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PREPARATION OF INSIDE-OUT MEMBRANE VESICLES FROM NEUTROPHILS CAPABLE OF ACTIVELY TRANSPORTING CALCIUM

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SUMMARY: We have prepared inside-out membrane vesicles from rabbit neutrophils. These vesicles are capable of actively transporting calcium. This active transport requires the presence of Mg ATP. Other nucleotides such as UTP, GTP, CTP, AMP, and ADP are not able to stimulate the active uptake of calcium in these vesicles. This transport system has a K_m for free calcium of 1.3 ± 0.7 μM and a V_{max} of 3.6 ± 0.5 nmole/mg protein, min. and is inhibited by the calmodulin inhibitor, trifluoperazine.

INTRODUCTION

Neutrophils are motile cells that respond to stimuli in a variety of ways. They are capable of directed or random movement, cell aggregation, phagocytosis, generation of hydrogen peroxide, and under appropriate conditions, of degranulation. All these events can be elicited by the various chemotactic factors, certain products of arachidonic acid metabolism, and other stimuli. Activation of the neutrophils begins with the binding of the stimulus to specific plasma membrane-located receptor sites. One result of this interaction is to raise, at least transiently, the intracellular level of a certain compound, one of the so-called second messen-

ABBREVIATIONS

EGTA:	Ethylene glycol-bis-(β-Amino ethyl Ether)N,N'-Tetra acetic Acid.
UTP:	Uridine 5'-Triphosphate
GTP:	Guanosine 5'-Triphosphate
CTP:	Cytidine 5'-Triphosphate
AMP:	Adenosine 5'-Monophosphate
ADP:	Adenosine 5'-Diphosphate
ATP:	Adenosine 5'-Triphosphate
HEPES:	(N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid)

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gers, which is then thought to trigger the cell into performing its appropriate function. At present, it is generally agreed that calcium ion is at least one, if not the primary, second messenger in neutrophil chemotaxis, enzyme secretion, aggregation, and O_2 consumption (1,2,3,4). One of the early events following cell stimulation appears to be a local rise in the concentration of ionized intracellular Ca^{2+}. Defining the mechanisms by which the cell regulates the level of intracellular ionized calcium and how these mechanisms may be modified following cell stimulation are essential components of understanding cell activation.

The regulation of the intracellular concentration of calcium ions in the neutrophils where mitochondria are relatively scarce is achieved by pump-leak systems at the plasma membrane and binding of Ca^{2+} by cytoplasmic constituents and plasma membrane (1). The presence of a specific calcium pump driven by the hydrolysis of ATP by Mg^{2+}, Ca^{2+} activated ATPase in the plasma membrane of neutrophils has been indirectly alluded to (5,6). It is not known whether or not these ATPases are directly involved in the active excretion of Ca^{2+} from the cell. Also, very little is known about the role of this pump in the regulation of intracellular calcium and how this role may be modified following cell stimulation. The difficulty has always been due to the inaccessibility of the system to direct experimental studies.

We wish now to report the results of experiments in which we have prepared inside-out membrane vesicles from neutrophils which are capable of actively transporting calcium.

MATERIALS AND METHODS

The basic principle involved in the preparation of inside-out plasma membrane vesicles from neutrophils or similar cell types is to induce the cells to ingest particles. The phagocytic vesicles thus produced can be readily isolated and are normally bound by plasma membrane whose cytoplasmic side is now facing the outside (7).

In the present studies, the particles to be phagocytized have been prepared from paraffin oil. One volume of paraffin oil and 4 volumes of modified Hanks' buffer containing 20 mg/ml of albumin were mixed and sonicated in a Bronson Sonicator, Model 185, with a micro tip for two minutes at full power. The modified Hanks' buffer contains (mM): NaCl, 124; KCl, 4.9; Na_2HPO_4, 0.66; KH_2PO_4, 0.64; $CaCl_2$, 0.5; $MgCl_2$, 0.74; $NaHCO_3$, 15; HEPES, 10; pH 7.4. At the end of the sonication period, the oil dispersion was centrifuged down for 5 minutes at 270 x g and the thin layer of coacervated droplets at the surface was removed with a spatula; the remaining stable suspension was kept on ice ready to be used.

