# STUDIES ON HEART SARCOLEMMA: VESICLES OF OPPOSITE ORIENTATION AND THE EFFECT OF ATP ON THE $Na^+/Ca^{2+}$ EXCHANGER

L. REINLIB, P. CARONI and E. CARAFOLI
*Laboratory of Biochemistry, Swiss Federal Institute of Technology (ETH), 8092 Zürich, Switzerland*

Received 16 February 1981

## 1. Introduction

Recent investigations on heart sarcolemma vesicles [1–6] have extended the knowledge of the $Na^+/Ca^{2+}$ exchanger which was available from in vivo studies. It now appears established that the exchange operates electrogenically, that it has a higher affinity for $Ca^{2+}$ than previously assumed ($K_m$ 1.5 $\mu$M), and that it has a considerable $V_{max}$ for $Ca^{2+}$ transport (up to 20 $\mu$mol . $mg^{-1}$ . $s^{-1}$). A study of the different mechanisms of charge compensation has established that $K^+$ and $Cl^-$ movements are probably both active in vivo [6,7].

A problem that is still open for study is that of the symmetric or asymmetric functioning of the exchanger. This would be optimally studied by separating the sarcolemmal vesicles into 2 populations: inside out and rightside out. This separation has been successfully performed [8] with the aid of wheat germ lectin covalently bound to a Sepharose 6MB column.

We have found it difficult to elute the sarcolemmal vesicles, once they have been bound to the gel. It was therefore decided to use 2 different gel affinity procedures to eliminate vesicles of a given polarity from a mixed population. The results indicate that a very good separation of vesicles of opposite polarity has indeed been achieved. It is important to emphasize that a highly purified preparation of sarcolemma was used as the starting material. The specific activity of the $Na^+$-$K^+$-ATPase typically exceeded 130 $\mu$mol phosphate liberated . mg $protein^{-1}$ . $h^{-1}$.

As ATP has been repeatedly reported to stimulate the exchange of $Na^+$ for $Ca^{2+}$ in axonal membranes [9–14], a study of the effect of ATP on the same transporter in sarcolemmal vesicles was carried out. ATP was indeed found to stimulate the initial rate of $Ca^{2+}$ uptake at low (10 $\mu$M) $Ca^{2+}$ levels.

## 2. Materials and methods

Sarcolemma from dog heart was prepared as in [6] and suspended in 160 mM NaCl, 20 mM Hepes (pH 7.40) (soln. 1). The protein concentration was determined by a modification of the method in [15].

$Ca^{2+}$ changes were measured with arsenazo III [16] at 685–660 nm in an Aminco DW-2 dual wavelength spectrophotometer. The composition of the medium is detailed in the legends. $Ca^{2+}$-ATPase activities were measured by a coupled enzyme assay as indicated in [17].

Inside out vesicles were prepared by a Sephadex–concanavalin A (con A) batch technique, essentially as in [18]. Sarcolemmal vesicles (400 $\mu$g) washed twice in soln. (1) to remove extraneous sugar residues, were gently shaken 20 min at room temperature with the gel. The gel was allowed to sediment to the bottom of the tube, and washed 3 times with soln. (1) on ice. All supernatants were combined, and centrifuged at 140 000 × $g$ for 30 min. The vesicles were resuspended in soln. (1) at 2 mg protein/ml.

To prepare rightside out vesicles, ATP bound to activated CH–Sepharose 4B was used. $Na_2ATP$ at 54 mg/g swollen, washed CH–Sepharose 4B, were coupled as recommended [19]; 0.5 ml swollen gel was washed with soln. (1) plus 0.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, and mixed with 400 $\mu$g sarcolemmal vesicles. The remainder of the ATP-coupled gel was stored at 2°C in the presence of 1 mM $NaN_3$. The sarcolemmal vesicles were gently shaken with the gel for 30 min, at room temperature. The supernatant was removed, and 3 successive washings with the same buffer were done on ice. The sarcolemma were centrifuged at 140 000 × $g$, 30 min, and resuspended in soln. (1) at 2 mg protein/ml.

To study the effect of ATP on the $Na^+/Ca^{2+}$

 *Elsevier/North-Holland Biomedical Press*

exchange, it was first necessary to remove endogenous ATP. Sarcolemmal vesicles were incubated 30 min at room temperature in 9 vol. 20 mM Hepes to render them somewhat leaky, thereby allowing accessibility of the reactants to the inside of the vesicles. Glucose and $MgCl_2$ were added to 20 $\mu$M final conc. before adding 1 unit (Sigma) hexokinase. The enzyme was allowed to react for 30 min at room temperature. Then the sarcolemma were washed in soln. (1) and centrifuged at 140 000 × $g$ for 30 min, and resuspended in soln. (1).

