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# THE $Ca^{2+}$-DEPENDENT ACTIVATION BY FLUORIDE OF HUMAN RED CELL MEMBRANE SODIUM PERMEABILITY: EVIDENCE FOR A CHEMICALLY ACTIVATED TETRODOTOXIN-SENSITIVE $Na^+$ CHANNEL

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Fluoride (NaF) (5-15 mM) activated the $^{22}Na^+$ uptake by human red blood cells (RBC). The effect was $Ca^{2+}$-dependent. $Sr^{2+}$, but not $Ba^{2+}$, $Mg^{2+}$, $Mn^{2+}$, substituted $Ca^{2+}$ in supporting the $^{22}Na^+$ uptake. The NaF-induced $^{22}Na^+$ uptake was sensitive to tetrodotoxin (TTX), pertussis toxin but not to amiloride nor valinomycin. The value of the influx was 8.6+/-5.0 $mmol/l_{cells}$. Thus, the TTX-sensitive $Na^+$-transport system is present in the RBC membrane in an inactive form which could be activated with NaF by a mechanism involving G-protein(s) but not the depolarization. 

Human RBC membrane is impermeable for monovalent cations and does not discriminate between $Na^+$ and $K^+$ [1, for review]. The activation of the RBC membrane permeability for $K^+$ observed by Gardos [2], was the first demonstration of the $Ca^{2+}$- activated $K^+$ channel (K(Ca)). The effect, triggered with NaF or by pre-incubation with inosine *plus* iodacetate [2] and, later, with propranolol [3], lead [4], A23187 [5], vanadate [6], and redox-mediators [7], all in the presence of $Ca^{2+}$ is regarded as specific for $K^+$ [see 1,8,9, for reviews, 10,11]. On the other hand, the RBC membrane permeability for $Na^+$ was shown to be increased in some experimental circumstances. Thus, Huestis [12] rendered the human red cell membrane permeable for $Na^+$ upon a pre- treatment of intact red cells with a phosphatidyl choline (PC) liposomes. The effect was blocked by TTX [12]. Szász and Gárdos [13] reported that the TTX - sensitive uptake of $Na^+$ by human RBCs could be induced by carbachol. Furthermore, the

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increase of $Na^+$ permeability was induced by A23187 [14] or by vanadate [15] which was sensitive to amiloride. Our results show that NaF-induced $^{22}Na^+$ uptake by human RBC with specific properties that occurs under the conditions used for the induction of the Gárdos effect.

**Methods**

Human blood was obtained from both healthy, and drug-free volunteers of both sexes, and was used within 3 days being stored at 0-4 $^oC$. As anticoagulant was used EDTA, K salt (5 mM). Red cells were prepared immediately before the experiment by differential centrifugation (500 x g, for 10 min at ambient temperature). Red cells were centrifuged, the buffy coat aspirated, and the pellet was washed three times, and, finally, suspended, in a medium containing (in mM): Tris-Cl-20, pH 7.3,; NaCl-130, KCl-5, glucose-10 (further referred to as the standard medium).

$^{22}Na^+$ uptake was measured as follows: RBC (40% hematocrit) suspended in medium as shown above, and kept at 25 $^oC$, were pipetted into the test tube containing unmixed aliquots of stock solutions of radionuclide $^{22}NaCl$ (Institute of Radionuclides of Academy of Sciences of Russia, Moscow), NaF, and $CaCl_2$ so that the final activity/concentration was $^{22}NaCl$ (about 100 MBq/ml), NaF (10 mM), $CaCl_2$ (2.5 mM), and vigorously vortexed. Aliquots were withdrawn, and centrifuged through a layer of a silicone oil in time indicated in the Figures (when not - 50 min). The individual phases were separated. The radioactivity of supernatants and pellets was measured by a liquid scintillation counting (corrected for quenching) after precipitating proteins with trichloracetic acid (5% (w/v) final). Control cells without NaF and without $Ca^{2+}$ were treated in parallel. When the effect of inhibitors was tested, these were added 5 min before the addition of the uptake inducers and radionuclide. Experimental points are represented by the average values of duplicate measurements +/- standard error. The latter is depicted by bars whenever it exceeds the dimension of symbol.

Identical conditions were used for the measurement of the Gárdos effect but no radioactivity was used and $K^+$ was measured in the supernatant by flame photometry.

