Biochimica et Biophysica Acta, 734 (1983) 363–367
Elsevier

BBA Report

BBA 70119

EFFECTS OF *p*-NITROPHENYLPHOSPHATE ON Ca^{2+} TRANSPORT IN INSIDE-OUT VESICLES FROM HUMAN RED-CELL MEMBRANES

ARIEL J. CARIDE, ALCIDES F. REGA and PATRICIO J. GARRAHAN

Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires (Argentina)

(Received May 17th, 1983)

Key words: Ca^{2+} transport; Ca^{2+}-ATPase; Ca^{2+}-phosphatase; Inside-out vesicle; (Erythrocyte membrane)

Ca^{2+}-ATPase activity and Ca^{2+} uptake in inside-out vesicles from human red cell membranes are changed in parallel by *p*-nitrophenylphosphate. This indicates that, unlike the Ca^{2+} pump of sarcoplasmic reticulum, the Ca^{2+} pump of the red cell membrane does not utilize *p*-nitrophenylphosphate hydrolysis to drive Ca^{2+} transport.

Kinetic data allow one to distinguish two classes of site with different affinities for ATP in the Ca^{2+}-ATPase from red-cell membranes [1,2]. The high-affinity site is that at which ATP phosphorylates the enzyme and the low-affinity site is that from which ATP increases the rate of hydrolysis of the phosphoenzyme and the overall ATPase reaction [3].

We have recently reported results suggesting that the Ca^{2+}-pNPPase activity of the Ca^{2+} pump requires ATP at the high-affinity site and that *p*-nitrophenylphosphate is hydrolyzed at the low-affinity site for ATP concomitantly with ATP hydrolysis at the high-affinity site [4].

With the aim of determining whether *p*-nitrophenylphosphate can support active transport of Ca^{2+} across the red cell membrane, in a previous study [5] we have compared the effect of *p*-nitrophenylphosphate on the transport of Ca^{2+} from resealed ghosts containing ATP, with that on Ca^{2+}-ATPase activity from fragmented membranes. Results suggested that Ca^{2+} transport and Ca^{2+} ATPase activity were inhibited to the same extent by *p*-nitrophenylphosphate [5]. This was taken as a first indication of the lack of ability of *p*-nitrophenylphosphate to drive Ca^{2+} transport in red cells, since, were *p*-nitrophenylphosphate to serve as energy source for active transport it should inhibit Ca^{2+} transport less than Ca^{2+}-ATPase activity. However, it is difficult to know the actual concentration of internal ATP and *p*-nitrophenylphosphate in resealed ghosts during Ca^{2+} transport and, moreover, it is also not known whether the Ca^{2+} pump has the same response to *p*-nitrophenylphosphate in resealed ghosts as in fragmented membranes.

For these reasons, and after knowing in more detail the mechanism of the Ca^{2+}-phosphatase, we have decided to reinvestigate the effects of *p*-nitrophenylphosphate on Ca^{2+}-transport using inside-out vesicles from erythrocyte membranes. Although it could be argued that vesiculization can alter the behaviour of the Ca^{2+} pump, we have found IOVs advantageous for this sort of study because they: (i) allow control of the concentration of ATP and *p*-nitrophenylphosphate at the cytoplasmic surface of the cell membrane; (ii) are free of most of the very active soluble phosphatase

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; Ca^{2+}-ATPase, Ca^{2+}-dependent ATPase; IOV, inside-out vesicle.

normally present in erythrocytes which remains in significant amounts inside released ghosts; and (iii) allow the measurement of Ca^{2+} transport and Ca^{2+}-ATPase activity in the same preparation.

Inside-out vesicles (IOVs) were prepared using red cells from fresh human blood following the procedure described by Lew et al. [6]. The vesicles were suspended in 100 mM KCl/10^{-3} M ouabain/50 mM Tris-HCl (pH 7.8 at 37°C), then passed through a 26-gauge needle and kept at 4°C until used. The proportion of IOVs measured according to Steck and Kant [7] by the acetylcholinesterase assay corresponded to about 25% of the total membrane protein in the preparation and was independent of the presence or absence of oxalate.

