# DEMONSTRATION OF NEW $Ca^{2+}$ BINDING SITES BY VESICULAR FRACTION OF SMOOTH MUSCLE CELL PLASMA MEMBRANES IN THE PRESENCE OF ALAMETHICIN

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Recent investigations using Scatchard plots, electrophoresis, and current-voltage characteristic curves have demonstrated that plasma membranes (PM) of skeletal, cardiac, and smooth muscles, leukocytes and hepatocytes, nerve and kidney tissue cells, and brush-border, placental, and other cells possess Ca-binding capacity [2, 5, 8]. However, until recently, no attention was paid in $Ca^{2+}$-binding studies to changes in the number of types of binding sites of PM and their affinity, observed in different incubation media. Yet the study of such changes is very important both in experimental biochemistry (for example, in the study of Ca channels, the Ca pump, and Na/Ca exchange) and in clinical medicine in the treatment of diseases of the muscular system and the alleviation of muscular dysfunctions with the aid of therapeutic agents acting on the myocyte by a Ca-dependent mechanism.

This paper analyzes the results of investigations into $Ca^{2+}$ binding by PM of smooth-muscle cells with dependence on external environmental conditions.

## EXPERIMENTAL METHOD

The PM fraction was isolated from smooth-muscle tissue of the rabbit small intestine by differential centrifugation, the free $Ca^{2+}$ concentration was determined by potentiometric titration with EGTA solution and by measuring fluorescence of Quin-2 (allowing for binding constants of the ions [7], binding of the cation ($^{45}CaCl_2$, 4-6 $\mu$Ci/ml) was determined by measuring radioactivity of the preparation on an Sh-4000 counter, and the concentration of ionized $Ca^{2+}$ was calculated on a Labtam-3003 computer [6]. Alamethicin was obtained from the Department of Biochemistry, Moscow University.

## EXPERIMENTAL RESULTS

As Table 1 shows, the PM fraction consisted of membrane vesicles with the correct (cytoplasmic side inward) and incorrect (outward) orientations. Using data for ouabain binding it was possible to determine not only the orientation of the vesicles, but the completeness of isolation of PM from a unit mass of tissue. On the basis of data given in Table 1 and determination of the number of isolated cells from 1 g wet weight of tissue ($2.1 \cdot 10^7$), the yield of PM protein was 0.44 mg/g tissue, and the completeness of isolation exceeded 96%. The extracellular space, determined as the difference between the volume of tissue and the total volume of isolated cells from a similar weighed sample, was $44.3 \pm 2.4\%$.

The data on the completeness of isolation and orientation of the vesicles of PM shown in Table 1 are essential for the study of $Ca^{2+}$ binding for at least two reasons. First, the number and affinity of binding sites for the cation, and also the number of binding sites obtained on the membrane fraction, must be compared with the corresponding results of tissue studies. Second, because of asymmetry of the membranes [5], $Ca^{2+}$ binding processes with the inner and outer surfaces of PM will differ. To ensure complete binding of the cation by membrane vesicles with different orientation, we added alamethicin to the medium, for this has

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TABLE 1. Determination of Orientation of Vesicles by Change in 5'-Nucleotidase and $Na^+,K^+$-ATPase Activity and Binding of $^3H$-Ouabain

| Parameter | | Value of parameter | Ratio of values of activity | Correctly oriented vesicles, % |
|---|---|---|---|---|
| 5'-Nucleotidase | | 130 | — | — |
| 5'-Nucleotidase in the presence of $7 \times 10^{-5}$ M sodium deoxycholate | | 250 | 0,510 | 51,0 |
| $Na^+,K^+$-ATPase | nmoles $R_i$/mg | 215 | — | — |
| $Na^+,K^+$-ATPase in the presence of 7.5 mM sodium cholate | protein/min | 440 | 0,489 | 51,1 |
| $Na^+,H^+$ATPase in the presence of 0.75 mg alamethicin/mg PM | | 365 | 0,569 | 41,1 |
| Binding of ouabain by homogenate, pulses/g tissue/min | | 1 600 | — | — |
| Binding of ouabain by PM fraction | pulses/mg protein/min | 151 200 | — | — |
| Binding of ouabain by PM fraction in the presence of alamethicin (0.75 mg/mg protein) | | 360 000 | 0,420 | 42,0 |

