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CALCIUM BINDING TO BRAIN PLASMA MEMBRANES

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SUMMARY

Ca^{2+} binding to two kinds of brain membranes is studied. The samples include neuronal-enriched plasma membranes from newborn rat brain and myelin from adult rat brain. Two or more apparent Ca^{2+} binding sites are detected in the two membranes. At $5 \cdot 10^{-2}$ M Ca^{2+} myelin binds 830 and plasma membranes 520 nmoles of Ca^{2+} per mg protein. The Ca^{2+}-binding component is examined using enzymes and extraction but the treatment reduces binding by only 50%, which is attributable to phospholipids and neuraminic acid. Incubation with pronase appears to increase Ca^{2+} binding suggesting unmasking of Ca^{2+}-binding sites. Monovalent cations (Na^+, K^+) decrease Ca^{2+} binding non-competitively while Mg^{2+} shows competitive inhibition. 5-Hydroxytryptamine and ATP significantly decrease Ca^{2+} binding to plasma membranes but not to myelin.

INTRODUCTION

Ca^{2+} is thought to be involved in a number of nerve functions at the membrane level. Ca^{2+} appears to participate in excitation of the neuronal membrane possibly by triggering the action potential [1]. The cation is reported to be involved in the release of transmitter substances [2], in the binding of acetylcholine to its receptor in the neuromuscular junction [2], and in the intracellular mediation of cathecolamine response through the cyclic AMP system [3]. Although Ca^{2+} is suggested to play such an impressive role in the nervous system, there are few direct observations on the interactions of Ca^{2+} with neuronal membranes.

In the present study two kinds of rat brain membranes were used in Ca^{2+} binding assays in vitro. These included plasma membranes from immature rat brain, presumably enriched in developing neuronal surface [4], and myelin, a specialized, nonexcitable plasma membrane from adult brain. The binding sites for Ca^{2+} in the membrane preparations as well as the interference of ions and neuroactive substances with the binding process were investigated.

MATERIALS AND METHODS

Plasma membranes were purified from cortices of rats (4–6 days of age)

as described elsewhere [4]. The preparation is thought to contain neuroblast plasma membranes and is simply referred to as "plasma membranes". The isolated membrane preparation was stored in 0.1 M Tris–HCl, pH 7.5 at −20 °C.

Myelin was purified from cortices of adult rats by homogenizing them in 0.25 M sucrose with 15 strokes of a loosely fitting Teflon–glass homogenizer. The homogenate was centrifuged at $800 \times g$ for 10 min; the supernatant was centrifuged further at $10\,000 \times g$ for 10 min. The pellet was once washed with 0.25 M sucrose by centrifuging as above, resuspended in 0.85 M sucrose and centrifuged in a Spinco SW-25 rotor at $63\,000 \times g$ for 60 min. The flotate was collected, diluted with 2 vol. of water and sedimented at $10\,000 \times g$ for 10 min. The pellet was suspended in a large volume of water for 15 min and centrifuged again at $10\,000 \times g$ for 10 min. The myelin pellet was stored at −20 °C until used.

The Ca^{2+}-binding assays were performed largely as described by Shlatz and Marinetti [5]. Samples containing 20–50 μg of protein (50 μl) were incubated in 400 μl of 0.1 M Tris–HCl, pH 7.5, containing 0.5 μCi of $^{45}CaCl_2$ (spec. act. 10–40 Ci/g Ca, Radiochemical Centre, Amersham) and $^{40}CaCl_2$ (normally at 1 mM) at 37 °C for 10 min. 3 ml of Tris were added to the assay tubes and the mixtures were pipetted (automated Finnpipette) onto Millipore filters (0.45 μm), previously soaked in 0.25 M KCl. The membranes were washed with 2×4-ml batches of Tris with vacuum section and counted in 5 ml of Bray's scintillation fluid.