The determination of ATP in sarcolemmal suspensions was performed with the luciferin–luciferase (Sigma, L-0633) method, using 2 mg enzyme in 3 ml $H_2O$ containing 5 $\mu$l of the sarcolemmal sample to be analysed, in a plastic scintillation vial. The vial was quickly shaken by hand, and immediately counted for 1 min in a Philips PW 4510/01 Liquid Scintillation Counter, with the coincidence circuit switched off. Log(cpm) were plotted *vs* –log[ATP] of standards and values for sarcolemma were determined from the curve.

Sephadex G-100 and activated CH–Sepharose 4B were obtained from Pharmacia; con A, hexokinase, ATP and luciferin–luciferase were obtained from Sigma Chemical Co. All other reagents were of the highest grade available.

## 3. Results and discussion

### 3.1. *Separation of oppositely polarized sarcolemmal vesicles*

The isolation of separate populations of sarcolemmal vesicles was carried out by exploiting the asymmetric distribution of carbohydrates and the ATP-binding sites. The $Ca^{2+}$-ATPase, like other ATPases, has its ATP-binding site on the intracellular side, while the carbohydrate groups of glycolipids and glycoproteins are located exclusively on the outer membrane face [20]. By attaching con A to a gel, a system was prepared which was used to remove vesicles with exposed carbohydrate groups from the mixed population. The gel, along with the attached sarcolemmal vesicles, was then discarded, leaving a suspension of vesicles, all of which should be inside out. The sidedness of the vesicles remaining was estimated by examining the specific activity of the $Ca^{2+}$-ATPase. Table 1 shows the results of typical separation experiments. A high recovery of vesicles, and a 96% increase in the specific activity of the $Ca^{2+}$-ATPase followed

Table 1
Comparison of the properties of the different populations of sarcolemmal vesicles

| Treatment | Protein recovery | $Ca^{2+}$-ATPase ($\mu$mol $P_i$ . mg protein$^{-1}$ . h$^{-1}$) | $Na^+/Ca^{2+}$ exchange (nmol $Ca^{2+}$ . mg protein$^{-1}$ . s$^{-1}$) |
|---|---|---|---|
| – | – | 2.70 | 4.4 |
| Sephadex–con A | 44.7% | 5.28 | 4.4 |
| CH-Sepharose–ATP | 53.5% | 0.23 | 3.0 |

Protein recovery is listed as % of applied protein. $Ca^{2+}$-ATPase was measured as in [17], $[Ca^{2+}]$ = 30 $\mu$M. $Na^+/Ca^{2+}$ exchange was measured as follows: 25 $\mu$g sarcolemmal vesicles were preloaded with NaCl in 160 mM NaCl, 20 mM Hepes (pH 7.40) for 20 min at 37°C, in 10 $\mu$l total vol. This was then diluted into 1.6 ml 160 mM KCl, 20 mM Hepes, 0.7 $\mu$M valinomycin, 50 $\mu$M arsenzo III, 8 $\mu$M $Ca^{2+}$. Initial rates were estimated from the first 3 s of $Ca^{2+}$ uptake. All values are average of ⩾2 expt.

treatment of the mixed population with Sephadex G-100–con A, indicating an enrichment in inside out vesicles in the fraction obtained. A similar procedure, treatment of a mixed population with CH–Sepharose 4B–ATP, was utilized to bind, and remove, rightside out vesicles. The vesicles obtained after the latter treatment had a specific activity of $Ca^{2+}$-ATPase which was <10% of that of the control population, indicating a large majority of rightside out vesicles. All fractions displayed a similar ability to take up $Ca^{2+}$ in response to a preformed $Na^+$ gradient. This can be considered an important control, in that it shows that the low $Ca^{2+}$-ATPase activity in the rightside out vesicles is not due to non-specific damage.

