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INTERACTION OF Ca^{2+} AND Mg^{2+} WITH SYNAPTIC PLASMA MEMBRANES

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SUMMARY

1. Synaptosomal fractions were isolated from sheep brain by sucrose density gradient centrifugation and were characterized by their succinate dehydrogenase and acetylcholinesterase activities. Five different fractions were collected. Succinate dehydrogenase activity increases from the top to the bottom of the gradients, while the acetylcholinesterase activity is maximal for the top and minimal for the bottom fractions.

2. Synaptic membranes bind Ca^{2+}, Mg^{2+} and other cations. We found two Ca^{2+} and Mg^{2+} binding components. The maximum Ca^{2+} binding capacities for each component are 115 and 90 nmoles/mg of protein, while for Mg^{2+} the corresponding values are 80 and 90 nmoles/mg protein. The affinity constants are 0.87 and 74 μM for Ca^{2+}, and 0.61 and 49 μM for Mg^{2+}.

3. Chlorpromazine at concentrations of 0.075 and 0.5 mM decreases the binding of Ca^{2+} by about 50 and 80%, respectively. Tetracaine has a much smaller effect; thus, at a concentration of 1 mM it displaces only 30% of the total Ca^{2+} bound. Procaine and the alkaloid caffeine were without effect on the Ca^{2+} bound. Acetylcholine displaces only 20% of the total Ca^{2+} bound when present at relatively high concentrations (5 mM).

4. Divalent cations increase the fluorescence of 8-anilinonaphthalene-1-sulfonic acid in aqueous suspensions of synaptic membranes. The order of efficiency of the cations is $Zn^{2+} \approx Cd^{2+} > Ca^{2+} \approx Sr^{2+} > Mg^{2+}$.

INTRODUCTION

The chemically-mediated synaptic transmission depends on Ca^{2+} in the extracellular medium[1–11]. In the absence of Ca^{2+}, the nerve impulse reaches the axon terminal but fails to release the chemical transmitter[8,9]. Other divalent cations such as Sr^{2+} and Ba^{2+} may substitute for Ca^{2+} in maintaining acetylcholine release at the neuromuscular junction[1,4,12], but normally they are less effective than Ca^{2+}. Other divalent cations such Mn^{2+} (ref. 13) and Mg^{2+} (refs 3, 7, 11) inhibit the release of the chemical transmitter even when Ca^{2+} is present in the medium.

The mechanism by which Ca^{2+} favors the release of transmitters by the nerve impulse is not yet known. Many workers have proposed that the Ca^{2+} action site is

Abbreviation: ANS, 8-anilinonaphthalene-1-sulfonic acid.

located at the membrane of the nerve terminal[3,7,12–14], since its action is too rapid to permit penetration. Ca^{2+} would combine with a specific site, X, leading to the formation of the complex CaX[6,7].

The ions which physiologically act as Ca^{2+} antagonists would compete with Ca^{2+} for the sames sites on the membrane avoiding the formation of the complex CaX needed for the release of the chemical transmitter. Some studies on end-plate and synaptic potentials provided information that a competition between Ca^{2+} and Mg^{2+} for the same sites may exist at nerve terminals[3,7] and different classes of sites have been suggested.

Quantitative studies relating the external Ca^{2+} concentration and the amount of transmitter released led to the suggestion that Ca^{2+} combine cooperatively with the membrane to form an "active release state"[6,9,11]. The action potential would produce a conformational change in the nerve terminal membrane and this change would result in a greatly increased activity of the proposed Ca^{2+} complex[7]. Conversely, Ca^{2+} would favor the action potential-dependent conformational change which would produce the release of neurotransmitters.

In the present study we isolated various fractions believed to contain synaptic plasma membranes from sheep brain. The fractions were characterized by their activities in acetylcholinesterase and succinate dehydrogenase. Furthermore, we studied the binding of Ca^{2+} and Mg^{2+} by synaptic membranes in order to establish the capacities and affinities of the membranes for these cations. On the other hand, we investigated possible conformational changes of the membrane macromolecules induced by those cations which may be involved in the cation-dependent mechanism of neurotransmitter release.