Rabbit peritoneal neutrophils were collected as previously described (8). The cells were spun down at 900 x g for 5 minutes and the supernatant removed. Thereafter the cells were washed twice by resuspending them in 40 ml of cold saline solution (150 mM NaCl, pH = 7.0), followed by centrifugation for 2 minutes at 1,200 x g. Finally the cells were resuspended at a concentration of 10^7 cells/ml in the modified Hanks' buffer containing 1 mg/ml dextrose and were incubated in a rotary shaker water bath for 15 minutes at 37°C. At the end of this period, a volume of freshly prepared oil dispersion was added to the cell suspension and the incubation was continued for another 15 minutes (the ratio of oil dispersion to cell suspension was 0.1). Following this, the suspension was centrifuged at 1500 x g for 5 minutes and the supernatant removed by suction. The cells were again resuspended and washed once in cold 150 mM NaCl and a second time in water at 4°C. Finally the cells were resuspended in 5 ml of cold medium containing: 0.34 M sucrose, 5 mM EGTA, 5 mM dithiothreitol, 20 mM Imidazole - HCl, pH 7.4. This medium also contained the following inhibitors: 1 mM phenylmethylsulfonyl fluoride, 0.25 mg/ml of α-1-antitrypsin, 0.25 mg/ml of soybean trypsin inhibitor, 0.1 mM of N-α-p-tosyl-L-lysine cholоromethyl ketone, 0.1 mM of L-1-tosylamide-2-phenylethylchloromethyl ketone. The cells were homogenized in a 10 ml Dounce homogenizer kept in ice, and at the end of this period, 5 mM of $CaCl_2$ was added to the homogenate. Aliquots of the homogenate (2 ml) were dispensed at the bottom of 5 ml cellulose nitrate tubes and 3 ml of cold solution containing 100 mM KCl, 30 mM Imidazole-HCl, pH 6.8 was overlaid on top. The samples were spun at 150,000 x g for one hour. At the end of the run, the packed phagocytic vesicles floating on the top of the tubes were carefully removed by a spatula. The vesicles were resuspended in a minimum volume in medium containing 100 mM KCl, 30 mM Imidazole - HCl, pH 6.8. Mixing was achieved by gentle homogenization. Each preparation was used twice, once immediately and another time the following day.

Calcium uptake was measured using standard filtration techniques (7,9). Unless otherwise specified, the vesicles used to measure calcium uptake were suspended in a medium containing 100 mM KCl, 1 mg/ml albumin, 30 mM Imidazole-HCl, pH 6.8, 0.1 mM EGTA, 5 mM ATP, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$ (1μ C$_i$/ml of ^{45}Ca) and when present 5 mM of ammonium oxalate. The concentration of cold $CaCl_2$ was changed when necessary and the free Ca^{2+} concentration was calculated as described previously (9).

Samples containing the phagocytic vesicles were incubated at 37°C for 1 minute, without oxalate. At the end of this time, ammonium oxalate was added and the incubation was continued for three more minutes. The reaction was started with the simultaneous addition of both cold and radioactive calcium. At a preset time interval a known volume (150 μl) was removed in a test tube and quickly diluted by 2 ml of ice cold incubation solution containing 100 mM KCl, 30 mM Imidazole-HCl, pH 6.8. The entire volume was then filtered through millipore filters, pore size 0.45 μm, and the filter was washed again with 2 ml of the same cold buffer. The filter was then removed and counted. The filters were washed with 2 ml of 250 mM KCl and 10 ml of cold H_2O before they were used (7).

RESULTS AND DISCUSSION

In intact neutrophils, the plasma membrane located calcium pump is responsible for the active transport of calcium from the inside to the outside of the cell. This pump is driven by the hydrolysis of ATP by membrane-associated Mg^{2+}, Ca^{2+}-activated ATPase. The phagocytic vesicles prepared by the method outlined in the previous section therefore should, if representing inside-out membrane preparations, be capable of accumulating calcium in an ATP-dependent manner.

