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ESTIMATION OF CARRIER DENSITY AND TURNOVER RATE OF THE Na^+/H^+ EXCHANGER IN HUMAN PLATELETS USING 5-(N-METHYL-N-[^{3}H] ISOBUTYL)- AMILORIDE

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Summary: We used the radiolabelled inhibitor of Na^+/H^+exchange 5-(N-methyl-N-[^{3}H]isobutyl)amiloride ([^{3}H]-MIA) for assessment of the amount of Na^+/H^+ exchanger in intact human blood platelets. The inhibition constant, K_I, of unlabelled MIA toward the antiport was determined at 100 nM. Washed platelets were incubated for 5 s with different concentrations of [^{3}H]-MIA in the presence or absence of an excess concentration of unlabelled amiloride (400 µM). The platelets were rapidly centrifuged and the radioactivity in the pellet was determined. Scatchard analysis revealed one single class of specific binding sites (K_D=63 nM) and a maximum binding capacity of 500 sites/cell. The turnover rate of the Na^+/H^+exchanger in unstimulated platelets was estimated at 800/s at 25°C.

The Na^+/H^+ exchanger is a ubiquitously expressed membrane transport protein. In resting blood platelets it extrudes metabolically produced protons, thereby keeping cytosolic pH, pH_i, constant. After stimulation of platelets by thrombin pH_i rises almost instantaneously from 7.1 to approximately 7.3 (1-3). This rise in pH_i is completed within 1 - 2 min. after addition of agonist. Thus, it is almost ten times faster than the pH_i elevation in agonist-stimulated neutrophils (4) or vascular smooth muscle cells (5). One reason for this rapid increase in pH_i may be a high number of active exchangers.

Recent studies suggest that platelets from patients suffering from primary hypertension exhibit an enhanced Na^+/H^+exchange activity which may be due to an increased number of Na^+/H^+antiporters (6,7). Therefore, we aimed at estimating the number and turnover rate of this ion transport system on intact human blood platelets.

Abbreviations

MIA, 5-(N-methyl-N-isobutyl)amiloride; BCECF, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein; pH_i, cytosolic pH; pH_o, extracellular pH.

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The radiolabelled high specific inhibitors of Na^+/H^+exchange MIA and 5-(N-ethyl-isopropyl)-amiloride have been successfully used to assess the amount of carriers in kidney membrane preparations (8,9) and on intact rat thymocytes (10).

MATERIALS AND METHODS

Chemicals

BCECF-AM was purchased from Molecular Probes (Eugene, OR, USA). [^{3}H]-MIA, specific activity 68.4 Ci/mM, was obtained from DuPont-NEN (Dreieich, FRG). Amiloride was a kind gift from MSD Sharp & Dohme (Munich, FRG). All other chemicals were of analytical grade and obtained from Sigma (Deisenhofen, FRG).

Preparation of Platelets

Human platelet concentrates, anti-coagulated with citrate-phosphate-dextrose (CPD), were purchased from the local transfusion service. After two centrifugation steps (30 min., 480 x g at room temperature) to remove residual plasma proteins, platelets were finally resuspended in Hepes-buffer consisting of (in mM) NaCl 140, KCl 5, KH_2PO_4 5, Hepes (free acid) 10, $MgSO_4$ 1, glucose 5, pH 6.5 at 25°C. For the binding assay NaCl was iso-osmotically replaced by N-Methyl-D-glucamine (NMG) chloride and the pH adjusted to 7.3. For the swelling assay, samples of platelet-rich-plasma (PRP) were directly used without further preparation.

Determination of Na^+/H^+exchange activity by a swelling assay

An aliquot of PRP (70 µl) was directly added to a cuvette containing a medium of (in mM) NaCl 140, Hepes (free acid) 20, glucose 10, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, pH 6.7 at 25°C. The influx of propionic acid leads to an intracellular acidification and stimulates the Na^+/H^+ exchanger which results in a gain in intracellular Na^+ and water. The accompanying volume gain is detectable as a decrease in optical density of such platelet suspension, the rate of which provides a measure of Na^+/H^+ exchange activity as recently described in detail (11,12).