MATERIALS AND METHODS

Preparation of the biological material

Synaptic membranes were obtained from sheep brain according to the method described by Cotman and Matthews[15]. Synaptosomes were osmotically shocked in 6 mM Tris at pH 8.1. The particulate material was concentrated by centrifugation at $100000 \times g$ for 15 min and the sediment was suspended in a small volume of 10% sucrose. The suspension was layered over a discontinuous sucrose density gradient (25, 32.5, 35 and 38%, w/v) and centrifuged at $100000 \times g$ for 1.5 h. As described by Cotman and Matthews[15], we found four bands located approximately at the interfaces of the sucrose layers, and a brown pellet. We called the various fractions, Fraction 1, Fraction 2, Fraction 3, Fraction 4 and Fraction 5 (pellet). These symbols represent the order of the fractions from the top to the bottom of the gradient. The fractions were washed in 0.1 mM EDTA at pH 7.0 and were finally suspended in 10% sucrose and kept in the cold (0–4 °C) at a protein concentration of 5–10 mg/ml.

Analyses of protein

The protein was determined according to the Folin–Ciocalteu method[16] using standards of bovine serum albumin. We always included, in the standards, all the reagents present in the samples.

Determination of enzyme activities

Succinate dehydrogenase was determined by the method of Bonner[17] at room

temperature and pH 7.4 in a medium (total volume 3 ml) containing 83 mM Tris, 0.83 mM KCN, 16.5 mM sodium succinate, 0.83 mM $K_3Fe(CN)_6$ and 0.5–1.0 mg protein. The reaction was followed by recording the change in absorbance at 400 nm using a spectrophotometer–recorder set up. Appropriate blanks without $K_3Fe(CN)_6$ were run in order to correct for changes in absorbance due to the swelling or shrinking of the particulate material. We could estimate the amount of $K_3Fe(CN)_6$ reduced per min and, therefore, the amount of succinate oxidized.

Acetylcholinesterase activity was measured at room temperature and pH 7.0 in a medium (10 ml) containing 10% sucrose and 1 mM acetylcholine (freshly prepared) using a Radiometer pH-stat system by following the production of acid which was continuously titrated with 10 mM KOH delivered from an autoburette. Measurements were taken at 1-min intervals after an initial equilibration period of 3 min. Appropriate controls without protein were run in order to rule out non-enzymatic hydrolysis of acetylcholine. Other controls were run by omitting acetylcholine in the medium.

Binding of cations

Suspensions of protein (3–5 mg) were incubated for 10 min with stirring in a medium (10 ml) containing 4 mM imidazole pH 7.0, and the tested substances in the desired concentrations. Suspensions were centrifuged at $200000 \times g$ for 30 min. The pellets were washed by resuspension in 10% sucrose and were centrifuged again as previously. The pellets were then suspended in 1.0 ml of water and aliquots were taken for protein analysis. To the remaining suspension we added 0.9 ml of 20% trichloroacetic acid and the denaturated protein was sedimented in a table centrifuge. The concentrations of cations present in the last supernatants and those of the first centrifugation were measured in a Perkin Elmer, Model 305, atomic absorption spectrophotometer. For the analysis of Ca^{2+} and Mg^{2+} we included 0.5% La^{3+} in the samples and standards to avoid interferences[18].

Fluorimetric studies and binding of ANS

Fluorimetric experiments were conducted in a medium (3 ml) containing 10 mM histidine (pH 7.3), 0.1 mM 8-anilinonaphthalene-1-sulfonic acid (ANS) and 0.6 mg of protein suspensions. Substances to be tested were added in very small volumes using microburettes. Changes in fluorescence were measured in a Perkin Elmer, Model MPF-3, fluorimeter, set at 388 nm for excitation and 500 nm for emission. The contents of the cuvette were always well mixed before taking readings (1 min after the additions). Appropriate controls, without protein, were run and no changes in fluorescence could be detected. We always ran controls without ANS to correct for any changes in fluorescence due to the interaction of the various substances with the protein. At the wavelengths used, no changes in fluorescence were found for the controls.

The binding of ANS was studied by incubating suspensions of protein for 10 min at room temperature and pH 7.3 in a medium (10 ml) containing 10 mM histidine at pH 7.3, 25 μM ANS and Ca^{2+} as desired. After the incubation, the suspensions were centrifuged at $200000 \times g$ for 30 min and the ANS concentration present in supernatants was determined at 335 nm against blanks (without ANS) treated in the same manner as samples. The amount of ANS bound to the membranes

was determined by the difference between the concentration of ANS added and that present in supernatants.

