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Calcium-Binding of Synaptosomes Isolated from Rat Brain Cortex

I. Effects of High External Potassium Ions

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Summary. Ca^{++} concentration in the synaptosomal suspension was measured to determine the liberation from and binding to isolated brain cortex synaptosomes by utilizing a dual wavelength spectrophotometer to monitor the absorbance changes of murexide raised by Ca^{++}. When synaptosomes were suspended in isotonic solutions of a NaCl-KCl mixture containing more than 30 to 40 mM of KCl, a marked liberation of calcium ion was observed in proportion to the rise in KCl concentration, whereas there was rarely any significant release of Ca^{++} observed with an external KCl concentration of less than 20 mM. Titration of the synaptosomal suspension with Ca^{++} revealed that, in the absence of external K^+, a part of added Ca^{++} was almost instantaneously bound to the synaptosomal particles so far as these particles had not been saturated with Ca^{++}. Such a Ca^{++}-binding was markedly depressed by a higher external K^+ concentration. Ouabain and cyanide did not have that effect on such a K^+-induced inhibition of Ca^{++}-binding. The present results indicate that the inhibition of Ca^{++}-binding by high external K^+ concentration probably results from a change in synaptosomal membrane which is of a cooperative nature.

The importance of calcium ion in the process of excitation of cells was already recognized in the late nineteenth century (Ringer, 1883; Locke, 1894). Many studies have been done to elucidate the essential role of Ca^{++}, particularly in relation to the synaptic transmission at the neuromuscular junction which has been studied in detail electrophysiologically since the studies of del Castillo & Stark (1952). del Castillo & Katz (1954) suggested that the effect of calcium ions on chemical transmission is due to a reaction between Ca^{++} and some critical receptive site, X, on the presynaptic terminal leading to a reversible formation of CaX complex which is essential for the release of the transmitter. Along this line of investigation, Jenkinson (1957) and Dodge & Rahamimoff (1967) observed Ca^{++}-binding with presynaptic membrane by correlating the external Ca^{++} concentrations with

end-plate potentials (e.p.p.), and suggested the cooperative nature of the action of Ca^{++} in transmitter release.

In a previous paper we reported that K^+ caused swelling of isolated synaptosomal particles, and it was proposed that this may be due to K^+-induced Ca^{++}-liberation from synaptosomal membrane (Kamino, Inouye & Inouye, 1973). Since these membranes may be regarded as presynaptic by nature, Ca^{++}-binding appeared to be worthy of investigation. The present paper presents direct evidence for the Ca^{++}-binding of synaptosomes, and the effects of external high K^+ on synaptosomal membranes are also discussed.

Materials and Methods

Preparation of Synaptosomes from Rat Brain Cortex

Essentially the same methods as those used in our previous work (Kamino *et al.*, 1973) were employed, this procedure being in principle based on that originally described by Gray and Whittaker (1962), with slight modification. Pooled cortical tissues (usually 2 or 3 brains) were homogenized in 0.32 M sucrose by a Teflon-glass homogenizer of Potter-Elvejem type, after which the crude mitochondrial fractions were subjected to sucrose density gradient centrifugation (64,000 $\times g$ for 60 min) using a Hitachi preparative ultracentrifuge (Type HU 40P). The material at the 0.8 to 1.2 M interface was used as the synaptosomal fraction after usually two washings with resuspension and centrifugation (12,000 $\times g$ for 20 min) in buffered 0.32 M sucrose. The final pellets were suspended in buffered 0.32 M sucrose as the stock suspension and were stored at 0 °C until use which was at least within 12 hr after preparation.

To prepare synaptosomal suspensions in isotonic solution of various NaCl/KCl ratio, the pellets separated from the stock suspension were suspended in an appropriate buffered salt solution after washing with suspending media as described. Giving due consideration to the osmotic coefficient of NaCl and KCl, 170 mM of these salts were used as the isotonic concentration, the solution being prepared by mixing an appropriate amount of both isotonic solutions. The pH of suspending media was adjusted to 7.3 with 20 mM Tris-HCl buffer.