RESULTS

The conditions of Ca^{2+}-binding assays were adjusted in studies, where the amount of sample and binding time were varied (Fig. 1). Binding of Ca^{2+} to plasma membranes appeared to be linear with increasing quantities of membrane at least up to 75 μg of membrane protein. The binding was practically completed within 10 min, which time was used in the assays to follow.

The number of binding sites and their relative affinities were estimated by the

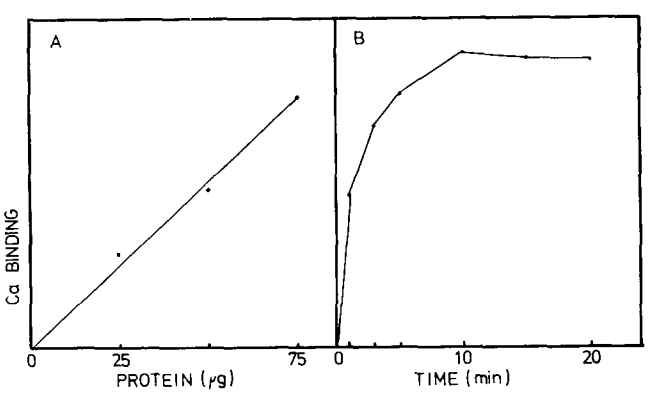

Fig. 1. Ca^{2+} binding to brain plasma membranes as a function of membrane concentration (A) and incubation time (B). 50 μl of membranes were incubated in 400 μl of 0.1 M Tris–HCl, pH 7.5, containing 1 mM $CaCl_2$ and 0.5 μCi $^{45}CaCl_2$ at 37 °C. Samples were collected on a Millipore filter and washed before liquid scintillation counting. The incubation time in A was 10 min.

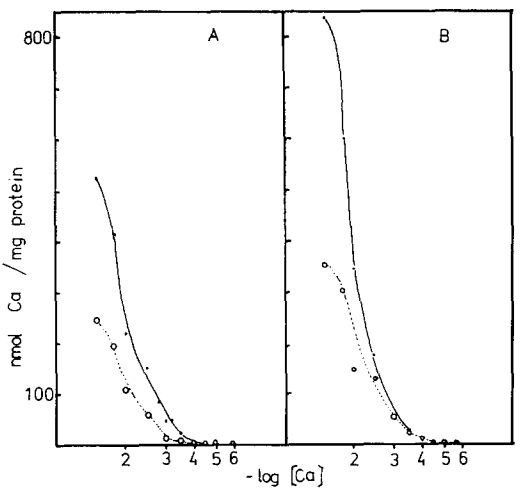

Fig. 2. Ca^{2+} binding to brain plasma membranes and myelin at different Ca^{2+} concentrations. (A) plasma membranes (●–●) and brain homogenate (○–○) from newborn rat cortex; (B) myelin (●–●) and homogenate (○–○) from adult rat cortex. Assays were performed by incubating samples as in Fig. 1 by varying the concentration of unradioactive $CaCl_2$. Means of 4–6 parallel determinations are shown.

graphic method established by Scatchard [6]. The concentration range tested varied from $4 \cdot 10^{-6}$ to $5 \cdot 10^{-2}$ M $CaCl_2$.

Immature plasma membranes bound more Ca^{2+} than the corresponding brain homogenate (Fig. 2A) and myelin more than adult brain homogenate (Fig. 2B). The binding capacity of the two membrane preparations was fairly similar at low concentrations of Ca^{2+}. At $5 \cdot 10^{-2}$ M Ca^{2+} myelin bound about 830 nmoles of