Reagents

Reagents used were always of a suitable grade for biochemical analysis. Salts and acetylcholine were obtained from Merck; procaine·HCl, DL-histidine·HCl and imidazole from Sigma; tetracaine·HCl from KK Laboratories (New York); chlorpromazine·HCl from Smith Kline and French Laboratories (Philadelphia); ANS from Eastman; EDTA and Tris from Fisher.

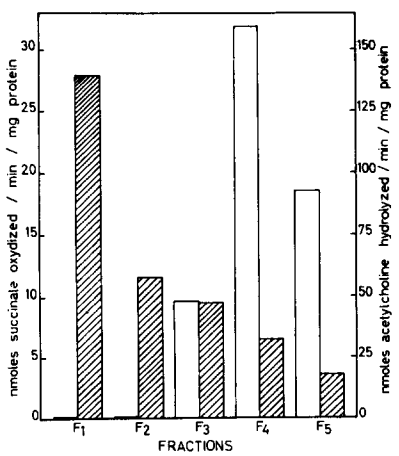
RESULTS

Biochemical characterization of the fractions

We characterized the various membrane fractions by using, as markers, succinate dehydrogenase and acetylcholinesterase activities.

In Fig. 1 we may distinguish the various fractions on the basis of their enzyme activities. Succinate dehydrogenase activity increases from the top to the bottom of the sucrose gradient and is maximal in Fraction 4 and almost zero in Fraction 1. Fraction 4 and Fraction 5 contain mitochondrial material and, possibly, Fraction 4 is the richest fraction in mitochondrial inner membrane.

Acetylcholinesterase activity decreases from the top to the bottom of the sucrose gradient (Fig. 1) being maximal for Fraction 1 and minimal for Fraction 5. The upper fraction of the gradient (Fraction 1) is extremely rich in acetylcholinesterase which represents an enrichment in synaptic membrane in this fraction.



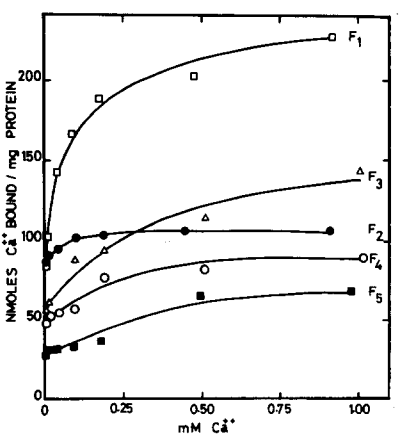


Fig. 1. Enzyme activities of synaptosomal fractions. The fractions are numbered in the order by which they appear in the sucrose density gradient after centrifugation (Fraction 1 near the top, Fraction 5 in the bottom). Acetylcholinesterase decreases from the top of the gradient to the bottom, and conversely for succinate dehydrogenase. Note the relatively high acetylcholinesterase activity of Fraction 1. Blank bars, succinate dehydrogenase; dashed bars, acetylcholinesterase.

Fig. 2. Binding of Ca^{2+} by synaptosomal fractions. The Fraction 1 is the most active fraction. In the abscissa we represent the free Ca^{2+} concentration in equilibrium with the complex membrane–Ca^{2+}.

Binding of cations

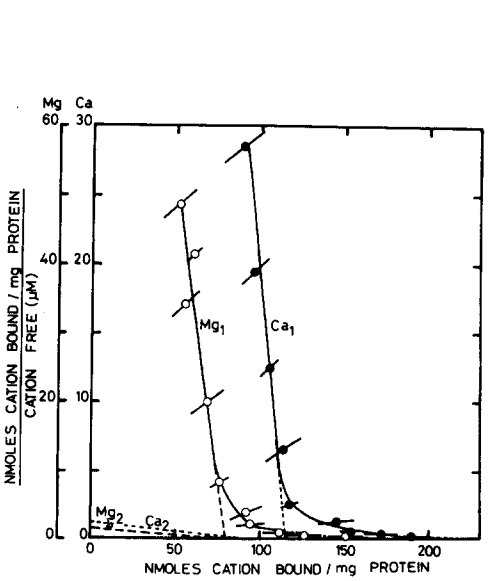
Synaptosomal fractions bind cations such Ca^{2+}, Mg^{2+}, Zn^{2+} and Cd^{2+}.