Measurement of pH_i

Cytosolic pH was determined using the intracellularly trappable fluorescent pH indicator 2',7'-bis(carboxyethyl)-5,6-carboxy-fluorescein, BCECF, exactly as described in detail elsewhere (1,2). Wavelengths for excitation and emission were set at 495 and 530 nm, respectively. Calibration of the BCECF signals was performed using the high K^+- nigericin method as described (1,2).
BCECF-loaded platelets were acidified by storage for 1 h in acidic Hepes-buffer, pH 6.7-7.2. Thereafter, platelets were rapidly diluted into a cuvette containing Hepes buffer, pH 7.4 at 25°C. This resulted in a prompt stimulation of the Na^+/H^+exchanger (cf. ref. 13). The velocity of pH_i recovery was recorded and the initial slopes of the pH_i recovery curves were taken as a measure of the antiport activity.

Binding assay

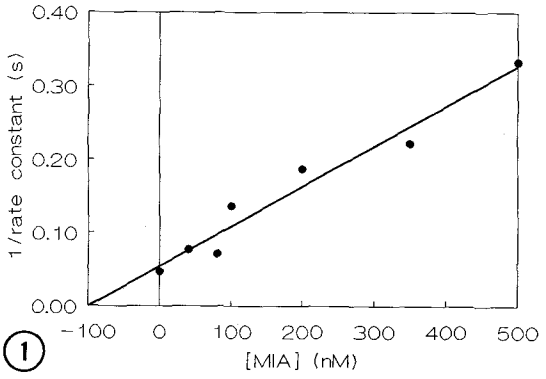
The assay conditions were chosen according to the method of Dixon et al. (10). In brief, 120 µl of platelets resuspended in NMG-chloride buffer at a concentration of 7×10^8 cells/ml, were transferred into the tip of a 400 µl microcentrifuge tube. One minute before the experiment either 10 µl of amiloride solution (in NMG-chloride buffer; 400 µM final)

or 10 µl of amiloride-free buffer were added. A 20 µl droplet containing the desired quantity of [^{3}H]-MIA was then carefully placed at the side of the microcentrifuge tube without mixing. At time zero the drop was mixed into the platelet suspension, stirred for 5 sec on a vortex mixer and immediately centrifuged for 5 sec at 9,000 x g in a Beckman microfuge B. The supernatant was rapidly aspirated and the tip of the microcentrifuge tube was cut into a counting vial. The platelet pellet was dissolved into 100 µl of NMG-chloride buffer. Radioactivity was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Amiloride analogs which are alkyl-substituted on their 5-amino nitrogen atom, such as MIA, are specific blockers of the Na^+/H^+ exchanger (14).

We first examined the inhibitory action of MIA toward the platelet Na^+/H^+ exchanger using a modified swelling assay (12). A Dixon plot of the obtained swelling rates (Fig. 1) revealed a K_I value of 100 nM which is in good agreement with those mentioned in the literature (10). We next ascertained the rapid onset of the inhibition by MIA. Platelets were activated by addition of thrombin, which resulted in an immediate rise in pH_i. This could be reversed instantaneously by the addition of MIA (10 µM; Fig.2 inset). Storage of



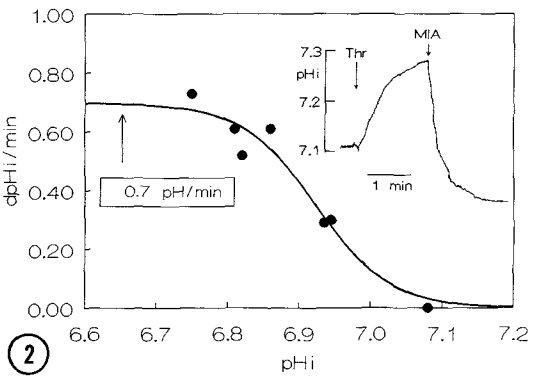


Fig.1. Inhibition by MIA of the platelet Na^+/H^+ exchanger.
Na^+/H^+exchange was activated by adding platelet-rich plasma into acidic (pH 6.7) Na^+-propionate medium. The subsequent swelling was recorded in an aggregometer. The graph is a Dixon plot of the swelling rates obtained at different concentrations of MIA. The straight line was calculated by a linear regression analysis (r=.9449). K_i is calculated at 100 nM.