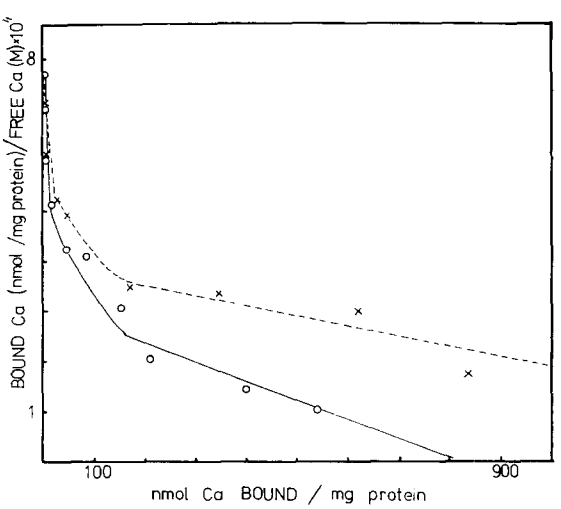

Fig. 3. A Scatchard plot for Ca^{2+} binding to brain plasma membranes (○–○) and myelin (· – ·). The binding values of Fig. 2 are plotted.

Ca^{2+} per mg protein as compared to 520 nmoles of Ca^{2+} per mg protein of plasma membranes. Each membrane showed two or more classes of binding sites as indicated by the biphasic shapes of the binding curves.

The data of Fig. 2 was plotted by the method of Scatchard [6] in Fig. 3 to extrapolate the number of binding sites (intercepts on the abscissa) and their association constants (intercepts on the ordinate/the number of binding sites). The binding sites of myelin, that of high and low affinity, were found to bind 70 and 1900 nmoles of Ca^{2+} per mg protein with association constants $1.1 \cdot 10^3\, l \cdot mole^{-1}$ and 20 $l \cdot mole^{-1}$, respectively. The high-affinity binding site of plasma membranes could accommodate 30 nmoles of Ca^{2+} per mg protein with an association constant of $2.8 \cdot 10^3\, l \cdot mole^{-1}$; the low-affinity binding site accommodated 820 nmoles Ca^{2+} per mg protein with an association constant of 37 $mole^{-1}$. In the two types of membranes medium affinity binding sites may also be present.

Characterization of the Ca^{2+}-binding components of brain plasma membranes and myelin was attempted by treating membranes with enzymes or selective extractions (Table I). A treatment of the membranes with pronase increased the binding activity: 2.7-fold in plasma membranes and 1.1-fold in myelin. This action suggests unmasking of Ca^{2+}-binding components in plasma membranes. Neuraminidase decreased Ca^{2+}-binding by 8 % in plasma membranes and by 29 % in myelin which indicates that neuraminic acid is a fairly important Ca^{2+}-binding component in myelin. An incubation of the membranes with phospholipase C failed to cause any large change in the Ca^{2+}-binding capacity. Another treatment, extraction of phospholipids with acetone, ammonia and water [7] caused a marked reduction in Ca^{2+} binding of plasma membranes, 43 %, and a moderate reduction in myelin, 20 %. The method of extraction appears very efficient in removing phospholipids [7] but it may remove other minor components as well. It is thus unclear whether the reduction in the binding capacity can entirely be attributed to phospholipids. The

TABLE I

THE EFFECT OF MEMBRANE MODIFICATIONS ON THEIR Ca^{2+} BINDING

Treatment	Ca^{2+} binding (% of control)			
	Plasma membranes	Homogenate	Myelin	Homogenate
Control	100	100	100	100
Pronase, 100 μg/ml*	273	290	110	120
Neuraminidase, 50 units/ml*	92	89	71	88
Phospholipase C, 100 μg/ml*	93	55	102	66
Phospholipid extraction**	57	43	80	56
Sonication***	91	41	92	79

* Enzyme incubations were performed at 37 °C for 45 min. The incubation media consisted of 0.1 M Tris, pH 7.5, and additionally 1 mM $CaCl_2$ with neuraminidase or 20 mM KCl and 0.5 mM $CaCl_2$ with phospholipase. After the incubation the samples were washed with 1 mM EDTA and 0.1 M Tris and Ca^{2+} binding was performed as normally at 1 mM $CaCl_2$.

** The samples were suspended on ice in 90 % acetone containing 10 % water and 0.01 % NH_3 for 10 min, centrifuged at 40 000 × *g* for 10 min and washed twice with 0.1 M Tris.