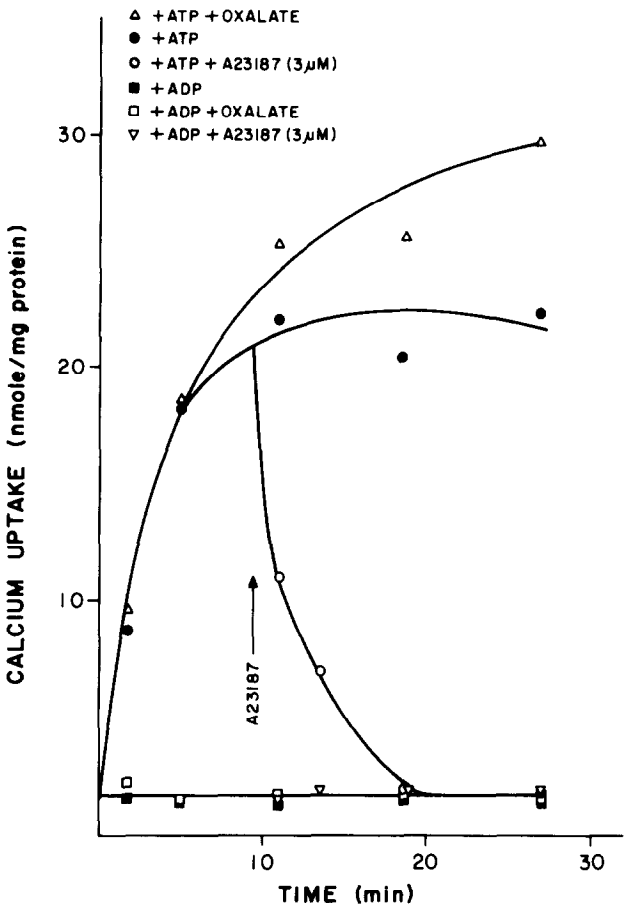

Figure 1. The time course of calcium uptake by phagocytic vesicles from rabbit peritoneal neutrophils. All measurements were carried out in the following medium: 100 mM KCl, 30 mM Imidazole-HCl, pH 6.8, 1 mg/ml albumin, 0.1 mM EGTA, 5 mM ATP, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$ (1 μC_i/ml of ^{45}Ca). The arrow indicates the time when 3 μM A23187 was added. The free calcium concentration in these experiments was 18.4 μM.

This is indeed the case as demonstrated in Figure 1 which shows a representative experiment of the time course of calcium uptake by these phagocytic vesicles from rabbit neutrophils. In the absence of ATP, approximately 1 nmole of Ca^{2+}/mg protein becomes associated with the vesicles within a very short time of incubation and thereafter does not change. In the presence of ATP, the vesicles were able to accumulate calcium. This accumulation continued for approximately 10 minutes

after which the system appeared to have reached steady-state. Continued incubation of the vesicles did not increase cell-associated calcium; in fact, a small but significant decline was observed. Addition of the calcium ionophore A23187 (3 μM) to the vesicles after 10 minutes of incubation with ATP and ^{45}Ca caused a rapid decrease of cell-associated calcium. This suggests strongly that the observed accumulation of calcium occurred against an electrochemical gradient (10). When oxalate (5 mM) was present in the vesicle as a trapping agent for calcium ions, the vesicles were able to continue accumulating calcium over the entire period (30 minutes) of incubation. It is worth noting that the initial rates of ^{45}Ca uptake into both systems (vesicles with or without oxalate) are approximately the same.

In intact human red cells, as well as in inside-out vesicles prepared from red cells, it is well documented that the splitting of ATP to ADP and P_i is necessary to produce active calcium transport (11). Other nucleotides such as ADP, AMP, pyrophosphate, and acetylphosphate cannot support the uphill movement of calcium in these systems (11). The data summarized in Table 2 demonstrate that the active accumulation of calcium by the neutrophil phagocytic vesicles resembles

TABLE 1

EFFECT OF VARIOUS NUCLEOTIDES ON CALCIUM UPTAKE BY THE PHAGOCYTIC VESICLES*

Nucleotide	calcium uptake (nmole/mg protein, 10 min)
Mg ATP	23.0 ± 4 (10)
Mg AMP	4.7 ± 0.5 (4)
Mg ADP	4.0 ± 0.2 (10)
Mg CTP	3.2 ± 0.3 (4)
Mg UTP	3.2 ± 0.5 (4)
Mg GTP	2.3 ± 0.8 (7)

* Errors are standard errors of the means. The number in parenthesis refers to number of determination. Temperature was 37°C. The incubation medium is identical to that given in the method section except for Mg ATP which was substituted by one of the test nucleotide (5 mM). The medium also contains 5 mM oxalate.