The intrinsic amount of cations (Ca^{2+} and Mg^{2+}) in synaptic membranes is very low. After the washing procedure in 0.1 mM EDTA we found that Fraction 1 contains about 10 nmoles Ca^{2+} and 5 nmoles Mg^{2+} per mg of protein. Since these values are relatively low, they do not affect the significance of the binding studies described below.

The binding of Ca^{2+} varied from fraction to fraction (Fig. 2). The total Ca^{2+} binding capacity of Fraction 1 is about 200–220 nmoles Ca^{2+}/mg protein which represents a very high value when compared with the maximal capacity of other membranous structures which bind Ca^{2+} such as mitochondria[19,20] and sarcoplasmic reticulum[21,22]. Thus, the high capacity of synaptic membranes for Ca^{2+} reinforces the idea that the Ca^{2+} receptor implicated in neurotransmitter release may be located on the synaptosomal plasma membrane.

Fraction 2 which is very close to Fraction 1 in the sucrose gradient has about half the capacity for Ca^{2+} of Fraction 1. The other fractions (Fraction 3, Fraction 4, and Fraction 5) have also low capacities for Ca^{2+}. For Fraction 4 and Fraction 5 we found a capacity (60–80 nmoles/mg protein) similar to that of mitochondria[19,20]. These results and the high succinate dehydrogenase activity present in these fractions (Fig. 1) suggest that the fractions are composed mainly of mitochondrial membranes.

For the studies described below, we chose Fraction 1 which probably is the richest in synaptic membranes.



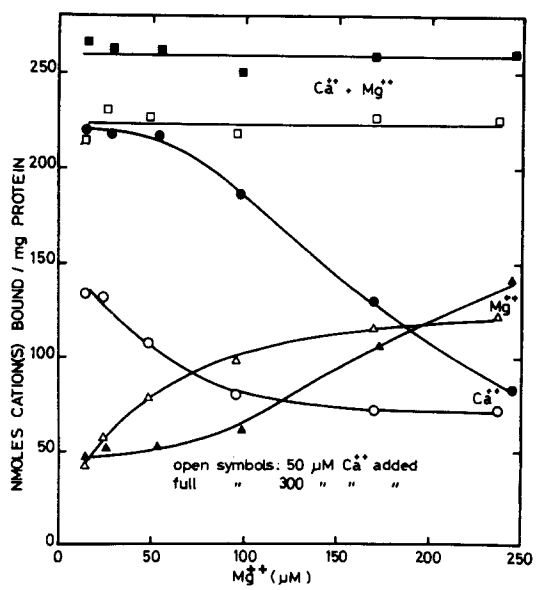


Fig. 3. Scatchard plots for the binding of Ca^{2+} and Mg^{2+} by Fraction 1. Each curve was resolved into two components. From these data we estimated the binding parameters reported in Table I.

Fig. 4. Binding of Ca^{2+} and/or Mg^{2+} by Fraction 1 as a function of free Mg^{2+} concentration, for two different concentrations of Ca^{2+} added (50 and 300 μM). The amount of Ca^{2+} *plus* Mg^{2+} bound is constant and when Mg^{2+} is increased there is a decrease of the amount of Ca^{2+} bound and conversely for Mg^{2+}.

Representing the results of binding in the form of a Scatchard plot[23] as done for other types of membranes[19,20,24], we found at least two components for the binding of both Ca^{2+} and Mg^{2+} (Fig. 3). The binding parameters are presented in Table I. The affinity of synaptic membranes for Mg^{2+} is greater than for Ca^{2+}, since the apparent dissociation constants are 0.87 and 74 μM for Ca^{2+} and 0.61 and 49 μM for Mg^{2+}.

The cations, Ca^{2+} and Mg^{2+} compete for the same binding sites in synaptic membranes as documented in Fig. 4. The Mg^{2+} displaces Ca^{2+} and is itself bound to the membranes, so that the amount of Ca^{2+} *plus* Mg^{2+} bound remains constant when the concentration of Mg^{2+} changes in the medium (Fig. 4).

TABLE I

Binding of Ca^{2+} and Mg^{2+} by synaptic membranes (Fraction 1). Constants for the binding were determined from a mass law treatment.