Assay of Synaptosomal Protein

To estimate the particulate concentration of synaptosomal suspension, the protein content of suspensions was assayed by the method of Lowry, Rosebrough, Farr and Randall (1951) with bovine serum albumin as the standard. In the titration experiments, the particulate concentration of synaptosomal suspension was usually adjusted to about 150 to 200 μg protein per ml. In the experiments of Ca^{++} liberation from synaptosomes however, much higher particulate concentrations (up to 0.8 ~ 1.2 mg protein/ml) were employed.

Spectrophotometric Measurement of Ca^{++} in Synaptosomal Suspensions

The use of murexide with a dual wavelength spectrophotometer to measure low Ca^{++} concentration of particulate suspensions was originally introduced by Ohnishi and Ebashi (1963). Further, Mela and Chance (1968) also utilized this same procedure for measurement of Ca^{++} concentration in mitochondrial suspensions and in the present

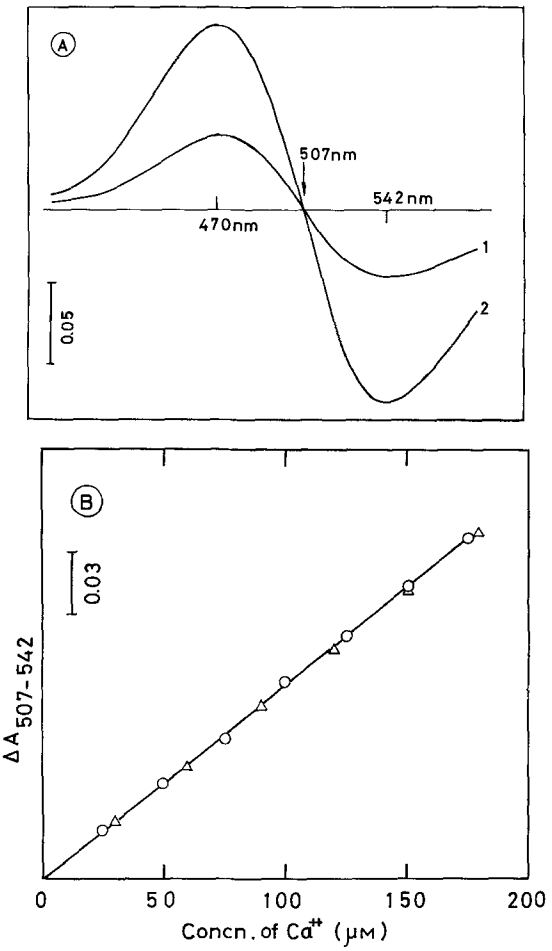

Fig. 1. (*A*) The difference spectrum of the Ca^{++}-murexide *vs.* the free murexide. Spectrum 1 was obtained by adding 100 μM $CaCl_2$ to the measuring cuvette, which contained 80 μM murexide in 170 mM NaCl solution containing 20 mM Tris-Cl buffer (pH 7.3). The reference cuvette contained 80 μM murexide, but no $CaCl_2$. Spectrum 2 was obtained by adding 220 μM $CaCl_2$ to the measuring cuvette, which contained 50 μM murexide in 170 mM KCl solution containing 20 mM Tris-Cl buffer (pH 7.3). The reference cuvette contained 50 μM murexide, but no $CaCl_2$. Temperature 20 °C. The difference spectra were entirely unaffected by replacing NaCl in 1 with KCl or KCl in 2 together with NaCl. (*B*) Relationship between changes in $\Delta A_{507-542}$ and Ca^{++} concentration. ○, in 170 mM NaCl solution; △, in 80 μM murexide and 20 mM Tris-Cl buffer (pH 7.3). Temperature 20 °C

study, a dual wavelength spectrophotometer (Hitachi, Type 354) was employed for the investigation. All measurements were carried out at a temperature of 20 to 24 °C. Murexide was purchased from Dojin Yakuchemical Company, Kumamoto, Japan.