*** Branson sonifier, 2 · 10 s at setting 8. Means of at least 2 parallel determinations.

TABLE II

THE EFFECT OF PHOSPHOLIPID EXTRACTION ON Ca^{2+} BINDING TO BRAIN MEMBRANES

Phospholipid extraction was performed with a mixture of acetone, water and NH_3 as described in Table I. Means of 2 to 4 parallel determinations.

Treatment	Ca^{2+} (M)	Ca^{2+} binding (% of control)	
		Plasma membranes	Myelin
Control	10^{-2}	100	100
Extraction	10^{-2}	15	68
Control	10^{-3}	100	100
Extraction	10^{-3}	57	80
Control	10^{-4}	100	100
Extraction	10^{-4}	34	94

concentration of protein was unchanged during the extraction excluding large removal of proteins. The discrepancy between these two methods applied may be explained by the inability of phospholipase C to attack intact brain plasma membranes and myelin, as has been reported to be the case with *Mycoplasma* membranes [8]. Mild sonication of membranes affected their Ca^{2+} binding only slightly.

Together the observed effects of neuraminidase and phospholipid extraction on Ca^{2+} binding to brain membranes could account for only about 50 % of the total binding. Structural reasons such as imbedding of the Ca^{2+}-binding component in the membrane making it unsusceptible to enzyme may contribute to this low figure. Ca^{2+}-binding properties of the homogenate were largely analogous to plasma membranes indicating that the same kinds of Ca^{2+}-binding components function in intracellular membranes and in plasma membranes. Yet, some differences could also be detected including susceptibility of intracellular membranes to phospholipase C.

As phospholipids appeared to be responsible for a large proportion of Ca^{2+} binding in plasma membranes and myelin, their removal was also investigated at various Ca^{2+} concentrations in order to assess the affinity of the binding site in question (Table II). In the case of plasma membranes binding was markedly influenced both

TABLE III

THE EFFECT OF IONS ON Ca^{2+} BINDING TO BRAIN MEMBRANES

Means of 2–6 parallel determinations.

Salt added	Ca^{2+} binding (% of control)					
	Plasma membranes			Myelin		
Concn Ca^{2+} (M):	$5 \cdot 10^{-2}$	10^{-3}	10^{-5}	$5 \cdot 10^{-2}$	10^{-3}	10^{-5}
Control	100	100	100	100	100	100
NaCl, 150 mM	35	33	30	85	27	8
KCl, 150 mM	33	40	44	56	22	26
$MgCl_2$, 2 mM	62	60	34	56	36	12

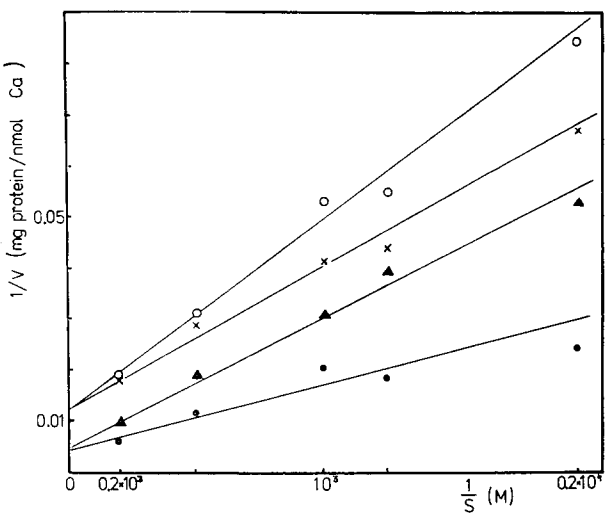

Fig. 4. A double-reciprocal plot for binding of Ca^{2+} to brain plasma membrane in the presence of ions. The incubations were performed at various concentrations of $CaCl_2$ with no addition (●–●), with 2 mM $MgCl_2$ (▲–▲), with 150 mM KCl (×–×) or with 150 mM NaCl (○–○). Means of two parallel determinations are shown.

at low and high Ca^{2+} concentrations indicating that phospholipids may be constituents of the low and the high affinity binding site. In the case of myelin, by contrast, the reduction of Ca^{2+} binding was preferentially observed at high Ca^{2+} concentrations, but even then phospholipids would not account for more than 38 % of Ca^{2+} binding.