**Results and Discussion**

The addition of NaF increased profoundly the $^{22}Na^+$ radioactivity of human red blood cells (RBC) pelleted by the centrifugation through the layer of silicone oil as compared with non-treated controls (Fig.1). The effect of NaF was discernible from about 5 mM but it became clearly pronounciated at and/or over 10 mM and culminated at about 15 mM. The effect of NaF was strongly dependent on the extracellular $Ca^{2+}$ when present at least in milimolar concentrations (Fig.1,2). In the presence of equivalent concentration of EGTA instead of $Ca^{2+}$ (Fig.1,2) or in the presence of $Ca^{2+}$ alone (Fig.2), no change in the $^{22}Na^+$ uptake was observed. The activating effect of $Ca^{2+}$ could be mimicked by

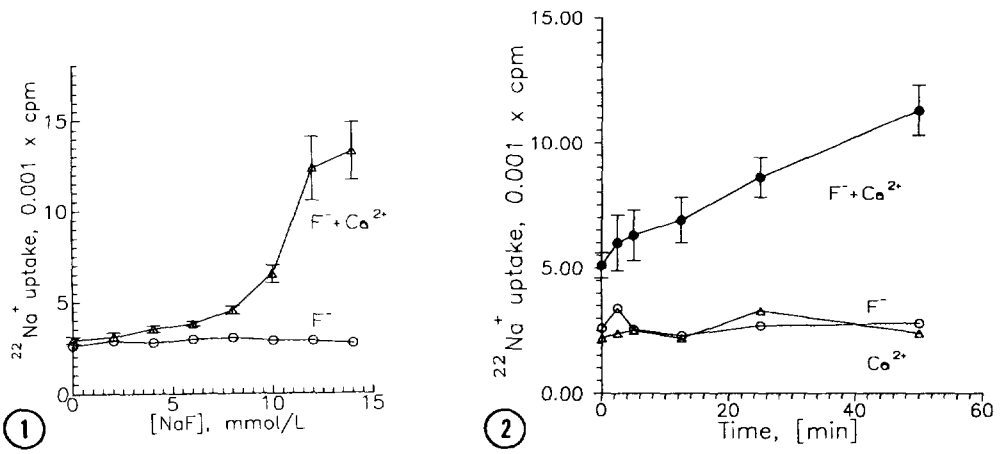


Fig.1. Effect of $Ca^{2+}$ on the $^{22}Na^+$ uptake by NaF-treated human RBCs.

The experiment was performed with NaF concentrations indicated in the Figure and containing either $Ca^{2+}$ (triangles) or EGTA (circles)(both 2.5 mM). One of three similar experiments.

Fig.2. Time course of the $^{22}Na^+$ influx by NaF and extracellular $Ca^{2+}$.

The $^{22}Na^+$ uptake was measured for the time indicated at 25 °C with NaF plus $Ca^{2+}$ (closed circles), NaF plus EGTA (open circles), or $Ca^{2+}$ alone (triangles). Typical of four experiments.

$Sr^{2+}$ (twice as effective as $Ca^{2+}$) but not by $Ba^{2+}$, $Mg^{2+}$ or $Mn^{2+}$ ions (Table I).

The $^{22}Na^+$ uptake was, generally, very rapid. In the Fig. 2 is shown a typical time course of the uptake at 25 °C. The rapid initial phase was folloved by a slower, linear, phase lasting for at least 50 min. As an exception, a slow time course of the $^{22}Na^+$ uptake with almost zero values at 0 min was observed (not shown). This could be ascribed to the variability among individual blood donors.

The changes in the $^{22}Na^+$ uptake have been accompanied by a massive $^{45}Ca^{2+}$ uptake or a $^{45}Ca^{2+}$ binding to the membranes (not shown) and, as expected, by the efflux of $K^+$ described by Gardos [2] several decades earlier. The extent of the $K^+$ efflux after 50 min of incubation was 35.2 +/- 9.7 $mmol/l_{cells}$ (n=7). The extent of the $Na^+$ influx could be calculated from the specific radioactivity of the medium and the red cell radioactivity assuming that the isotopic equilibrium was established. The value 8.6 +/- 5.0 $mmol/l_{cells}$ (n=9) was obtained.

The $Na^+$ transport pathway was characterized by pharmacological tools. Both $Na^+/H^+$ [16], and $Na^+/Ca^{2+}$ [17]

Table I
Effects of divalent ions (1) and inhibitors (2,3) on the NaF *plus* $Ca^{2+}$-induced $^{22}Na^{+}$ uptake by human RBC

| Expt. | Additions | cpm | % of control |
|---|---|---|---|
| 1 | $Ca^{2+}$, NaF | 27306+/-521 | 100 |
| | $Sr^{2+}$, NaF | 56512+/-704 | 207 |
| | $Ba^{2+}$, NaF | 2543+/-311 | 9.3 |
| | $Mg^{2+}$, NaF | 5511+/-843 | 20.2 |
| | $Mn^{2+}$, NaF | 45+/-125 | 0.2 |
| 2 | $Ca^{2+}$, NaF, DMSO | 26841+/-798 | 100 |
| | $Ca^{2+}$,NaF, 0.3 mM AMD | 29768+/-353 | 110.9 |
| 3 | $Ca^{2+}$, NaF | 8847+/-457 | 100 |
| | $Ca^{2+}$, NaF, 10 µg/ml VM | 7732+/-585 | 87.4 |