Since a NaCl-KCl mixture was used as the suspending media, effects of NaCl and KCl on the Ca^{++}-murexide *vs.* murexide difference spectrum were examined. As shown in Fig. 1*A*, the wavelength 507 nm is near the isosbestic point and can be used as a

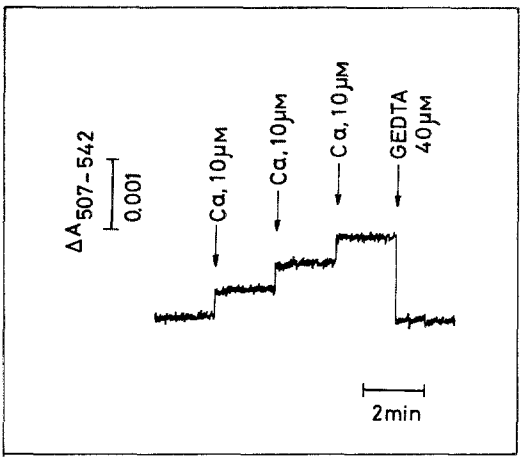

Fig. 2. Ca^{++}-induced increase in $\Delta A_{507-542}$ and its reversible change by adding GEDTA. $MX = 10$ μM, pH 7.3

reference wavelength, which is neither affected by murexide concentrations (at least in the range of $10 \sim 100$ μM) nor dependent on a medium of, NaCl or KCl solution. This figure demonstrates that decreasing absorbance at 542 nm indicates an increase in the concentration of Ca^{++}-murexide complex in salt (170 mM) solution. On the other hand, it can be stated that in our results of the light-scattering studies on synaptosomal suspension, the scattering at a wavelength greater than 500 nm was hardly affected by K^+-induced particulate swelling. As a measure for Ca^{++} concentration, therefore, we used the difference in absorbance at 507 nm and at 542 nm, $\Delta A_{507-542}$, instead of $\Delta A_{472-542}$. Indeed, as shown in Fig. 1*B*, a single linear correlation of $\Delta A_{507-542}$, with the Ca^{++} concentration was observed both in the NaCl and the KCl media.

Addition of glycoletherdiaminetetraacetate (GEDTA) in excess of chelating all Ca^{++} resulted in a return to the initial level of $\Delta A_{507-542}$ in the presence of Ca^{++}, thus demonstrating the rapid reversible change in $\Delta A_{507-542}$ due to Ca^{++} (Fig. 2). Elevating the sensitivity of the spectrophotometer, even 1 μM of Ca^{++} could be determined utilizing such a procedure.

The range of Ca^{++} concentrations which shows such a linear correlation with $\Delta A_{507-542}$ depends on the concentration of murexide applied. In the present experiment, a concentration up to 100 μM was utilized.

In such a range of murexide concentration, the absorbance at 507 nm measurement on supernatants from murexide-containing synaptosomal suspensions confirmed, as reported by Mela and Chance (1968), that the murexide remained chiefly (more than 98%) in the aqueous phase.

These observations indicate that the difference spectrophotometry with murexide is also a satisfactory method to determine Ca^{++} concentration external to the synaptosomes in a NaCl-KCl medium.

Results

Liberation of Ca^{++} from Synaptosomal Particles in the Presence of High Potassium Ions

In a previous report we suggested that K^+ liberated Ca^{++} from synaptosomal membrane (Kamino *et al.*, 1973). To determine whether or not

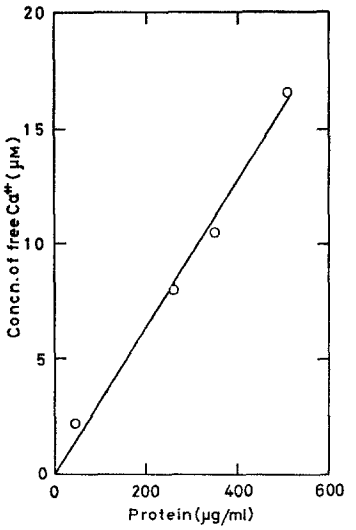

Fig. 3. Correlation of the amount of liberated Ca^{++} to the concentration of synaptosomal protein in 170 mM KCl solution

