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INFLUENCE OF CALCIUM AND OUABAIN UPON THE POTASSIUM INFLUX IN HUMAN ERYTHROCYTES

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Abstract—The influence of calcium and ouabain upon the potassium influx was studied in human erythrocytes. Changing the Ca concentration of the suspension medium from 1·1 to 8·2 μEq/ml did not influence the K influx or the inhibitory action of ouabain ($1{\cdot}36 \times 10^{-5}$ M) on K influx. Potassium influx was inhibited, however, by increasing the Ca concentration within the erythrocytes from 0·7 to 8·4 μEq/ml cells. The inactivation of the K transport by ouabain was diminished by an increase of the Ca concentration in the erythrocytes. It seems justified to conclude that the site of action of Ca and of ouabain upon the K/Na transporting system is on the *inside* of the erythrocyte membrane.

THE system responsible for the uphill transport of K and Na is located at the cell membrane. The activity of this system depends on the outside concentration of K and is inhibited by cardiac glycosides.[1–8] The enzymatic activity of the ATP-splitting system which can be prepared from red cell ghosts shows a striking similarity, under various conditions, to the system transporting K and Na. It depends, in addition, on the presence of K and Na ions and can also be inhibited by cardiac glycosides.[9–11] The degree of this inhibition is determined by the concentration of Ca.[9]

If Ca were to act on the transport system of the intact red cells, variations of the Ca concentration should be able to influence the inhibitory action of cardiac glycosides on the uphill transport of K and Na. Moreover, it should be possible to localize the part of the transporting system that is sensitive to cardiac glycosides by changing the Ca concentration at the inner and outer sides of the membrane.

Some information concerning the site of action of the glycosides at the cell membrane may be obtained from the literature. Experiments carried out by Caldwell and Keynes[12] on the squid giant axon suggest that cardiac glycosides act upon the outside of the membrane. The possibility of an action at the inside of the membrane of the red blood cell has been discussed[13] but not without objection.[14]

After it became possible to vary stepwise the inside concentration of Ca within erythrocytes,[15] it seemed promising to study the influence of ouabain upon the K influx when the Ca concentration was varied inside as well as outside the erythrocyte membrane.

METHODS

All experiments were done with human blood, which was obtained by puncture of the cubital vein and then defibrinated by gently beating with a wooden stick. After centrifugation (3 min at 4,000 rev/min) the upper cell layer was sucked off in order to remove the leukocytes, and the erythrocytes were washed thrice with 10 to 15 times

their volume. The washing solution had the same composition as the solution in which the cells were finally suspended, in a proportion of 1:1·5.

The influence of the inside concentration of Ca upon the ^{42}K-influx

In these experiments erythrocytes containing between 0·7 and 8·2 μEq Ca/ml were prepared by preincubation at 3° for 24 hr in isotonic solutions of different Ca concentrations (isotonicity maintained by choline chloride; see Rummel *et al.*[15]) To reduce hemolysis resulting from preincubation in $CaCl_2$ solutions, the erythrocytes were washed twice with a hypotonic NaCl solution (0·6%) before the second incubation. During the second incubation at 37°, this procedure reduced hemolysis from 20 to 6% maximally by removing most of the prehemolytic erythrocytes. After two more washings with Tyrode's solution the erythrocytes were suspended in a proportion of 1:1·5 in Tyrode's solution. From each suspension (n = 16) two samples of 3 ml were incubated at 37°. To each sample were added 0·1 ml of an isotonic NaCl solution containing ^{42}K (corresponding to 40,000 counts/min) and 0·1 ml isotonic NaCl solution with 30 μg ouabain (corresponding to a ouabain concentration in the sample of 10 μg/ml or $1{\cdot}36 \times 10^{-5}$ M). The control samples were run without ouabain.

At the beginning of the incubation period the hematocrit values and the hemoglobin concentration of the suspended erythrocytes were determined. The rate of hemolysis was calculated from the hemoglobin concentration of the suspension fluid measured at the end of the incubation. The degree of hemolysis amounted at most to 6% in the preincubated and to less than 0·1% in the erythrocytes not preincubated.