Data were obtained from independent experiments and were corrected for the radioactivity present in the corresponding controls without NaF. Average data from parallel samples are presented +/- standard error. AMD-amiloride, DMSO-dimethylsulphoxide, VM-valinomycin. Concentrations of ions-2.5 mM, of NaF-10 mM.

antiporters are sensitive to amiloride (AMD) and its derivatives while the $Na^{+}$ channel is sensitive to TTX. The NaF-induced $^{22}Na^{+}$ uptake was resistant to AMD (tested up to 0.3 mM) (Table I) but sensitive down to $10^{-5}$ mol/l TTX in a dose-dependent manner (Fig.3). Thus, the NaF treatment elicits the TTX - sensitive $Na^{+}$ permeability in human red cells in addition to procedures described by Szász and Gárdos [13] and Huestis [12].

The question arises about the mechanism(s) underlying the observed effect of NaF on the RBC $Na^{+}$ permeability. NaF is known to affect G-proteins [18], therefore the effects of inhibitors interfering with G-proteins, like pertussis toxin (PTX) and cholera toxin (ChTX) were tested. The former was found the powerfull inhibitor of the NaF-induced $^{22}Na^{+}$ uptake (Fig.3).

The second question raised by our observation was one about the role of membrane depolarization in the opening of the $Na^{+}$ membrane permeability. It is known that the RBC membrane potential ($V_m$) is about -8 to -12 mV [19]. In these conditions the $Na^{+}$ channel with properties of that found in excitable tissues would be either permanently open or inactivated. In order to find out whether our transport system is activated with NaF by

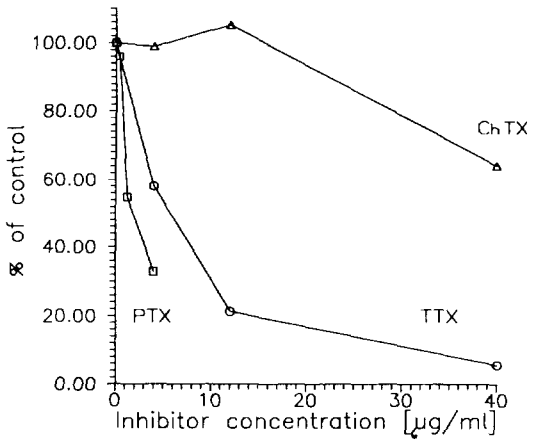


Fig.3. Effects of inhibitors on the NaF - induced $^{22}Na^{+}$ uptake The uptake was measured for 50 min with 10 mM NaF and 2.5 mM $Ca^{2+}$ and indicated concentrations of tetrodotoxin (TTX), pertussis toxin (PTX) and cholera toxin (ChTX). One of two similar experiments.

a mechanism that involves a short depolarization shifted to more positive $V_m$ values preceding the activation of the K(Ca) we measured the NaF-induced $^{22}Na^{+}$ uptake in the presence of valinomycin (VM) ($K^{+}$-specific ionophore) which clamps the membrane on the $K^{+}$ diffusion equilibrium potential. We found that VM had only a marginal effect on the NaF-induced $^{22}Na^{+}$ uptake (Table I). Thus, the TTX- sensitive $Na^{+}$ channel present in the RBC membrane is activated by a chemical mechanism which involves G-proteins found earlier in human RBC [20]. This conclusion is in agreement with results of [$^{3}$H]-Tetraphenylphosphonium$^{+}$ ([$^{3}$H]-$TPP^{+}$) distribution during the NaF *plus* $Ca^{2+}$- induced permeability changes. We observed only the monophasic [$^{3}$H]-$TPP^{+}$ uptake (unpublished results).

The significance of our observation for the RBC physiology is unknown. A clue for its understanding could be the early observation of Singer and Tasaki [21] who showed that the internal perfusion of the squid axon with KF (but not KCl) re-activated the inactivated action potential and prevented its inactivation for many hours. The activation by NaF of the cryptic, TTX-sensitive $Na^{+}$ transport in RBC is phenomenologically identical. The functional significance of these observations could be elucidated by future biochemical and biophysical studies.

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