K^+ has such a releasing action on Ca^{++} bound with synaptosomes, an attempt was made to calculate concentrations of Ca^{++} in isotonic NaCl or KCl solutions which were used for washing of the pellets isolated from a stock suspension of synaptosomes: the pellets from a stock suspension were resuspended in isotonic salt solutions at a rather higher concentration and spun down (12,000 × *g* for 20 min) after incubation at 25 °C for 10 min. The supernatants thus obtained were then subjected to the measurement of $\Delta A_{507-542}$ after addition of murexide.

When NaCl solution was applied, no significant amount of Ca^{++} was detected in the supernatant from suspensions of high particulated concentration (0.8 to 1.2 mg protein/ml) with the highest sensitivity of the photometer and high murexide concentration (100 μM); addition of an ample amount of GEDTA had hardly any effect on $\Delta A_{507-542}$. With isotonic KCl, however, a significant decrease in $\Delta A_{507-542}$ was observed on addition of GEDTA which, after application of an appropriate amount of the chelating agent, reached a steady level and remained unaltered even with further addition of GEDTA. Using the calibration curve shown in Fig. 1*B*, the Ca^{++} concentration of KCl washing solution could therefore be determined from decreases in $\Delta A_{507-542}$ produced by GEDTA. When the Ca^{++} concentration was compared to the synaptosomal concentration submitted to washing with KCl solution, a linear correlation was obtained (Fig. 3). These findings indicate that Ca^{++} was liberated from synaptosomal

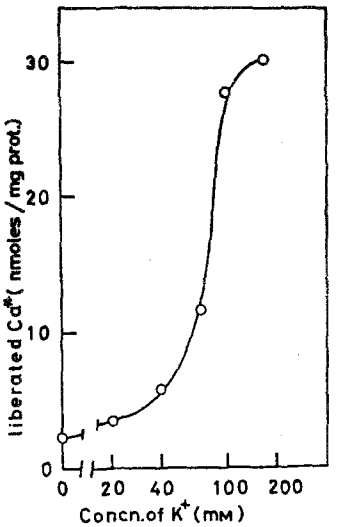

Fig. 4. Dependence on external K^+ concentration of Ca^{++} liberation from synaptosomes. *Ordinate:* liberated Ca^{++} (nmoles/mg protein). *Abscissa:* external K^+ concentration (mM) in log scale

particles in the KCl medium and the Ca^{++} concentration observed in KCl supernatants indicates the amount of Ca^{++} which is liberated.

Liberation of Ca^{++} from synaptosomes in isotonic NaCl-KCl mixture was thus found to depend upon their K^+ concentration. The higher the external K^+ concentration the larger was the amount of Ca^{++} liberated as seen in Fig. 4. It is noteworthy that Ca^{++} liberation becomes pronounced when the external K^+ concentration exceeds 40 mM, this being nearly the same concentration at which significant K^+-induced synaptosomal swelling begins to occur (Kamino *et al.*, 1973). Such a finding provides indirect evidence that the K^+-induced swelling is related to Ca^{++} liberation from synaptosomal membrane.

Titration of Murexide in Synaptosomal Suspensions with Sequential Addition of $CaCl_2$

In this series of observations, the synaptosomes were incubated in 170 mM KCl for 10 min to liberate as much Ca^{++} as possible from the particles, then were spun down at 12,000 × *g* for 20 min and washed twice with the solution to be used as the suspending medium.