The effects of ions on Ca^{2+} binding to brain plasma membranes and myelin were shown in Table III. Na^+, K^+ and Mg^{2+} appeared to decrease Ca^{2+} binding to the membrane preparations. Na^+ inhibited Ca^{2+} binding more than K^+ at low Ca^{2+} concentration in plasma membranes as well as in myelin, while at high concentrations their effectiveness was reversed. At a much lower concentration than Na^+ or K^+, Mg^{2+} inhibited Ca^{2+} binding roughly to the same extent. The interaction of ions with Ca^{2+} binding to plasma membranes is shown in Fig. 4. The inhibition by Mg^{2+} appeared to be competitive, while that of Na^+ and K^+ noncompetitive. No cooperativity of binding was detected.

Considering the large number of interactions of Ca^{2+} in the reactions of transmitter substances, it was of interest to perform the Ca^{2+}-binding assays in the presence of some putative transmitters and other neuroactive agents (Table IV). Significant effects were observed with plasma membranes only in the presence of 5-hydroxytryptamine and ATP, both eliciting inhibitory effects by 20 and 46 %, respectively. The effects may be caused by binding of Ca^{2+} to these compounds, as discussed below. The changes observed, significant and insignificant, were usually analogous in plasma membranes and myelin, and it is questionable, whether any specific properties of nerve plasma membranes were probed. Such changes are perhaps too small to be detected with the entire neuronal plasma membrane. Shlatz and Marinetti [5] observed a marked increase in Ca^{2+} binding to liver plasma mem-

TABLE IV

EFFECTS OF NEUROACTIVE SUBSTANCES ON Ca^{2+} BINDING TO BRAIN MEMBRANES

The concentration of Ca^{2+} was 1 mM. Means ±S.E. of at least 3 duplicate assays.

Addition	Ca^{2+} binding			
	Plasma membranes		Myelin	
	nmoles Ca^{2+}/mg protein	%	nmoles Ca^{2+}/mg protein	%
Control	34.2±1.6	100	23.1±2.7	100
Acetylcholine, 1 mM + eserine	38.5±2.6	113	25.9±7.7	112
γ-Aminobutyrate, 5 mM	33.1±2.0	98	21.7±2.1	94
Noradrenaline, 0.5 mM	35.2±2.5	103	18.6±4.6	81
5-Hydroxytryptamine, 1 mM	27.5±0.7*	80	18.8±3.4	82
ATP, 0.5 mM	18.5±1.3*	54	19.7±9.6	85
Cyclic AMP, 1 mM	37.2±1.4	109	25.7±0.5	111
EDTA, 5 mM	2.4±0.3*	7	–	

* Significant at $P < 0.05$ in a two-tailed Student's t-test.

branes in the presence of cyclic AMP, which could not be shown with the present membranes. EDTA, which was used as a check, abolished Ca^{2+} binding to plasma membranes by 93 %.

Numerous contradictory reports have been published on the influence of ATP on Ca^{2+} binding to plasma membranes [5, 9]. In the present study ATP was found to decrease calcium binding to brain plasma membranes and myelin (Table IV). This reaction was examined further, as Mg^{2+} has been reported to be an important variable in the system [9]. Table V shows that ATP appeared to abolish the inhibitory effect of Mg^{2+} on Ca^{2+} binding at a low Ca^{2+} concentration. At a high Ca^{2+} concentration the addition of ATP was without an effect. As both Ca^{2+} and Mg^{2+} are known to bind to ATP [10], it is likely that the effect of ATP is explained by its interference with these ions rather than by an active binding mechanism. The inhibitory effects of calcium on ATPase activity is a further complication.

TABLE V

THE EFFECTS OF Mg^{2+} AND ATP ON Ca^{2+} BINDING TO PLASMA MEMBRANES

Means of 2 determinations.