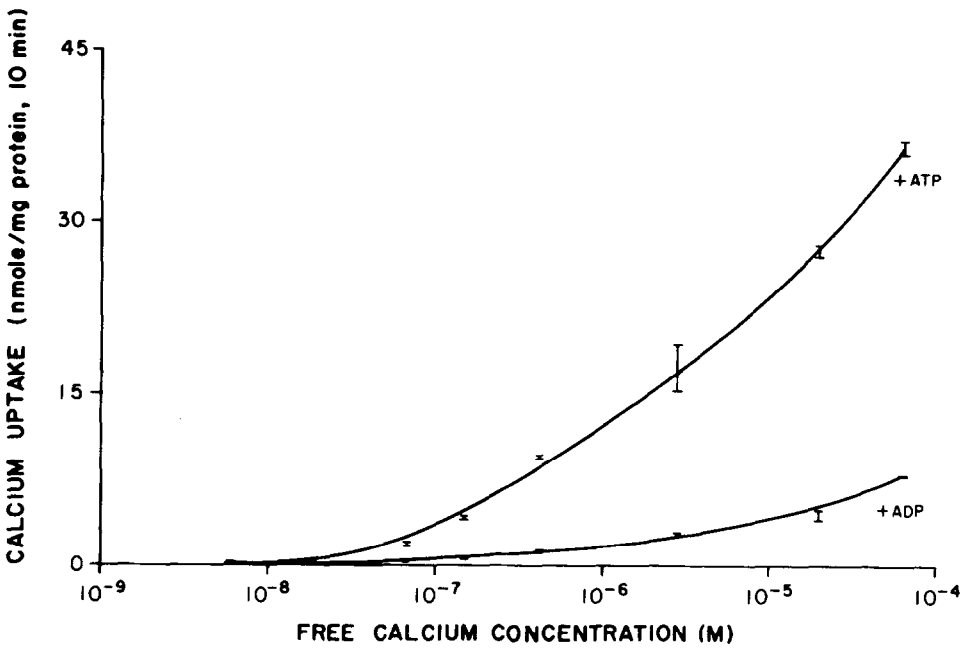

Figure 2. Variation of calcium uptake with the concentration of free calcium in the bathing medium. The suspending medium is identical with that given in Figure 1 except for the concentration of calcium. Calcium uptake was measured in the presence of 5 mM ammonium oxalate. Each point represents the average of two determinations.

greatly that of inside-out vesicles prepared from human red cells with respect to the requirements for Mg ATP (Table 1).

In order to study the kinetic characteristics of the calcium uptake in these vesicles, we have investigated the effect of varying the concentration of free calcium on the rate of ^{45}Ca uptake into the phagocytic vesicles. The results summarized in Figure 2 demonstrate that the rate of ^{45}Ca uptake into the vesicles is dependent on the concentration of free calcium in the medium. Note that at high concentrations of calcium, there is a slight increase in cell associated calcium in the absence of ATP. In order to calculate the values of the apparent K_m and V_{max} for this system, it is necessary to subtract the values of ATP independent from those of ATP-dependent uptake of ^{45}Ca. If we do that, then the calculated K_m for free calcium is 1.3 ± 0.7 μM and the V_{max} is equal to 3.6 ± 0.5 nmole/mg protein, min. The present value for K_m is similar to that for calcium transport in human red cells and other blood cells (7,9,11). It is also in the same