Cation	*Maximum binding capacities (nmoles/mg protein)*	*Dissociation constants (μM)*
Ca^{2+}	$B_{max1} = 115 \pm 10$ (8)	$K_{D1} = 0.87 \pm 0.10$ (8)
	$B_{max2} = 90 \pm 6$ (8)	$K_{D2} = 74.00 \pm 5.10$ (8)
Mg^{2+}	$B_{max1} = 80 \pm 7$ (7)	$K_{D1} = 0.61 \pm 0.10$ (7)
	$B_{max2} = 90 \pm 10$ (7)	$K_{D2} = 49.00 \pm 6.50$ (7)

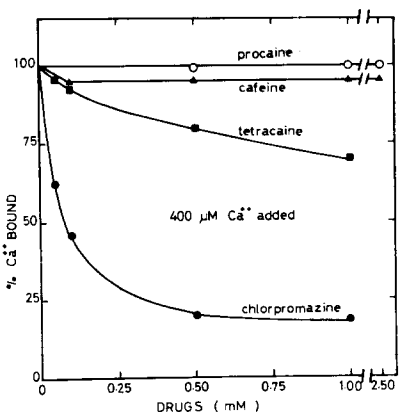


Fig. 5. Effect of local anesthetics and caffeine on Ca^{2+}-binding by Fraction 1. Only chlorpromazine significantly inhibits the Ca^{2+} binding. When present at concentration of 75 μM it decreases the maximum binding capacity by 50%.

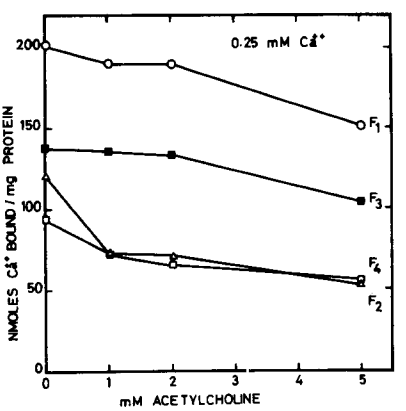


Fig. 6. Effect of acetylcholine on Ca^{2+} binding by synaptosomal fractions. The drug only displaces the bound Ca^{2+} when present at relatively high concentrations (5 mM).

Effect of drugs on Ca^{2+} binding

The local anesthetic, procaine, has no effect on Ca^{2+} binding, but tetracaine (1 mM) decreases the maximum binding capacity of Fraction 1 by 30% (Fig. 5).

Chlorpromazine inhibits the maximum Ca^{2+} binding by 50 and 80% when present at concentrations of 0.075 and 0.5 mM, respectively, (Fig. 5).

The alkaloid, caffeine, does not significantly modify the binding of Ca^{2+}, decreasing the maximum capacity by only 5% (Fig. 5).

Acetylcholine does not interfere appreciably with the Ca^{2+} binding by synaptic membranes (Fig. 6). The binding is decreased by 20% in Fraction 1 when the drug is present at relatively high concentrations (5 mM).

Fluorimetric studies

These studies were conducted by observing the change in fluorescence of the fluorochrome anilinonaphthalenesulfonate which is believed to bind to many biological membranes[25–32]. There is an enhancement of the dye fluorescence when its environment undergoes a transition from hydrophilic to hydrophobic[26–30].

In Fig. 7 we report the change in fluorescence of ANS as a function of the concentration of various cations added to the medium. The order of efficiency of cations in causing an increase in fluorescence is $Cd^{2+} \approx Zn^{2+} > Ca^{2+} \approx Sr^{2+} > Mg^{2+}$.

Representing data in the form of a Scatchard plot (Fig. 8), as done for other types of membranes[25], we were able to observe that Ca^{2+} has higher potency to increase ANS fluorescence than Mg^{2+} does. Data in Table II reveal that Ca^{2+} has an affinity 2.9 and 1.55 times greater than Mg^{2+} for components 1 and 2, respectively. Values obtained for Sr^{2+} are intermediate between those for Ca^{2+} and Mg^{2+}. For Zn^{2+} and Cd^{2+} it was impossible to evaluate the data, since Scatchard plots for these cations did not give informative figures.