Incubation conditions	Ca^{2+}-binding (% of control)	
Concn Ca^{2+} (M):	10^{-3}	10^{-5}
Control	100	100
+ $MgCl_2$ (2 mM)	47	34
+ $MgCl_2$ (2 mM), ATP (0.5 mM)	53	109

DISCUSSION

Ca^{2+} participates in a number of membrane-associated physiological functions including maintenance of cell shape, involvement in transport mechanisms, release of secretory products, contraction in muscle and excitation in nerve [11, 12]. In nervous tissue Ca^{2+} is additionally involved in many steps of transmission. Biochemical work on the role of Ca^{2+} in nervous tissue has included purification of Ca^{2+}-binding proteins from brain [13], adrenal medulla [14] and squid axons [15]; Ca^{2+}-activated ATPase has been characterized from brain [16] and from actomyosin like proteins isolated from nerve ending constituents [17]; artificial membrane models have been constructed to study Ca^{2+} transport [18] and Ca^{2+} displacement [19]. Yet the author is unaware of any in vitro Ca^{2+}-binding assays with brain membranes, although such assays have been performed with retinal outer segments [20], liver [5] and erythrocyte plasma membranes [9].

Brain membranes used in this study, including plasma membranes, isolated from immature rat cortex, and myelin, isolated from adult brain, showed two or more classes of apparent Ca^{2+} binding sites each. The high-affinity binding site of plasma membranes with an association constant of $2.8 \cdot 10^3\,l \cdot mole^{-1}$ accommodated 30 nmoles of Ca^{2+} per mg protein and the low-affinity site with association constant of $37\,l \cdot mole^{-1}$ accommodated 820 nmoles Ca^{2+} per mg protein. The Ca^{2+}-binding sites of myelin had association constants of $1.1 \cdot 10^3$ and $20\,l \cdot mole^{-1}$ accommodating 70 and 1900 nmoles of Ca^{2+} per mg protein, respectively. The total number of binding sites was about 6-fold higher in brain plasma membranes and 13-fold higher in myelin as compared to liver plasma membranes, where lower Ca^{2+} concentrations were used in the assays [5]. It is thought that the values obtained largely represent true binding rather than transport or trapping, as the concentration curves (Fig. 2) were constructed with a constant amount of radioactive Ca^{2+} and showed no extensive background radioactivity.

In the present study Ca^{2+} binding was calculated per protein. As membranes extensively differ in their protein content, the absolute binding values vary according to the unit of reference selected. Myelin is a lipid-rich membrane with a protein/lipid ratio of 0.25 [21] as compared to brain plasma membranes of about 1.0 [22] and liver plasma membranes of 1.0–2.3 [21, 23]. If binding capacity is calculated per dry weight of membrane, brain plasma membranes appear to bind more Ca^{2+} than myelin. Plasma membranes and myelin differed in their Ca^{2+}-binding properties in several respects, in addition to the amounts of Ca^{2+} bound. The major Ca^{2+}-binding component in plasma membranes appeared to be phospholipid, while in myelin neuraminidase-sensitive carbohydrates and phospholipids were roughly equally important. It is of interest that one of the major polypeptides of myelin is reported to be a glycoprotein [24]. Ca^{2+} binding to the two kinds of membranes appeared to be differentially sensitive to ions. Na^+, K^+ and Mg^{2+} inhibited Ca^{2+} binding to plasma membranes more than to myelin at a high Ca^{2+} concentration, while the opposite was to case at low Ca^{2+} concentrations. The interference of ions in thepresent Ca^{2+}- binding assays contradicted with a report on liver plasma membranes, where hardly any effect was noted [5]. Ca^{2+} binding to plasma membranes and myelin also quantitatively differed in respect to the effects of 5-hydroxytryptamine and ATP, which caused a significant decrease in Ca^{2+} binding in plasma membranes only. The

presence of ATP in Ca^{2+} binding assay requires special caution, as it binds to Ca^{2+} [10].

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