When a small amount of $CaCl_2$ was added to a NaCl solution containing synaptosomes thus prepared, $\Delta A_{507-542}$ attained a higher steady level

within 10 sec and remained practically unaltered for more than 3 min, whereas such an increase in $\Delta A_{507-542}$ for a given amount of added Ca^{++} was always less than that observed on the synaptosome-free isotonic NaCl solution. Moreover, such an effect on synaptosomes following a change in $\Delta A_{507-542}$ induced by adding Ca^{++} was never observed in the presence of ouabain or cyanide which had been added to the suspension at the concentration of $10^{-4} \sim 10^{-3}$ M about 30 min prior to addition of Ca^{++}. It is obvious, therefore, that synaptosomal membranes almost instantaneously bind a part of Ca^{++} added, attaining an equilibrium, or a quasi-equilibrium quite rapidly with free Ca^{++} remaining unbound in the medium. The difference in the $\Delta A_{507-542}$ of suspensions from the particle-free control indicates the amount of Ca^{++} which was bound to synaptosomal membrane, a compartment not detected by murexide. To examine effects of K^+ on Ca^{++}-binding with synaptosomal membrane, titration curves of murexide in synaptosomal suspension of isotonic NaCl or KCl were constructed by sequential addition of $CaCl_2$ at an interval of about 3 min.

Fig. 5 shows an example of such titration curves in synaptosomal suspension of isotonic NaCl and KCl. Isotonic NaCl and KCl titrated in the absence of synaptosomes was utilized as the control. Both control curves coincided and served as a calibration curve of the murexide change in synaptosomal suspensions.

As seen in Fig. 5, the titration curve of synaptosomes in a NaCl solution thus constructed rises less steeply than does the control curve when the concentration in the medium of added Ca^{++} remains less than 0.3 mM. In a higher concentration the curve runs parallel to that of the control which indicates that synaptosomes were saturated with Ca^{++} and further binding of Ca^{++} does not take place despite increases in free Ca^{++} concentration in the medium. The extrapolation of the last linear part of this curve to the abscissa thus indicates the amount of Ca^{++} required to saturate binding sites of synaptosomal membrane. Ca-binding capacity and the mean values on four different preparations were found to be 139 ± 1.36 nmoles/mg protein. Since the free Ca^{++} concentration (x) in synaptosomal suspensions is easily obtained from the control curves used as a calibration curve, the fractional saturation with Ca^{++} ($\bar{X}$) at a given x can be computed from such a titration curve. Thus, as shown in Fig. 6, Ca^{++} binding curves of synaptosomes as $\bar{X}$ *vs.* x were drawn.

On titrating synaptosomes in a KCl medium, it was found that circumstances were, however, quite different; no appreciable uptake of Ca^{++} occurs until the free Ca^{++} concentration in the medium reaches a certain level (A in Fig. 5), beyond which the curve runs nearly parallel to that in

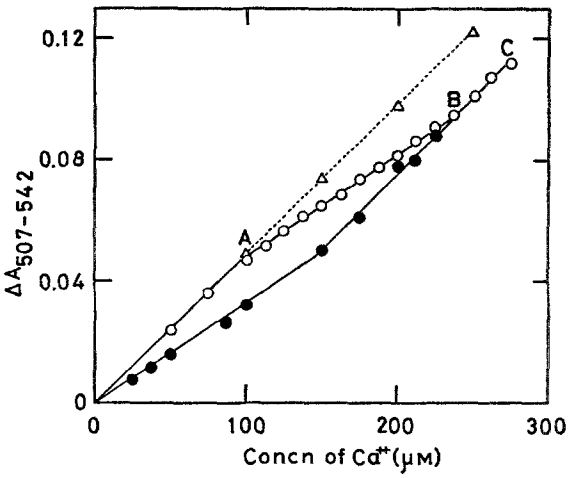

Fig. 5. Titrations of murexide (75 μM) in synaptosomal suspensions (200 mg protein/ml) with Ca^{++}. *Ordinates:* $\Delta A_{507-542}$, *Abscissae:* concentration of Ca^{++} added. •, synaptosomal suspension in 170 mM NaCl solution; ○, synaptosomal suspension in 170 mM KCl solution; △, in 170 mM NaCl solution without synaptosomes