The hematocrit value of the preincubated erythrocytes differed from that of the non-preincubated erythrocytes; preincubated, HK = 27·4 $\pm$ 3·10 (n = 16); non-preincubated, HK = 34·6 $\pm$ 0·11 (n = 16). The hemoglobin concentration, however, was the same: preincubated, 343 mg Hb/ml erythrocytes ($\pm$ 6·96; n = 16); non-preincubated, 349 mg Hb/ml erythrocytes ($\pm$ 3·27; n = 16). This indicated that a change of volume had taken place during the preincubation. The two experimental groups, therefore could only be compared by relating the flux rates to the hemoglobin content (given in grams). The flux rates were corrected for hemolysis.

After an incubation period of 1 hr, samples of 1 ml were withdrawn from each suspension. The erythrocytes were washed three times with 10 ml Tyrode's solution, and resuspended in 3 ml Tyrode's solution; 2 ml of each of these resuspensions were dried in plates (1-inch diameter) at 70°. The activity of the dried samples was measured with an end-window flow counter (Frieseke & Hoepfner, type FH 407*). In order to determine the total activity, 1 ml of the original suspension was mixed with 2 ml Tyrode's solution, and 2 ml of this mixture was dried in plates for measurement of the ^{42}K activity. The specific activity of the suspension fluid at the beginning of the experiment, calculated from the potassium content of the Tyrode's solution and the added ^{42}K activity, provided the basis for the calculation of the flux rates.

The influence of the outside concentration of Ca upon the ^{42}K-influx

Fresh erythrocytes were washed in Tyrode's solution. The erythrocytes of each of four blood samples were divided into four parts and were then resuspended in Tyrode's solutions containing the following Ca concentrations: 1·1, 2·6, 5·6, and 8·2 μEq/ml. Two samples of 3 ml each were taken from these suspensions and, after addition of

* We are indebted to Deutsche Forschungsgemeinschaft for lending us the counting equipment.

^{42}K and ouabain as indicated above, they were incubated at 37° for 1 hr, and the ^{42}K activity of the erythrocytes was measured.

Determination of lactate

In one experimental group the formation of lactate in erythrocytes, which had been preincubated in the cold, was compared with that of fresh, non-preincubated erythrocytes. For this purpose, erythrocytes which had been preincubated in isotonic solutions of choline chloride or $CaCl_2$ at 3° for 24 hr were suspended with fresh erythrocytes in Tyrode's solution. The lactate concentration in the suspension fluid was determined after an incubation period of 1 hr at 37°, according to the method of Barker and Summerson.[16] From this value and from the hematocrit, the amount of lactate produced by 1 ml erythrocytes during 1 hr was calculated.

At the end of the incubation period the pH-value was measured in all samples with a glass electrode; it was, on the average, 7·68 (± 0·07) in 64 single measurements.

RESULTS

Figure 1 shows the K influx, i.e. the uphill transport of K as a function of the Ca concentration within the erythrocytes. Ca itself inhibited the uphill transport of K

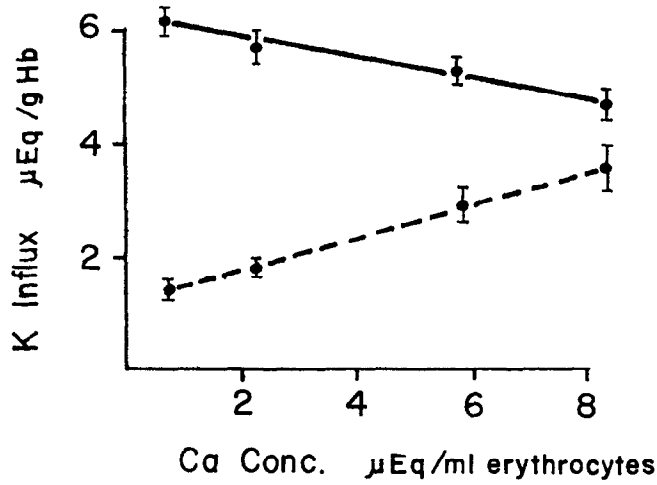

FIG. 1. Relationship between K influx and Ca concentration within human erythrocytes. Abscissa: concentration of Ca in erythrocytes; ordinate: influx of K per g hemoglobin during 1 hr at 37°. Solid line, control samples; broken line, samples with ouabain $1{\cdot}36 \times 10^{-5}$ M. Each point is the mean of four experiments. The vertical lines show the standard deviation.