TABLE 2. Calcium Binding by Plasma Membrane of Smooth-Muscle Cells

| Incubation medium | Affinity of binding sites | Dissociation constant $K_d$, M | No. of binding sites, nmoles/mg PM protien | |
|---|---|---|---|---|
| | | | affinity sites | total number |
| 50 µg/ml of PM protein, 250 mM sucrose, 50 mM imidazole-HCl, 5 mM ATP, 5 mM $MgCl_2$, 0.1 mM oxytocin, 5 mM $NaN_3$ (18) | High | $4,0 \cdot 10^{-5}$ | 220 | 280 |
| | Low | $8,0 \cdot 10^{-4}$ | 60 | |
| The same medium but without ATP and ATPase inhibitors (11) | High | $7,6 \cdot 10^{-5}$ | 23 | |
| | Low | $9,4 \cdot 10^{-4}$ | 198 | 501 |
| | Low | $1,2 \cdot 10^{-3}$ | 280 | |
| GTP, CTP, UTP, and ADP instead of ATP (20) | High | $3,8—4,1 \cdot 10^{-5}$ | 54—62 | |
| | Low | $7,9—8,5 \cdot 10^{-4}$ | 213—247 | 132 |
| The same medium, but without $Mg^{++}$ (13) | High | $9,1 \cdot 10^{-6}$ | 28 | |
| | High | $9,3 \cdot 10^{-5}$ | 76 | 344 |
| | Low | $8,6 \cdot 10^{-4}$ | 240 | |
| The same medium with albumin, 200 µg/mg PM protein(5) | High | $4,3 \cdot 10^{-5}$ | 46 | 131 |
| | Low | $5,1 \cdot 10^{-3}$ | 185 | |
| 125 mM NaCl instead of sucrose (11) | High | $3,7 \cdot 10^{-5}$ | 51 | 211 |
| | Low | $1,1 \cdot 10^{-3}$ | 160 | |
| 125 mM KCl instead of sucrose (11) | High | $4,4 \cdot 10^{-5}$ | 70 | |
| | Low | $4,6 \cdot 10^{-3}$ | 292 | 362 |
| Tissue of small instestine, Krebs' solution (7) | High | $4,3 \cdot 10^{-5}$ | 32 | |
| | Low | $5,0 \cdot 10^{-4}$ | 100 | 132 |
| Tissue of small intestine, Krebs' solution + 1-5 mM ATP (9) | High | $4,3 \cdot 10^{-5}$ | 40 | 164 |
| | Low | $4,8 \cdot 10^{-4}$ | 124 | |
| Tissue of guinea pig taenia coli, Krebs'solution | High | $4,4 \cdot 10^{-5}$ | 40 | |
| | Low | $7,7 \cdot 10^{-4}$ | 140 | 180 |

**Legend.** Number of experiments shown in parenthesie.

a detergent-like action on membranes [4] and, as our investigations showed, it does not change their enzyme activity or the free $Ca^{2+}$ concentration in the medium.

As Table 2 shows, PM of smooth-muscle cells have two types of $Ca^{2+}$ binding sites, whose affinity for the cation is similar in value to the affinity of analogous sites obtained in tissue studies. As regards the number of $Ca^{2+}$ binding sites with membrane vesicles in the presence of alamethicin, which was twice the number found in tissue studies, this can easily be explained. First, in investigations on the PM fraction binding of the cation with both surfaces of the plasma membrane was recorded. Second, in tissue studies a certain proportion of Ca-binding sites was screened by intercellular protein, as is confirmed by changes in $K_d$ and the number of binding sites in the presence of albumin in the incubation medium of the PM fraction.