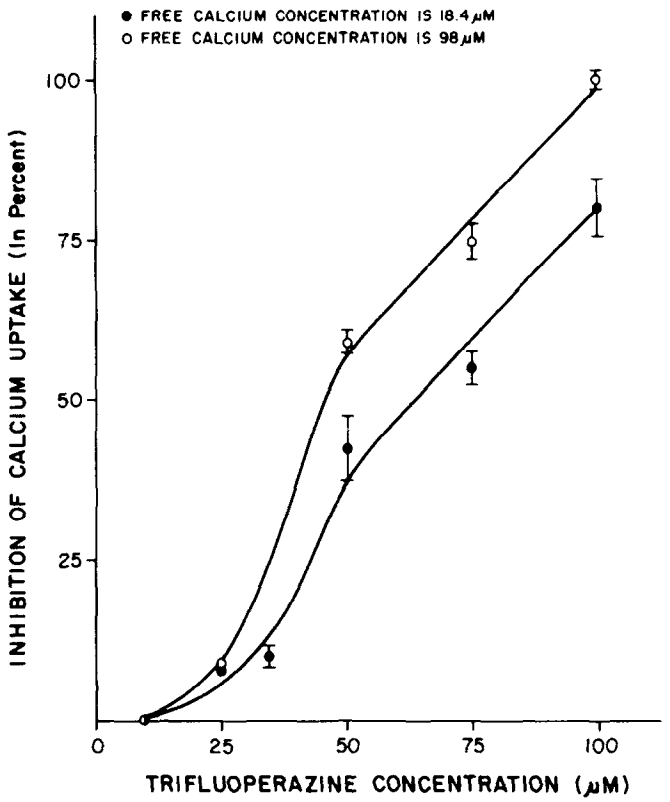

Figure 3. Effect of trifluoperazine on calcium uptake in the phagocytic vesicles. The composition of the suspending medium is identical to that given in Figure 1 except for the free calcium concentration in these experiments. Two free calcium concentrations were used (18.4 and 98 μM). Calcium uptake was measured in the presence of 5 mM ammonium oxalate and at 10 minutes after the initiation of the reaction. Each point represents the average of at least two determinations.

order of magnitude as the value of 3.3 μM for Mg, Ca-activated ATPase in membrane preparation obtained from rabbit neutrophils (5).

Calcium tranport in intact red cells or inside-out vesicles prepared from human red cells under conditions that do not deplete the membrane preparations of calmodulin such as those used in this study can be inhibited by antipsychotic agents such as trifluoperazine. The results which are summarized in Figure 3, clearly show that the trifluoperazine significantly inhibits the ATP-dependent calcium accumulation in the neutrophil phagocytic vesicles. Trifluoperazine

at concentrations as high as 100 μM did not affect the non-ATP dependent, small amounts of cell associated calcium. It is interesting to note that the concentration of trifluoperazine required for 50% inhibition of calcium accumulation by the phagocytic vesicles is significantly higher than the value required to inhibit by 50% the chemotactic factor dependent neutrophil activation (12). This difference suggests that the inhibition by trifluoperazine of the chemotactic factor induced cell activation (aggregation, oxygen consumption and lysosomal enzyme release in rabbit neutrophils) may not be mediated through the action of trifluoperazine on the calcium pump.

It is important to point out that this preparation will open the way to examining directly the effect of various stimuli on the neutrophil calcium pump. Since the cytoplasmic side of the plasma membrane is now accessible to direct biochemical and biophysical modification, the system can be used to fully characterize the kinetic parameters of the calcium pump and the role of calmodulin on its activity. It will also provide a unique opportunity to investigate the interesting questions dealing with the similarities and differences in the biochemical and biophysical properties of the different membrane vesicles induced by various phagocytic stimuli. These vesicles in conjunction with cell granules can be used to investigate the fusion process which is an essential step in degranulation.

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REFERENCES

1. Sha'afi, R.I. and Naccache, P.H. (1981) Advances in Inflam. Research, G. Weissmann (editor), Raven Press, 2: 115-148
2. Simchowitz, L. and Spilberg, I. (1979) J. Lab. Clin. Med. 93: 583-593.
3. Weissmann, G., Korchak, H.M., Perez, H.D., Smolen, J.E., Goldstein, I.M. and Hoffstein, S.T. (1979) Advances in Inflam. Research, G. Weissmann, B. Sanuelsson and R. Paoletti (editors), Raven Press, 1: 95-112.
4. Romeo, D., Zabucchi, G. and Soranzo, M.R. (1975) In: Calcium Transport in Contraction and Secretion, E. Carafoli, C.E. Draikowski and A. Margreth, North-Holland, P. 195.
5. Jackowski, S., Petro, K. and Sha'afi, R.I. (1979) Biochim. Biophys. Acta 558: 348-352.
6. Schneider, C., Mottola, C. and Romeo, D. (1979) Biochem. J. 182: 655-660.
7. Lew, P.D. and Stossel, T.P. (1980) J. Biol. Chem. 255: 5841-5846.
8. Showell, H.J., Williams, D., Becker, E.L., Naccache, P.H. and Sha'afi, R.I. (1978) J. Reticuloendo. Soc. 25: 139-150.
9. Volpi, M., Sha'afi, R.I and Feinstein, M.B. (1981) Mol. Pharmacol. 20: 363-370.
10. Scarpa, A., Baidassare, J. and Inesi, G. (1972) J. Gen. Physiol. 60: 735-749.
11. Sarkadi, B. (1980) Biochim. Biophys. Acta 604: 159-190.
12. Alobaidi, T., Naccache, P.H. and Sha'afi, R.I. (1981) Biochim. Biophys. Acta 675: 316-321.