the total uptake of K^+ ($^{86}Rb^+$) by the ouabain-resistant, * $P>0.1$, ** $P<0.05$.

the rat and human leucocytes, respectively. These values were not significantly different from the uptake by cells incubated in the presence of calcium (0.1–10 mM) ($P>0.1$ or higher). In contrast, calcium did not inhibit the pump activity in intact human erythrocytes ($P>0.1$ or higher) when comparing the uptake in a calcium-free medium (1335 ± 74 pmol/10^9 cells/min) with the uptake in the presence of calcium (0.1–10 mM) (Fig. 3). As observed with the leucocytes calcium did not change the ouabain-resistant uptake of K^+ ($^{86}Rb^+$) into human erythrocytes ($P>0.1$ or higher, control value in calcium-free medium: 689 ± 70 pmol/10^9 cells/min).

The ouabain-sensitive K^+($^{86}Rb^+$)-uptake by intact human leucocytes was 358 ± 30 and 328 ± 30 pmol/10^6 cells/min after 5 min temperature equilibration at 37°C in a potassium-free medium in the absence and presence of calcium (1.2 mM), respectively. The values were not significantly different ($P>0.1$). After 60 min incubation in the presence of potassium but without calcium the uptake was 146 ± 14 pmol/10^6 cells/min. If calcium (1.2 mM) was present in addition to potassium the ouabain-sensitive uptake was decreased significantly to 96 ± 5 pmol/10^6 cells/min ($P<0.01$). The ouabain-resistant uptake was 18.7–26.5 pmol/10^6 cells/min, and this was not influenced by the various combinations of potassium and calcium.

4. DISCUSSION

This investigation demonstrates that the activity of the Na^+-K^+ pump of human polymorphonuclear leucocytes and mixed peritoneal leucocytes from rat is sensitive to the extracellular concentration of calcium. In both cases almost maximal inhibition of the pump activity was observed with 0.1 mM calcium. In contrast, the pump activity in human erythrocytes was insensitive to extracellular calcium unless in high concentration beyond the physiological range. A sensitivity to extracellular calcium similar to our observations with the leucocytes was reported for the Na^+-K^+ pump activity in rat peritoneal mast cells [1]. Inhibition by extracellular calcium of the Na^+-K^+ pump activity in isolated myocytes from guinea pigs have also been reported [3], and omission of extracellular calcium increased the ouabain-sensitive uptake of $^{86}Rb^+$ into cultured rat vascular smooth muscle cells [4].

Isolation of the cells was performed at 4°C, and at this temperature the pump activity is considered to be blocked allowing accumulation of sodium to occur inside the cells. This isolation procedure is responsible for the high level of pump activity found in the human

polymorphonuclear leucocytes after the temperature equilibration (Fig. 4). Incubation of the cells with potassium for 60 min reduces the pump activity considerably due to transport of intracellular sodium to the extracellular space, and this decreases the stimulation of the pump from inside the cell. The significant difference in pump activity in the presence and absence of calcium may be due to differences in the permeability of the plasma membrane to sodium. The presence of calcium in the medium seemed to decrease the permeability and thus to decrease the stimulation of the pump by sodium. This view is supported by the observation that calcium did not change the pump activity when the cells were loaded with sodium (Fig. 4). Similarly, in rat peritoneal mast cells extracellular calcium seemed to regulate the permeability of the plasma membrane to sodium [2]. Since in these cells the activity of the ouabain-sensitive Na^+-K^+ pump is mainly regulated through stimulation of the pump from inside the plasma membrane by sodium [1,2,5], extracellular calcium has a major influence on the pump activity. This mechanism is possibly also relevant concerning the regulation of the pump activity in the leucocytes. However, extracellular calcium may be less important for the regulation of the pump activity in leucocytes than in rat mast cells due to the fact that the maximal pump inhibition by calcium in mast cells was about 80%, while the maximal inhibition in the leucocytes was 25% and 30%.

In addition to our observations above on leucocytes and rat mast cells data in the literature support the view that extracellular calcium may participate more generally in regulation of the permeability of plasma membranes. Increased sodium permeability of the plasma membrane upon removal of extracellular calcium has been observed in amphibian oocyte [6], and in a calcium- and magnesium-deficient medium the permeability of the plasma membrane to monovalent cations increased [7]. Increased turnover rate of the Na^+-K^+ pump of isolated myocytes from guinea pigs in the absence of calcium is likely to be caused by an increased stimulation of the pump due to an increased sodium permeability of the plasma membrane [3]. The omission of extracellular calcium increased the intracellular concentration of sodium in cultured rat vascular smooth muscle cells [4]. Evidence for increased sodium permeability of rat heart myocytes due to a decrease of calcium in the medium was provided by Hohl et al. [8]. A decreased sodium permeability measured as decreased $^{22}NaCl$ efflux due to extracellular calcium in the micromolar to millimolar range has been observed in outside out orientated plasma membrane vesicles from cat pancreas [9].

In conclusion, we have provided evidence that calcium plays an important role in the regulation of the pump activity in leucocytes from man, and this is likely to be relevant also in leucocytes from rat. The mechanism of regulation is considered to be the same as that previously observed to affect the pump activity in rat mast cells. The present data and the data in the literature support the view that extracellular calcium may have a major influence on the sodium permeability of plasma membranes and hence their Na^+-K^+ pump activity. However, this effect of calcium does not include the human erythrocyte.

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