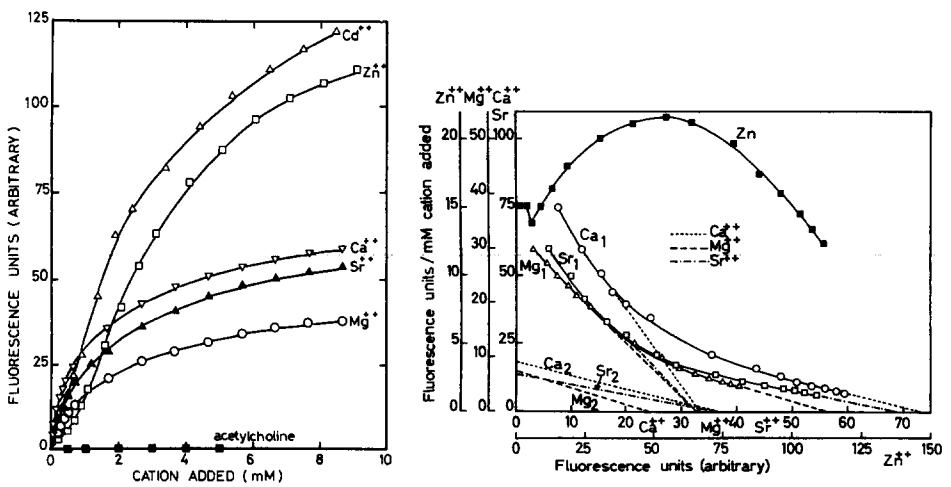


Fig. 7. Effect of cations on ANS fluorescence in aqueous suspensious of Fraction 1. Fluorescence was measured in a Perkin Elmer MPF-3 fluorimeter regulated at 388 nm for excitation (Slit 2) and 500 nm for emission (Slit 5) with sensitivity at 30 (see text for details). Fluorescence readings were taken 1 min after the additions. At the end of this time, fluorescence intensity remains constant.

Fig. 8. Scatchard type of plots for fluorescence data. From similar data we evaluated the parameters of Table II. The curves for Ca^{2+}, Mg^{2+} and Sr^{2+} were resolved into two components. For Zn^{2+} and Cd^{2+} (not shown), the shapes of the curves of the Scatchard plots were uncommon, and it was not possible to determine the affinity parameters.

TABLE II

Cation-dependent ANS fluorescence in suspensions of synaptic membranes (Fraction 1).

Cation	*Maximal fluorescence (arbitrary units)*	*Affinity constants (mM)*
Ca^{2+}	$Fl_{max1} = 33.5 \pm 5.0$ (4)	$F_{D1} = 0.34 \pm 0.05$ (4)
	$Fl_{max2} = 36.5 \pm 5.0$ (4)	$F_{D2} = 1.97 \pm 0.10$ (4)
Mg^{2+}	$Fl_{max1} = 33.0 \pm 3.5$ (4)	$F_{D1} = 1.00 \pm 0.07$ (4)
	$Fl_{max2} = 23.0 \pm 4.3$ (4)	$F_{D2} = 3.07 \pm 0.30$ (4)
Sr^{2+}	$Fl_{max1} = 33.0 \pm 5.4$ (4)	$F_{D1} = 0.46 \pm 0.06$ (4)
	$Fl_{max2} = 36.5 \pm 6.5$ (4)	$F_{D2} = 2.70 \pm 0.30$ (4)

In Fig. 9 we show that Ca^{2+} increases the amount of ANS bound by synaptic membranes. The amount of ANS bound increases considerably in the range 0.25 to 1.0 mM of Ca^{2+} added. This study was conducted using only 25 μM ANS instead of 100 μM which we used for the fluorimetric studies. The reason for this was the impossibility of detecting differences in ANS bound for 100 μM ANS in the medium, *i.e.*, the limitations of the analytical method forced us to use a lower ANS concentration.

The interpretation of the fluorimetric data may be done in two different ways: (1) the cations may induce conformational changes increasing the hydrophobicity of the membrane; (2) or cations may induce the binding of ANS to the membranes and its fluorescence increase. This last situation may also involve conformational changes which may lead to the exposition of groups involved in ANS binding. Thus the fluorimetric data may be interpreted as an index of the conformational state of the membrane macromolecules. Furthermore, as ANS is anionic it may be carried with Ca^{2+} to the inside of the synaptic membrane vesicles. In this way, part of ANS may remain inside the vesicles and not bound to the membrane material.