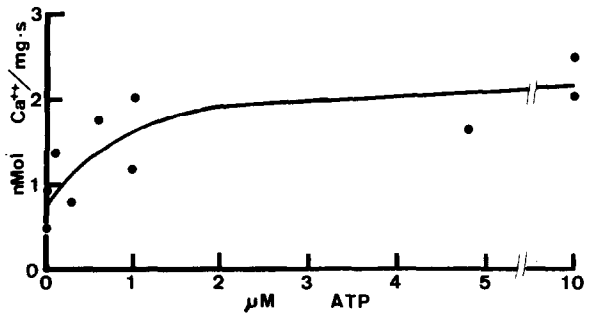


Fig.1. Effect of ATP on the $Na^+/Ca^{2+}$ exchange. In all cases, $MgCl_2$ was added in equal concentration to $K_2ATP$. $Na^+/Ca^{2+}$ exchange measured as in table 1. In addition, the medium contained: 8 $\mu$M Na vanadate; 0.2 $\mu$M digitoxigenin; 3 $\mu$M oligomycin; 0.52 $\mu$M $[Ca^{2+}]_{free}$.

### 3.2. *Effect of ATP on the $Na^+/Ca^{2+}$ exchange*

ATP was eliminated from the sarcolemmal vesicles as indicated in section 2. The assay indicated that ATP was reduced in the stock suspension of sarcolemmal vesicles from 4.0–0.4 $\mu$M (av. 3 expt).

The effect of increasing medium [ATP] on the rate of $Na^+/Ca^{2+}$ exchange is shown in fig.1. $Ca^{2+}$ uptake due to the possible activity of interfering ATPases was prevented by the addition of digitoxigenin, oligomycin and Na-vanadate. A definite stimulation of up to 4-times could be seen at ~1 $\mu$M added ATP, which failed to increase further at higher ATP concentrations.

Thus, in agreement with what has been found in axonal membranes [10,13,21], the $Na^+/Ca^{2+}$ exchange of heart sarcolemma is stimulated by low [ATP]. A question remaining is that of the mechanism of the ATP effect, i.e., whether ATP hydrolysis is required or not. Future work will investigate this problem as well as the question of the symmetric or asymmetric behaviour of the exchange.

**Acknowledgement**

This work was made possible by the financial contributions of the Swiss Nationalfonds (grant 3.282-0.78).

**References**

[1] Reuter, H. and Seitz, N. (1968) J. Physiol. 195, 451–470.
[2] Jundt, H., Prozig, H., Reuter, H. and Stucki, J. W. (1975) J. Physiol. 246, 229–253.
[3] Reeves, J. P. and Sutko, J. L. (1979) Proc. Natl. Acad. Sci. USA 76, 590–594.
[4] Pitts, B. J. R. (1979) J. Biol. Chem. 254, 6232–6235.
[5] Mas-Oliva, J., Williams, A. and Nayler, W. (1979) Biochem. Biophys. Res. Commun. 87, 441–447.
[6] Caroni, P., Reinlib, L. and Carafoli, E. (1980) Proc. Natl. Acad. Sci. USA 77, 6354–6358.
[7] Philipson, K. and Nishimoto, A. (1980) J. Biol. Chem. 255, 6880–6882.
[8] Mas-Oliva, J., Williams, A. and Nayler, W. (1980) Analyt. Biochem. 103, 222–226.
[9] Baker, P. F. (1976) Fed. Proc. Fed. Am. Soc. Exp. Biol. 35, 2589–2595.
[10] Baker, P. F. (1972) Prog. Biophys. Mol. Biol. 24, 177.
[11] Baker, P. F. and McNaughton, P. A. (1976) J. Physiol. 259, 103–144.
[12] Baker, P. F. and Glitsch, H. G. (1973) J. Physiol. 233, 44P–46P.
[13] DiPolo, R. (1974) J. Gen. Physiol. 64, 503–517.
[14] DiPolo, R. (1978) Nature 274, 390–392.
[15] Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.
[16] DiPolo, R., Regrena, J., Brinley, F. J., Mullins, A., Scarpa, A. and Tiffert, T. (1976) J. Gen. Physiol. 67, 433–467.
[17] Niggli, V., Penniston, J. and Carafoli, E. (1979) J. Biol. Chem. 254, 9955–9958.
[18] Edelman, G. M., Rutishauser, U. and Millete, C. F. (1971) Proc. Natl. Acad. Sci. USA 68, 2153–2157.
[19] Pharmacia Fine Chemicals (1979) Affinity Chromatography, pp. 24–27, Ljungföretegen AG, Örebro, Sweden.
[20] Bretscher, M. S. and Raff, M. C. (1975) Nature 258, 43–49.
[21] Blaustein, M. P. (1976) Fed. Proc. Fed. Am. Soc. Exp. Biol. 35, 2574–2478.