The two sites of the Ca^{2+} pump for ATP have been well documented in isolated membranes [1] and in resealed ghosts [2], but the published data on IOVs are controversial. Mollman and Pleasure [8] have reported curves of Ca^{2+} transport in IOVs as a function of ATP which are clearly biphasic, while Sarkadi et al. [9] reported experiments of the same sort in which the velocity curves appear to follow simple Michaelis-Menten kinetics. Since the two sites of the Ca^{2+} pump for ATP are involved in *p*-nitrophenylphosphate hydrolysis we decided to see first if they were apparent in the preparation of IOVs used for the experiments reported here. For this purpose, we measured the uptake of Ca^{2+} as a function of the concentration of ATP in the external medium. A double-reciprocal plot of the experimental points (not shown) can be fitted by two straight lines of different slopes, indicating that the response to ATP of the rate of Ca^{2+} uptake is biphasic. In the graph in Fig. 1, Ca^{2+} uptake is plotted against the concentration of ATP together with a theoretical curve for the sum of two Michaelis-like equations. It can be concluded, therefore, that the two sites for ATP of the Ca^{2+} pump are preserved in IOVs, although it would seem that in this preparation V_1 and K_{m2} have higher values than those reported for other preparations [1,2].

Fig. 2 shows an experiment in which the rate of Ca^{2+} uptake by oxalate-loaded IOVs was measured at different *p*-nitrophenylphosphate concentrations in media with and without ATP. In the absence of ATP and *p*-nitrophenylphosphate there

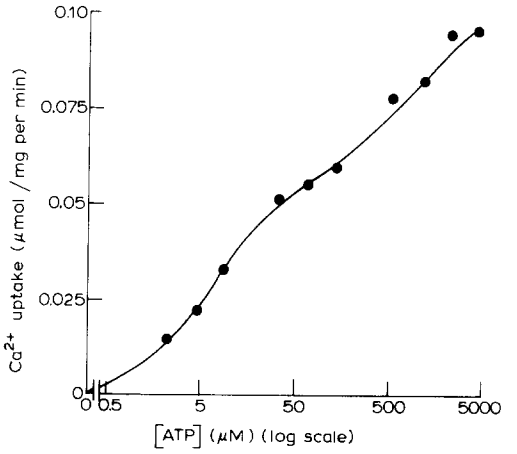

Fig. 1. The rate of Ca^{2+} uptake by oxalate-loaded IOVs as a function of ATP concentration. The curve drawn represents the sum of two Michaelis-Menten curves with K_{m1} 6.44 μM and V_1 0.0578 μmol Ca^{2+}/mg protein per min and K_{m2} 803 μM and V_2 0.0472 μmol Ca^{2+}/mg protein per min calculated by a non-linear regression procedure (Garrahan, P.J. and Rossi, R.C., unpublished data) based on Wilkinson's method [10]. Ca^{2+} uptake was measured at 37°C in 100 mM KCl, 5 mM potassium oxalate, 2 mM $MgCl_2$, 4.2 mM $^{45}CaCl_2$, 10^{-3} M ouabain, 5 mM phosphocreatine, 5 U/ml creatine phosphokinase, 50 mM Tris-HCl (pH 7.8 at 37°C) and different concentrations of MgATP. IOVs were loaded with oxalate just before use by incubating a suspension of vesicles (0.5–0.7 mg protein/ml) in 150 mM KCl/75 mM Tris-HCl (pH 7.8 at 37°C)/7.5 mM potassium oxalate/10^{-3} M ouabain at 37°C for 25 min [8]. After loading, the IOVs received a sufficient volume of a concentrated solution of $CaCl_2$ to give a final concentration of 6.3 mM and were incubated at 37°C for 5 min more. Immediately after, the flux experiment was started by mixing 2 vol. of the IOV suspension with 1 vol. of a solution containing 1 μC/ml of $^{45}CaCl_2$ without added carrier plus 6 mM MgCl, 10^{-3} M ouabain, 15 mM phosphocreatine, 15 U/ml creatine phosphokinase and different amounts of MgATP. Every 0.5 min, 0.25 ml of the final IOV suspension (0.3–0.4 mg protein/ml) was removed and mixed with 1 ml of 100 mM KCl/50 mM Tris-HCl (pH 7.8 at 37°C)/2 mM $MgCl_2$/5 mM EGTA in a conical polyethylene tube at 0°C. The tubes were centrifuged 3 min at 10000 × *g* in an Eppendorf centrifuge and the pellet washed once with 1 ml of the same solution and then twice with 1 ml of the same solution to which 0.6 mM $CaCl_2$ and 0.5 mM EGTA had been added. The pellet was dissolved in 0.5 ml of 1% sodium dodecyl sulfate and the radioactivity was counted in a Beckman LS-100C liquid scintillation counter. An aliquot of the dissolved pellet was kept aside to measure protein concentration by the procedure of Lundahl [11]. The rate of Ca^{2+} uptake by the IOVs was estimated from the linear initial part of plots of the amount of $^{45}Ca^{2+}$ per mg protein in the pellet against incubation time. Provided the IOVs had been preincubated in the presence of $CaCl_2$ for 5 min as stated above, the rate of Ca^{2+} uptake was constant during at least the first 3.5 min of incubation at 37°C.