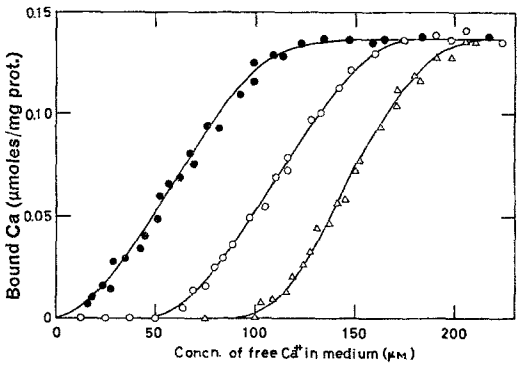

Fig. 6. Relationship between synaptosome-bound Ca^{++} and the free Ca^{++} concentration in suspending media in the presence and absence of external K^+. •, in 170 mM NaCl solution (n=4); ○, in 100 mM NaCl-70 mM KCl mixture solution (n=2); △, 170 mM KCl solution. These solutions contained Tris-Cl buffer (pH 7.3, 15 mM); n, number of independent observation in different samples

the NaCl medium. In other words, it is at much higher Ca^{++} concentrations that synaptosomes in isotonic KCl solution take up Ca^{++} from the medium in nearly the same way as is observed in isotonic NaCl solutions. On the other hand, the last portion of the curve in the KCl medium (*BC* in Fig. 5) coincides with that in the NaCl medium, which indicates that saturation with Ca^{++} of synaptosomes also occurs. The mean value of the amount of Ca^{++} maximally bound with synaptosomes in isotonic KCl media was

141 ± 1.12 nmoles/mg protein ($n = 4$). In other words, there was no significant difference from the value in isotonic NaCl media. The Ca^{++}-binding curves of synaptosomes in isotonic KCl media are also presented in Fig. 6.

Ca^{++}-Binding Curves of Synaptosomes in NaCl and KCl Media

The results shown in Fig. 6 demonstrate that the Ca^{++}-binding curves in both KCl and NaCl media are sigmoid in shape. Moreover, as already stated, the binding capacity for Ca^{++} of synaptosomal particles is not affected by the presence of K^+ in the suspension media; however, K^+-induced inhibition of Ca^{++}-binding is, as clearly demonstrated by the Hill plot, of a noncompetitive nature.

As a result of the sigmoid nature of these curves, the binding of Ca^{++} with receptive sites on synaptosomes may be formally expressed as

$$M + n\,Ca^{++} \rightleftharpoons Ca_n M \tag{1}$$

and so

$$\log [\bar{X}/(1-\bar{X})] = n \log x + \log K \tag{2}$$

where M denotes a binding site of synaptosomal membrane, K the dissociation constant of synaptosome-Ca^{++} complex, $Ca_n M$, while x and $\bar{X}$ stand for Ca^{++} concentration in a medium and fractional saturation of the binding sites with Ca^{++}, respectively. Plotting $\log [\bar{X}/(1-\bar{X})]$ against $\log x$,

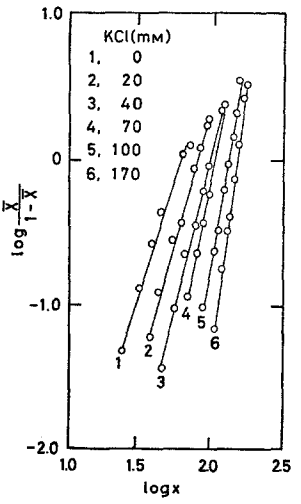

Fig. 7. Hill plots of Ca^{++}-binding by synaptosomes in the various external K^+ concentrations. *Ordinate:* $\log [\bar{X}/(1-\bar{X})]$, where $\bar{X}$ is fractional saturation of bound Ca^{++}. *Abscissa:* logarithm of free Ca^{++} concentration (x) in μM

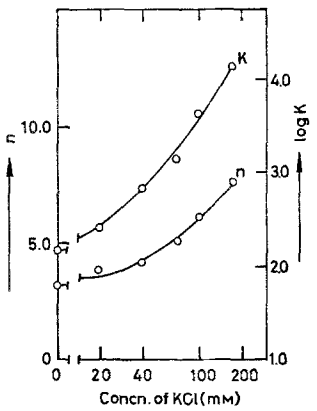

Fig. 8. Correlation of Hill's exponent (n) and apparent dissociation constant (K) with external K^+ concentration. *Ordinates:* on the left, n; on the right, log K. *Abscissas:* external K^+ concentration (mM) in log scale

the value of n and K is easily obtained. Such is known as the Hill plot which provides a linear relation over a wide range of x in the study of macromolecules capable of combining two or more different ligands.