Fig.2. Properties of the platelet Na^+/H^+ exchanger.
Main Panel: Acidified platelets were added to Hepes buffer, pH 7.4 and the velocity of pH_i recovery was recorded. The plot shows initial recovery rates as a function of initial pH_i immediately after resuspension of platelets. The line was calculated assuming a sigmoidal relationship between dpH_i and pH_i.
Inset: Effect of addition of thrombin (Thr, 0.1 U/ml) to BCECF-loaded platelets. The pH_i rise is immediately reversed after addition of MIA (10 µM).

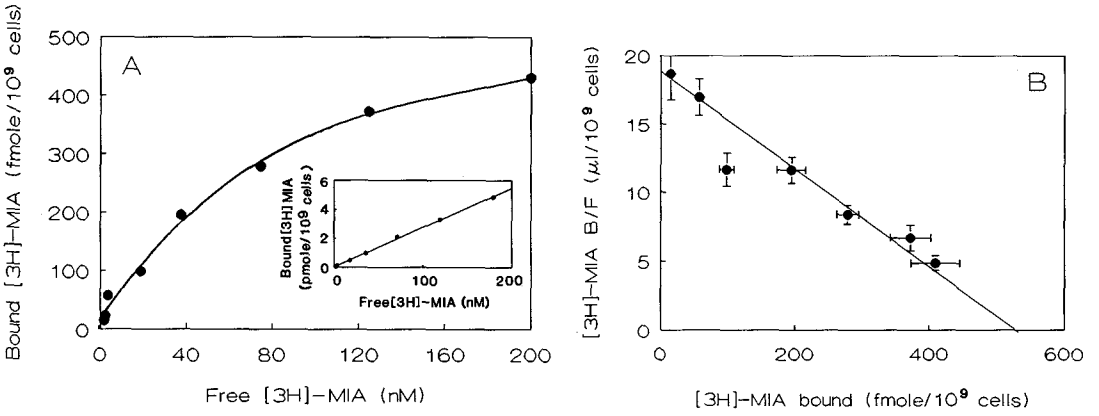


Fig.3. Binding of [^{3}H]-MIA to human platelets.
A: Specific binding of [^{3}H]-MIA. The data are representative of three independent experiments. Inset: Nonspecific binding of [^{3}H]-MIA determined in the presence of an excess (400 µM) of unlabelled amiloride.
B: Scatchard plot of the data of Fig.3A. Error bars indicate ±S.D. The straight line was obtained by linear regression analysis (r=.967) yielding a K_d of 63 nM and a maximum number of binding sites of 500/platelet.

platelets at different pH_o values lower than pH 7.4 led to a pH_o-dependent cytosolic acidification (data not shown), which confirms earlier findings (15). After dilution into Hepes buffer pH 7.4 (25°C), a rapid pH_i recovery could be observed. The initial slope of each pH_i recovery curve was plotted against the initial pH_i after acidification. As shown in Fig. 2 (main panel) the resulting curve could mathematically best be described by a sigmoidal function resulting in an estimate for the maximum velocity of Na^+/H^+exchange in quiescent platelets of 0.7 pH/min. Using this value and a buffer capacity for $H^+$of 17 mmol/(l x pH) (11,16) we calculated a maximum velocity of proton transfer of 11.9 mmol H^+/l of cytosol.

As outlined in detail by Dixon et al. (10), the rapid reversibility of the inhibition of the Na^+/H^+ exchanger by MIA prevents such binding assays as equilibrium binding or successive membrane filtration techniques. Having ascertained the rapid interaction of MIA with the exchanger (Fig.2) we therefore reduced the incubation time to 5s in order to minimize unspecific binding.