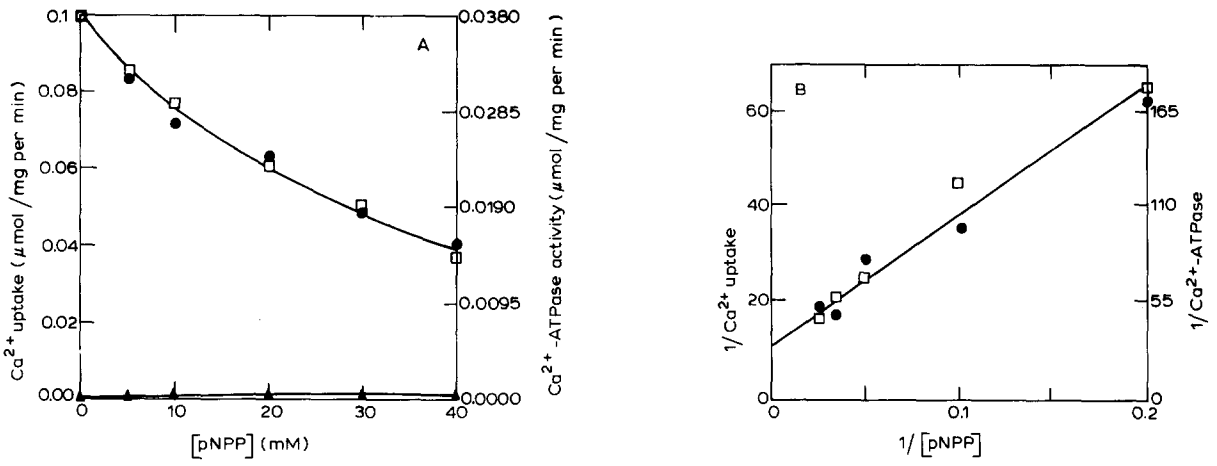

Fig. 2. Ca uptake in the presence (●) and absence (▲) of 5 mM ATP and Ca^{2+} ATPase activity (□) in oxalate-loaded IOVs as a function of *p*-nitrophenylphosphate (pNPP) concentration. Ca^{2+} uptake was measured as in Fig. 1 in the absence of phosphocreatine and creatine phosphokinase. When present, *p*-nitrophenylphosphate was added together with ATP and enough $MgCl_2$ to give a final concentration of 2 mM free Mg^{2+} calculated on the basis of an association constant for complexing Mg^{2+} of 300 M^{-1}. Ca^{2+}-ATPase activity was measured on the same batch of IOVs at 37°C in medium of composition identical to those used for Ca^{2+} uptake except that the $CaCl_2$ was non-radioactive, ATP was [γ-^{32}P]ATP and the concentration of IOVs was 0.6–0.8 mg protein/ml. The ATPase reaction was started as described for the measurement of Ca^{2+} uptake. Every 0.5 min, 0.25 ml f the IOV suspension was taken and mixed rapidly with 0.75 ml of 0.5% (w/v) ammonium molybdate in 5% percloric acid contained in a 3 ml glass tube at 0°C. The amount of [^{32}P]orthophosphate present was measured as described previously [1]. ATPase activity was estimated from the linear initial part of plots of the amount of [^{32}P]orthophosphate released from ATP per mg IOV protein against incubation time. Ca^{2+}-ATPase activity was the difference between the activities in media with $CaCl_2$ and in media without $CaCl_2$. Part B is a double-reciprocal plot of the difference between the rate of Ca^{2+} uptake or the Ca^{2+}-ATPase activity at zero *p*-nitrophenylphosphate and at each of the *p*-nitrophenylphosphate concentrations tested.