Replacement of ATP in the sucrose incubation medium by other nucleoside phosphates (Table 2) had no significant effect either on the affinity of the $Ca^{2+}$ sites or on the total number of binding sites. However, removal of ATP from the incubation

medium led to the appearance of a third binding site for $Ca^{2+}$ by the membrane and to a considerable increase in the total number of binding sites. An outwardly similar picture of an increase in $Ca^{2+}$ binding sites on PM also was observed after removal of $Mg^{2+}$ from the medium. In that case, however, a new binding site appeared with high affinity for the cation.

The observed effects can be explained as follows. In the absence of ATP the increase in the number of $Ca^{2+}$ binding sites with low affinity is the result of binding of the cation by those sites which bind the substrate of membrane ATPases. This is all the more probable because the substrates for these enzymes are Mg-ATP$^{--}$ complexes [3] and the affinity of enzymes for substrates, as has been shown for Ca,Mg-ATPase of PM of smooth-muscle cells [1], is comparatively low, namely $K_m = 10^{-4}$ M. Energy-yielding processes do not affect $Ca^{2+}$ binding after removal of ATP from the medium, as shown by calculations based on the experimental results: $\Delta F < 0$ (during binding of 1 M $Ca^{2+}$ 6-8 kcal of heat energy is given out), $\Delta S > 0$ (44-46 kcal $\cdot$ mole$^{-1}$ $\cdot$ deg$^{-1}$). So far as the appearance of new $Ca^{2+}$ binding sites in the absence of $Mg^{2+}$ ions is concerned, it can be postulated that those sites with which $Mg^{2+}$ ions were bound became accessible to $Ca^{2+}$ ions, in conformity with the well-known views on competitive relations between these cations on PM [2, 5]. However, the decrease in affinity of both types of its binding sites for $Ca^{2+}$ after removal of $Mg^{2+}$ from the medium (Table 2) can hardly be explained by competitive relations between the cations. It is quite possible that the presence of $Mg^{2+}$ bound with PM is responsible for the higher specificity of the Ca-binding sites. Replacement of the $Mg^{2+}$ ions by calcium in these sites can no longer ensure the initial specificity of the membranes for $Ca^{2+}$. Consequently, the appearance of new high-affinity binding sites for $Ca^{2+}$ and the decrease in specificity of calcium binding sites existing in the presence of $Mg^{2+}$ are evidence that these cations can exhibit not only competition, but also synergism in relation to PM of smooth-muscle cells.

The results described above were obtained in a "sucrose" medium. If sucrose was replaced by KCl and NaCl (Table 2) the appearance of new types of $Ca^{2+}$ binding sites was not observed. However, the characteristics of the binding sites and their number changed significantly, in agreement with the results of investigations on aortic microsomes [8]: If $K^+$ ions in the incubation medium were replaced by sodium, the affinity of PM for $Ca^{2+}$ was modified, the number of high-affinity binding sites was reduced by 30%, and the number of low-affinity sites by 45%. The changes observed can be explained on the grounds that sodium, activating the Na/Ca carrier, increases the affinity of PM for $Ca^{2+}$, and this is shown as a decrease in $K_d$ of the sites of the first type. $K^+$ ions do not possess this property, as shown by the increase in $K_d$ of these sites. So far as the decrease in affinity of sites of the second type for $Ca^{2+}$ in the presence of $K^+$ or $Na^+$ ions is concerned, this may be the result of competition, although the binding constants of monovalent cations with different anionic groups are 1-2 orders of magnitude less than those for $Ca^{2+}$ [3].

After removal of ATP from the incubation medium new type of binding sites of low affinity was thus found on PM, whereas after removal of $Mg^{2+}$, new type of high-affinity sites for calcium was found. These changes in the character of $Ca^{2+}$ binding by PM of myocytes must be taken into account both in biochemical investigations of Ca-dependent phenomena on membrane fragments and also in medical practice, during the use of therapeutic substances which exert their own influence on the cell by Ca-dependent mechanisms.

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