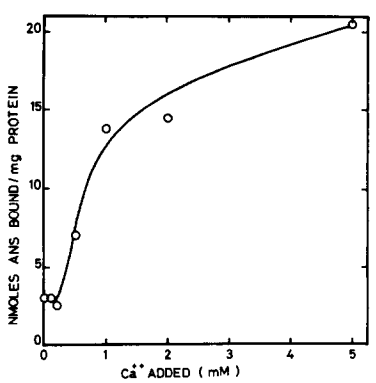


Fig. 9. Ca^{2+}-dependent binding of ANS by Fraction 1. Ca^{2+} favors the binding of ANS. The binding may be partially apparent since the method used (see text) does not account for the portion of ANS which may be drawn to the inside of the vesicles with Ca^{2+}. This portion may remain inside the vesicles in the free form and not bound to the membranes.

DISCUSSION

Biochemical characterization of the fractions

The results are in close agreement with those of Cotman and Matthews[15] and other investigators[33]. The synaptosomal fractions obtained by sucrose density gradient centrifugation are located at about the same sucrose densities as described by Cotman and Matthews[15]. The distribution of acetylcholinesterase among the fractions is as previously reported[15,33] and succinate dehydrogenase follows the same pattern described for cytochrome *c* oxidase[15].

Various authors[15] have exhaustively characterized similar brain fractions by studying the distribution of various enzyme markers for plasma membranes, *e.g.*, (Na^+–K^+)-ATPase, 5′-nucleotidase and others. Their results suggest that fractions corresponding to our Fraction 1 and Fraction 2 are particularly rich in synaptic plasma membranes. The main contamination appears to be mitochondrial outer membrane which may represent as much as 10% of the total protein[33]. Thus, it is believed that the fractions may be about 90% pure plasma membrane[33].

Our yield in synaptic membranes (Fraction 1) was about 1.5 mg of protein per g of brain wet weight which is very similar to Cotman's yield[15].

Binding of cations

As stated previously in the results, the high binding capacity and affinity of synaptic membranes for the cations, Ca^{2+} and Mg^{2+}, is compatible with the hypothesis that the sites which regulate the neurotransmitter release dependent on these cations are located in the synaptic plasma membrane.

It is commonly thought that Ca^{2+} associated with its receptor in the membrane induces an "active release state"[7,11,12] which may represent conformational changes operating in the macromolecular core of the membranes[7]. On the other hand, Mg^{2+} would be unable to induce the same changes as Ca^{2+}. The antagonistic effect of Mg^{2+} has been interpreted in terms of a competition with Ca^{2+} for the same sites. This interpretation was based on observations of the effect of both Ca^{2+} and Mg^{2+} on the amplitude[7,8,10] and frequency[3] of the miniature end-plate potentials which were used as indices of the amount of transmitter released. Although many of these studies have been done in neuromuscular junctions, there is also evidence that the same type of regulation exists in most synapses between neurons, and the same considerations are generally applied for both synapse and neuromuscular junction[1].

The existence of a competitive interaction between Ca^{2+} and Mg^{2+} was always suggested by indirect studies since the synaptic regulatory sites have not been isolated so far. We propose that the Ca^{2+}-binding sites of the synaptic membrane may represent part of the system which regulates the Ca^{2+}-mediated neurotransmitter release. We found that Ca^{2+} and Mg^{2+} compete for the same binding sites and basically the functional antagonism between these cations may be primarily mediated by such a competition at specific sites.

Studies relating the effect of Ca^{2+} and Mg^{2+} to end-plate potentials revealed that three sites appear to exist in nerve terminals for which Ca^{2+} and Mg^{2+} could compete[7]. Our data were evaluated from Scatchard plots which revealed that at least two types of independent sites may exist. However, we accept that three or more sites may be involved, since Scatchard plots give only a relatively crude information

about the number of independent classes of binding sites. Furthermore, other sites, in addition to the membrane sites, may be involved in the regulation of neurotransmitter release.