is almost no uptake of Ca^{2+} as is to be expected from the low permeability to Ca^{2+} which is characteristic of the red cell membrane. Under these conditions, no change in the rate of Ca^{2+} uptake can be detected when the concentration of *p*-nitrophenylphosphate in the incubation medium is raised to 40 mM (Fig. 2A). It seems, therefore, that *p*-nitrophenylphosphate neither alters the passive permeability of the vesicles to Ca^{2+} nor promotes Ca^{2+} transport in the absence of ATP. Absence of Ca^{2+} transport under these conditions confirms previous findings in the absence of oxalate [5,9] and is not surprising, since ATP is necessary for the Ca^{2+} pump to catalyze *p*-nitrophenylphosphate hydrolysis [4,5]. Results in Fig. 2A also show that in media containing 5 mM ATP, the vesicles transport Ca^{2+} at a good rate. As *p*-nitrophenylphosphate concentration raises, the rate of Ca^{2+} uptake observed in the presence of 5 mM ATP decreases. The inhibition curve can be fitted by a rectangular hyperbola, since a double-reciprocal plot of the difference between the rate of Ca^{2+} uptake at zero *p*-nitrophenylphosphate and at each *p*-nitrophenylphosphate concentration tested, against the *p*-nitrophenylphosphate concentration gives a straight line (Fig. 2B). In the experiment in Fig. 2, the effect of *p*-nitrophenylphosphate on Ca^{2+}-dependent ATP hydrolysis by the vesicles was also measured. It can be seen that the experimental points of ATPase activity can be fitted by the same curve that fits Ca^{2+} transport. This result was confirmed in three independent experiments and indicates that, within the range of *p*-nitrophenylphosphate concentration tested, the stoichiometry of the transport reaction is independent of *p*-nitrophenylphosphate and hence that *p*-nitrophenylphosphate hydrolysis is not associated with Ca^{2+} transport.

We have reported elsewhere that inhibition of Ca^{2+}-ATPase from disrupted membranes by *p*-nitrophenylphosphate takes place along an S-shaped curve (see Fig. 9 in Ref. 4). This contrasts

with the simple hyperbolic inhibition shown in Fig. 2. We have no explanation for this discrepancy. It should be mentioned, however, that in the experiment of Fig. 2 we could have missed an S-shaped curve because ATPase activity was measured from the amount of P_i released by the vesicles after incubation at 37°C during 3 min, a procedure which is less accurate than that used for the experiments in disrupted membranes in which the incubation at 37°C to measure ATPase activity lasted 30 min. However, differences in the behaviour of the Ca^{2+} pump depending on whether it belongs to disrupted membranes or IOVs and/or an effect of oxalate might also be responsible for the difference in the kinetics of inhibition. Because of this, we decided to test the effects of *p*-nitrophenylphosphate on Ca^{2+} transport and Ca^{2+}-ATPase activity in IOVs which have not been loaded with oxalate. Fig. 3 shows the results of a typical experiment with these vesicles. The rate of Ca^{2+} uptake in the absence of *p*-nitrophenylphosphate is about 20-times and Ca^{2+}-ATPase activity about 2-times lower than in the oxalate-loaded IOVs. The effect of *p*-nitrophenylphosphate is biphasic. At low concentration, *p*-nitrophenylphosphate increases the rate of Ca^{2+} uptake by the vesicles, but as its concentration rises, the uptake of Ca^{2+} is inhibited upon reaching 65% of the control at 40 mM *p*-nitrophenylphosphate. Fig. 3 also shows that, along the range of *p*-nitrophenylphosphate concentration tested, the experimental points of Ca^{2+}-ATPase activity follows the biphasic response of Ca^{2+} uptake. It seems, therefore, that to some extent the shape of the inhibition curve of Ca^{2+}-ATPase and Ca^{2+} transport by *p*-nitrophenylphosphate depends on the membrane preparation and the assay condition. In spite of this, under all the condition used here, the effect of *p*-nitrophenylphosphate on Ca^{2+}-ATPase activity followed step-by-step that on Ca^{2+} uptake, indicating that up to 40 mM, *p*-nitrophenylphosphate does not change the stoichiometry of the transport process. Results in this paper are therefore consistent with the idea that *p*-nitrophenylphosphate does not serve as the energy source for Ca^{2+} transport by the Ca^{2+} pump of human red cells.