increasingly with increasing concentrations. When the potassium influx amounts to 6·2 µEq/g Hb at a Ca concentration of 0·7 µEq/ml erythrocytes, it is already significantly reduced ($P < 0{\cdot}01$) to 5·7 µEq/g Hb at a concentration of 1·7 µEq Ca/ml erythrocytes—i.e. by 8%. An increase of the Ca concentration within the erythrocytes up to 8·4 µEq/ml reduced the K influx to 4·7 µEq/g Hb—i.e. by 24% of the value at the lowest concentration of Ca. Although the sodium concentration of these erythrocytes remained unchanged, the potassium concentration was reduced to about half since it had decreased during the previous incubation in isotonic $CaCl_2$ solution at 3°.[15] Thus the gradient against which potassium ions had moved was less steep in

erythrocytes with a calcium concentration of 8·4 μEq/ml than in erythrocytes with 0·7 μEq Ca/ml.

In order to find out whether the metabolic activity had been impaired by the incubation of the erythrocytes in isotonic $CaCl_2$ solution at 3°, the lactate production of preincubated erythrocytes was compared with that of fresh erythrocytes in Tyrode's solution at 37°. Table 1 shows the results of these experiments.

TABLE 1. COMPARISON OF LACTATE PRODUCTION BY PREINCUBATED AND NON-PREINCUBATED ERYTHROCYTES

Lactate production in μEq lactate produced by 1 ml erythrocytes during 1 hr at 37° in Tyrode's solution. Preincubation: 24 hr at 3°; SD = standard deviation; n = 12; pH 7·68 (± 0·06).

	Preincubated in isotonic: $CaCl_2$	Choline chloride	Non-incubated
Lactate (μEq/ml)	2·62	2·66	2·51
SD	±0·34	±0·20	±0·18

The lactate production of preincubated erythrocytes did not differ from that of the non-preincubated erythrocytes. On the one hand this means that the metabolic activity of the erythrocytes was not impaired by preincubation, and on the other hand that the glycolysis was not increased after considerable loss of potassium (erythrocytes incubated in isotonic $CaCl_2$ solution lost about 50% of their potassium.)

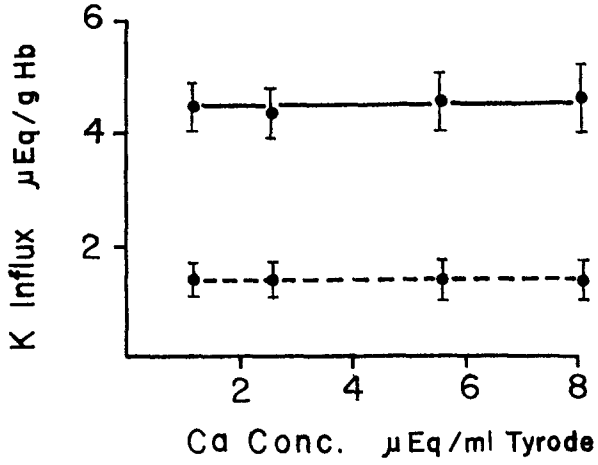

FIG. 2. Relationship between K influx and Ca concentration of the medium. Abscissa: Ca concentration of the medium; ordinate: K influx per g hemoglobin during 1 hr at 37°. Solid line, controls; broken line, samples with ouabain $1{\cdot}36 \times 10^{-5}$ M. Each point is the mean of four experiments; the vertical lines show the standard deviation.

Ouabain inhibited the uphill transport of K by 78% at a Ca concentration of 0·7 μEq/ml erythrocytes. However, with increasing concentrations of calcium within the erythrocytes, the inactivating effect of ouabain on the potassium transport disappeared more and more. At a Ca concentration of 8·4 μEq/ml erythrocytes it amounted to no more than 25%.

Figure 2 represents the potassium influx as a function of the calcium concentration in the medium. A change in the concentration outside was without effect on the K influx. The range of Ca concentrations investigated was the same as that found effective within the erythrocytes. The K influx was between 4·4 and 4·7 μEq/g Hb in the range investigated. Similarly, the inhibition of the uphill transport of K by ouabain was not influenced by a change of the Ca concentration within the suspension medium. The inhibition amounted to about 70% over the range studied.