Wolff and Siegel have isolated a pure phosphoprotein[34–36] from pig brain which binds Ca^{2+}, and in which Mg^{2+} competes with Ca^{2+} for the same binding sites[35]. The authors postulated a role for the protein as a Ca^{2+}-receptor molecule involved in the release of neurotransmitters[35]. However, from the results of those authors it is not possible to ascertain the cellular source of the protein. The affinity constant of the protein is 25 μM[34] which differs from the constants we obtained for Ca^{2+} (0.87 μM for component 1 and 74 μM for component 2). The difference, although large, does not at all exclude the possibility that the protein may arise from synaptic plasma membranes, since proteins once isolated from membranes may not behave in the same manner as when they are arranged together with other macromolecules which may cooperatively interact and modify their own chemical activities. The same considerations may be applied for sarcoplasmic reticulum from which a Ca^{2+}-binding protein was isolated (calsequestrin) which has an affinity for Ca^{2+} (ref. 37) lower than that of intact sarcoplasmic reticulum[38].

The components which bind Ca^{2+} may involve glycoproteins, since there is a significant amount of sialic acid in synaptic plasma membranes[39]. Glycoproteins with high affinity and capacity for Ca^{2+} were isolated from mitochondria[40] and cartilage[41]. The dye ruthenium red, which is believed to be somewhat specific for polysaccharides[42], strongly inhibits the binding of Ca^{2+} by those proteins[40,41] and also by our synaptic plasma membranes (Fig. 10). Thus, a glycoprotein could be involved in Ca^{2+} binding by synaptic membranes.

Conformational changes

Evaluating the fluorimetric data, it seems that Ca^{2+} induces the membrane macromolecules to suffer a transition from a hydrophilic to a more hydrophobic state. Mg^{2+} also has a similar effect, but its affinity is much lower than that of Ca^{2+} as evaluated by the affinity constants (Table II).

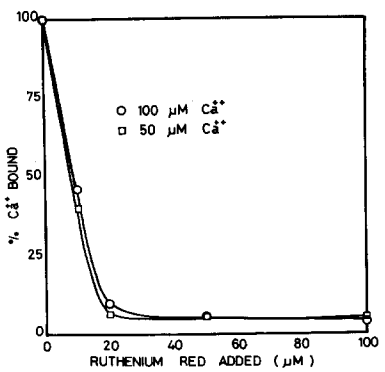


Fig. 10. Effect of ruthenium red upon the Ca^{2+} binding by synaptic membranes (Fraction 1). Reactions were carried out at pH 7.0 and room temperature in media (10 ml) containing 4 mM imidazole, 50 or 100 μM $CaCl_2$ and ruthenium red as desired. The amount of Ca^{2+} bound was determined by atomic absorption spectrophotometry as described in Methods. Ruthenium red (20 μM added) inhibits the Ca^{2+} binding by 90–95%.

The change from a hydrophilic state to a hydrophobic one is frequently interpreted as a conformational change of membrane macromolecules[27–30,32] in which the increase in hydrophobicity is due to the exposition of hydrophobic groups initially located inside the proteins. Fluorimetric data led us to conclude that Ca^{2+} is much more potent than Mg^{2+} in inducing conformational changes of membrane macromolecules.

The mechanism by which Ca^{2+} favour the release of neurotransmitters may involve a phase transition in membrane macromolecules leading to the "active release state". Assuming such a mechanism we would be able to explain the antagonist effect of Mg^{2+}. As discussed previously, Mg^{2+} and Ca^{2+} compete for the same binding sites in the membrane and the affinity for Mg^{2+} is greater than that for Ca^{2+}. Therefore, Mg^{2+} can substitute for Ca^{2+} in the membrane, but it is not able to induce the same conformational change as Ca^{2+} which may partially inhibit the effect of Ca^{2+}.

Local anesthetics

The stabilizing effect of local anesthetics in nerve and muscle by blocking the action potential[43–45] is often interpreted in terms of a membrane–Ca^{2+} displacement by the drugs[43,45,46], since Ca^{2+} play an important role in regulating the excitability and electric conduction of excitable membranes[47].

Local anesthetics displace Ca^{2+} from axon membrane[43], muscle sarcolemma[48] and other biological membranes[45,49,50]. These findings stimulated us to investigate the effect of the local anesthetics on the Ca^{2+} binding by synaptic membranes. As shown in Fig. 5, procaine does not displace Ca^{2+} as it does in axon[43] and muscle membrane[48], and tetracaine has a relatively small effect. Obviously the blocking effect of these local anesthetics cannot be explained at the synapse level by a Ca^{2+}-displacement mechanism. On the other hand chlorpromazine strongly inhibits the Ca^{2+} binding by synaptic membranes. Therefore, we have to consider this effect as a possible mechanism by which chlorpromazine acts as a tranquilizer.

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