Comparison of results in Fig. 2 and 3 shows that in oxalate-loaded IOVs the ratio of Ca^{2+} uptake to ATP hydrolysis is 2.6, whereas in oxalate-free IOVs the ratio is 0.35. It is likely that this difference expresses the ability of oxalate to retain intravesicular Ca^{2+} rather than an actual change in the stoichiometry of the Ca^{2+}-pump.

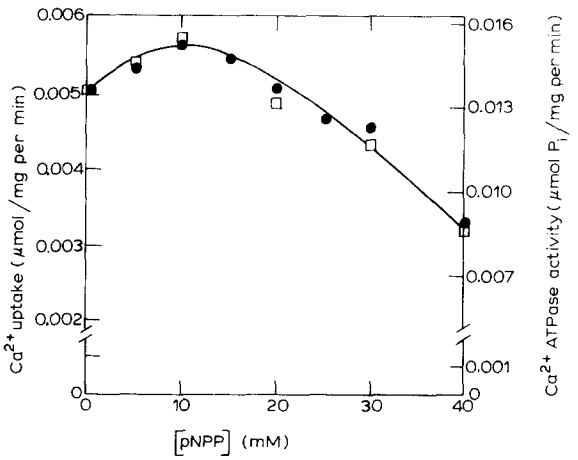

Fig. 3. Ca^{2+} uptake (●) and Ca^{2+}-ATPase activity (□) in IOVs without oxalate in media with 5 mM ATP as a function of *p*-nitrophenylphosphate (pNPP) concentration. Ca^{2+} uptake was measured at 37°C in 100 mM KCl/0.5 mM EGTA/2 mM $MgCl_2$/0.6 mM $^{45}CaCl_2/10^{-3}$ M ouabain/50 mM Tris-HCl (pH 7.8 at 37°C)/5 mM MgATP. When present, *p*-nitrophenylphosphate was added with $MgCl_2$ as in Fig. 2. Ca^{2+}-ATPase activity was measured in media of identical composition except that they contained [γ-^{32}P]ATP and $CaCl_2$ was non-radioactive. The measurements were made as in Fig. 2 except that the experiments were started by mixing 1 vol. of IOVs (1.0–1.5 mg protein/ml) in 100 mM KCl/10^{-3} M ouabain/50 mM Tris-HCl (pH 7.8 at 37°C) with 2 vol. of the same suspending medium containing adequate concentrations of ATP, $CaCl_2$, EGTA, ouabain, $MgCl_2$ and *p*-nitrophenylphosphate to give the composition of the incubation medium. The rate of Ca^{2+} uptake was constant during at least the first 7 min of incubation at 37°C. For other details, see legend of Fig. 1.