DISCUSSION

The experiments showed that an increase up to 8·2 μEq/ml of the Ca concentration outside the cells had no influence upon the uphill transport of K or on the effect of ouabain on this transport. A similar observation has been made with erythrocytes incubated in the cold, in which case Ca concentrations up to 20 μEq/ml suspension fluid had been used.[17] Kahn,[18] however, has found that the K uptake at 37° was inhibited in erythrocytes, preincubated in the cold, by increasing the outside concentration of Ca up to 64 μEq/ml. A comparison of these results with ours is difficult because only net fluxes were measured, and data referring to the duration of the incubation in the cold were not given.

An increase of the calcium concentration inside the cells instead of outside led to a reduction of the potassium influx and diminished the inhibitory activity of ouabain upon the potassium transport (Fig. 1). It is conceivable that the potassium influx was influenced by a change in the K/Na ratio in the preincubated erythrocytes because it is known that with a high potassium concentration, small changes of the sodium concentration alter the potassium transport of erythrocytes as well as the K/Na-dependent ATPase activity. On the other hand, changes of the potassium concentration may greatly influence the K/Na-dependent ATPase activity.[9, 10] However, after incubation in the cold the sodium concentration of the erythrocytes remained within the normal range. The decrease of the inner concentration of potassium from 95 to 50 μEq/ml, which was measured after incubation of the erythrocytes in the cold, does not lie within the range in which changes of the activity of the K/Na-dependent ATPase system are to be expected. Thus it is improbable that a change of the K/Na ratio within the erythrocytes influenced the potassium influx.

Another factor to be discussed is the level of metabolic activity that could be changed by the preincubation in isotonic $CaCl_2$ solution in the cold, and which thus might influence potassium transport. The comparison of the lactate production in preincubated and not preincubated erythrocytes shows that the glycolytic activity was not changed by incubation in the cold. This also is interesting because an increased glycolytic activity might have been expected in connection with the reabsorption of potassium after a previous loss of about 50%.

It is well known that Ca inhibits the K/Na-activated ATPase system.[9, 10, 19, 20] Considering that there is a linkage between this ATPase system and the K/Na transporting system, one would have expected that Ca would inhibit the transport of K

and Na. As shown by our experiments an increase of the Ca concentration *within* the erythrocytes did indeed inhibit the uphill transport of potassium. Yet, increased concentrations of Ca in the *medium* had no effect upon the K influx. These results suggest that the site of action of Ca is at, or on the inside, of the erythrocyte membrane; in other words, that the part of the K/Na transporting system which is sensitive to Ca is located at the inside of the erythrocyte membrane.

Dunham and Glynn[9] studied the effect of strophanthin upon the ATPase system of human red cell ghosts under various Ca concentrations. They found that on the one hand the total ATPase activity was increased with Ca concentrations up to 0·3 mM. This Ca concentration, which is very close to the Ca concentration within erythrocytes (0· to 1·4 μEq/ml;[21] our own measurements by emissions-spectrography gave values of 0·28 $\pm$ 0·10 μEq/ml), had a maximal activating effect upon the total ATPase. A further increase of the Ca concentration resulted, however, in a decrease of the activating effect—i.e. a decrease of the ATPase activity. Dunham and Glynn demonstrated, on the other hand, that the part of the ATPase system which is sensitive to glycosides is diminished with increasing Ca concentrations. There is a quite similar response in the intact erythrocyte. As shown by Fig. 1, at a Ca concentration of 0·5–0·6 μEq/ml erythrocytes there was a 78% decrease of the potassium influx by ouabain; increasing the Ca concentration to 8·2 μEq/ml erythrocytes reduced the ouabain effect to 25%. This indicates that the sensitivity of the potassium pump to ouabain is diminished by increasing Ca concentration *inside* the erythrocytes. A change of the Ca concentration of the medium did not influence the action of ouabain upon the potassium influx.

The Ca concentrations that inhibited the effect of the glycoside in the ATPase preparation were lower than those used in our experiments on erythrocytes. The cause of this difference may be that the Ca concentration reported by Dunham and Glynn for their experiments with suspended erythrocyte ghosts was very close to the concentration of ionized calcium. In the erythrocytes, however, the total calcium is most likely not present in a free form; hence the effective calcium concentration will be lower than the measured one.

If we assume that the inhibitory action of ouabain consists in the displacement of calcium, as is indicated by the results of Lüllman and Holland[22] and Klaus and Kuschinsky[23] in the cardiac muscle, we could explain that calcium diminishes the inhibitory effect of ouabain on the potassium uphill transport. But this is rather speculative and needs experimental confirmation.

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