Binding of [^{3}H]-MIA to intact platelets could be resolved into two portions: an unspecific binding (70-80% of total) , that was not displaced by excess (400 µM) amiloride (Fig.3A, inset), and a second saturable component (Fig.3A, main panel) which was sensitive to amiloride added in excess. A Scatchard analysis (Fig. 3B) of these data suggests a single class of binding sites with a binding constant, K_D, of 63 nM. This values is in good agreement with the K_I value of 100 nM obtained from the swelling assay (cf. Fig.1). Furthermore, this analysis leads to a maximum number of specific binding sites of 500/platelet.

This implies the presence of three times more carriers on the same surface area than on rat thymocytes.

Together with the data for maximum velocity of the exchanger in blood platelets of 11.9 mmol H^+/l of cytosol and the assumption that only 50% of the cytosol is actually involved in rapid pH_i regulating processes we calculate the turnover number of the platelet Na^+/H^+ exchanger at 800/s. This value compares to 2000/s as computed for rat thymocytes (10). This latter difference in the maximum turnover number may mainly result from the fact that our assay for determination of maximum velocity was conducted at 25°C, whereas the data of Dixon et al. we derived at 37°C (10). An increase in Na^+/H^+ exchange activity at higher temperatures has been reported by Livne et. al. (11). In addition, differences in the species and cell type investigated must be considered.

The present report appears to be the first attempt to quantify the amount of Na^+/H^+ exchanger protein in human platelets. Although the derived values fit excellently with those reported for thymic lymphocytes, it should be mentioned that a considerable portion of the total [^{3}H]-MIA binding was nonspecific. Furthermore the incubation time had to be kept rather short for reasons outlined above, which may produce an additional experimental error both in the results reported here as well as in previous publications (10). Nevertheless, determination of the amount of Na^+/H^+ exchangers in human cells remains of importance, with respect to the finding that the Na^+/H^+ exchange activity is almost doubled in blood cells of patients with primary hypertension. The development of more specific tools to address this problem appears of major significance.

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REFERENCES

1. Siffert,W., Jakobs,K.H., and Akkerman, J.W.N. (1990) J.Biol.Chem. 265, 15441-15448
2. Siffert, W., Siffert,G., and Scheid,P.(1987) Biochem.J. 241, 301-303
3. Zavoico, G.B., Cragoe, E.J., and Feinstein, M.B. (1986) J.Biol. Chem. 261, 13160 - 13167
4. Weisman, S.J., Punzo,A., Ford, C., and Sha'afi, R.I. (1987) J.Leukocyte Biol. 41, 25 - 32
5. Berk, C., Brock,T.A., Gimbrone,M.A., and Alexander, R.W. (1987) J.Biol.Chem. 262, 5065-5072
6. Livne, A., Balfe, J.W., Veitch, R., Marquez-Julio, A., Grinstein, S., and Rothstein, A. (1987) Lancet i, 533 - 536
7. Schmouder, R.L., and Weder, A.B. (1989) J.Hypertens.7, 325-330
8. Vigne, P., Frelin, C., Audinot, M., Borsotto M., Cragoe,E.J., and Lazdunski,M. (1984) EMBO J. 3, 2647 - 2651
9. Talor,Z., Ng,S.C., Cragoe, E.J., and Arruda,J.A.L. (1989) Life Sci. 45, 517-523

10. Dixon, S.J., Cohen, S., Cragoe, E.J., and Grinstein, S. (1987) J. Biol. Chem. 262, 3626 - 3632
11. Livne, A., Grinstein, S., and Rothstein, A. (1987) Thromb. Haemost. 58, 971 - 977
12. Rosskopf, D., Morgenstern, E., Scholz, W., Osswald, U., and Siffert, W. (1991) J. Hypertens., In press
13. Conlin, P.R., Kim, S.Y., Williams, G.H., and Canessa, M.L. (1990) Endocrinology 127, 236-244
14. Benos, D.J. (1988) in Na^+/H^+-Exchange (S.Grinstein, Ed.) pp. 121-138, CRC Press Inc, Boca Raton, Fl
15. Horne, W.C., Norman, N.E., Schwartz, D.B., and Simons, E.R. (1981) Eur.J.Biochem. 120, 295-302
16. Clemens, N., Siffert, W., and Scheid, P. (1990) Eur.J.Physiol. 416, 68-73