It could be argued that *p*-nitrophenylphosphate failed in activating Ca^{2+} uptake because IOVs do not hydrolyze *p*-nitrophenylphosphate. Although control experiments (not shown) demonstrated that the IOV preparations as a whole hydrolyze *p*-nitrophenylphosphate at a good rate, it is difficult to assess that point, since IOVs represented 25% of the membranes in the preparation. Nevertheless, there seems to be no reason to suppose that the IOVs could be impeded in hydrolyzing *p*-nitrophenylphosphate, because during phosphatase activity the sites for *p*-nitrophenylphos-

phate, Ca^{2+} and ATP are located on the inner surface of the red cell membrane [5,12], so that in IOVs they should be exposed to the incubation media, giving free access to all these ligands. If this is so, from previous experiments in disrupted membranes [4], it can be calculated that in media with 5 mM ATP, at the highest *p*-nitrophenylphosphate concentration tested in this paper (40 mM) the phosphatase activity is at least 0.012 μmol *p*-nitrophenylphosphate/mg protein per min. This means that, were *p*-nitrophenylphosphate and ATP equally effective in promoting Ca^{2+} transport, under the conditions used 40 mM *p*-nitrophenylphosphate should have increased 25–50% the rate of Ca^{2+} transport, a change too high to pass unnoticed. Furthermore, from the maximum concentration gradient attained during Ca^{2+} transport in sarcoplasmic reticulum vesicles, Inesi [13] has calculated the Gibbs energy of hydrolysis of *p*-nitrophenylphosphate and ATP to be respectively -10.5 kcal/mol and -12.4 kcal/mol. From these values and the Ca^{2+} gradient imposed on the IOVs, it is clear that in the experiments shown here, the transport of Ca^{2+} by *p*-nitrophenylphosphate has not been energetically limited.

Lack of ability to use *p*-nitrophenylphosphate for active transport is not unique to the Ca^{2+} pump of red cells. In fact, Brandley and Mullins [14] and Garrahan and Rega [15] have demonstrated in perfused axons and resealed ghosts from human red cells, respectively, that *p*-nitrophenylphosphate is unable to induce either Na^+-K^+ or Na^+-Na^+ exchange through the Na^+ pump.

In contrast with this, in sarcoplasmic reticulum *p*-nitrophenylphosphate drives active calcium transport in the absence of ATP, with the coupling of 2 mol of Ca^{2+} per mol of *p*-nitrophenylphosphate hydrolyzed [13]. The findings in this paper together with the need of ATP at the high-affinity site of the Ca^{2+} pump of red cells for phosphatase activity [4] suggest that the mechanism of the Ca^{2+}-phosphatase of red cells may be substantially different from that of sarcoplasmic reticulum.

This work was supported by grants from CONICET and SUBCYT (Argentina), the PNUD-UNESCO RLA 78/24 and the Programa Regional de Desarrollo Científico y Tecnológico of the OEA. A.J.C. is recipient of a fellowship from CONICET. P.J.G. and A.F.R. are established investigators of CONICET.

References

1 Richards, D.E., Rega, A.F. and Garrahan, P.J. (1978) Biochim. Biophys. Acta 511, 194–201
2 Muallem, S. and Karlish, S.J.D. (1979) Nature 277, 238–240
3 Garrahan, P.J. and Rega, A.F. (1978) Biochim. Biophys. Acta 513, 59–65
4 Caride, A.J., Rega, A.F. and Garrahan, P.J. (1982) Biochim. Biophys. Acta 689, 421–428
5 Rega, A.F., Richards, D.E. and Garrahan, P.J. (1973) Biochem. J. 136, 185–194
6 Lew, V.L., Muallem, S. and Seymour, C.A. (1982) Nature 296, 742–744
7 Steck, T.L. and Kant, J.A. (1974) Methods Enzymol. 31, 172–177
8 Mollman, J.E. and Pleasure, D.E. (1980) J. Biol. Chem. 255, 569–574
9 Sarkadi, B., Szász, I. and Gárdos, G. (1980) Biochim. Biophys. Acta 598, 326–338
10 Wilkinson, G.N. (1961) Biochem. J. 80, 324–332
11 Lundahl, P. (1975) Biochim. Biophys. Acta 379, 304–316
12 Rega, A.F., Garrahan, P.J. and Wainer, S.R. (1972) Experientia 28, 1158–1159
13 Inesi, G. (1971) Science 171, 901–903
14 Brinley, F.J. and Mullins, L.J. (1968) J. Gen. Physiol. 52, 181–211
15 Garrahan, P.J. and Rega, A.F. (1972) J. Physiol. 223, 595–617