Indeed, as seen in Fig. 7, the Hill plots derived from one and the same synaptosome sample obtained on aliquots are almost linear over a wide range of the free Ca^{++} concentration with $n > 1$, the higher the external K^+ concentration the greater the slope and the shift towards the right of the line. In consequence, the value of n and log K increases almost linearly with logarithm of K^+ concentration when it exceeds 40 mM (Fig. 8), the dependence on K^+ concentration being quite similar to that observed on K^+-induced Ca^{++}-liberation from synaptosomes (Fig. 4). Thus, it is obvious that K^+-induced inhibition of synaptosomal Ca^{++}-binding is not of simple competition. If K^+ competitively inhibits Ca^{++}-binding, n would remain unaltered with increasing K.

Discussion

The results presented above clearly demonstrate that isolated synaptosomes are capable of taking up Ca^{++} from an isotonic saline medium, while the presence of high concentrations of K^+ in a suspension medium not only inhibits such a synaptosomal Ca^{++} uptake but also liberates Ca^{++} from the particles. Such a synaptosomal uptake of Ca^{++} appears to reach an equilibrium, or at least a quasi-equilibrium with free Ca^{++} within one minute. Moreover, ouabain and cyanide had no effect on synaptosomal

Ca^{++} uptake. These findings indicate that the divalent cation binds with and liberates from receptive sites on synaptosomal membrane, the formation of the Ca-receptor complex being quite rapid. The relation of the inhibitory effect on Ca^{++}-binding of raised external K^+ with its concentration is quite similar to that observed on K^+-induced swelling of synaptosomes (Kamino *et al.*, 1973), a finding which provides evidence for our speculation that K^+-induced swelling results from some changes in membrane structures due to liberation of membrane-bound calcium.

Binding between Ca^{++} and presynaptic membranes has already been investigated by several workers who employed the amplitude of the end-plate potential (e.p.p.) as the indicator for Ca-receptor complex formed (CaX), the same of which presumably determines the amount of transmitter released. The scheme put forward by del Castillo and Katz (1954) is quite the same as the relation shown in formulas (1) and (2) except that in their experiments $n=1$. Jenkinson (1957) tested this hypothesis quantitatively, showing that the competitive antagonism between Ca^{++} and Mg^{++} fitted reasonably well with the equation expected for this type of reaction. Dodge and Rahamimoff (1967) re-examined the problem in detail and suggested a cooperative action of Ca^{++} because of the sigmoid nature of the dose-action curves. During the course of preparing this manuscript, a paper by Cooke and Quastel (1973*b*) was published which stated that the frequency of miniature e.p.p. was applied instead of the amplitude of e.p.p., but interaction of Ca^{++} with binding sites on membrane was assumed, both in their "log" and "linear" model, to be the same as that put forward by the earlier investigators. Just as in these studies, Eq. (2) and the Hill plot based on this relation were utilized in the present study and were found to be applicable to interaction of Ca^{++} with synaptosomal membrane. The following points should however, be taken into consideration: the present experiments were done on synaptosomes which were isolated, and the amount of Ca^{++} bound with synaptosomal membranes was measured while the earlier investigators used e.p.p. *in situ* for estimation of CaX formed, for which Eq. (2) was assumed as a function linking CaX to the transmitter release. Thus, in our observations, the exponent n is directly involved in the process of Ca^{++} binding with the membrane, but in both Dodge & Rahamimoff's study (1967) and the study of Cooke and Quastel (1973*b*), the exponent n or θ is related to the number of Ca^{++} ions that appear to cooperate for release of each quantum of transmitter.

The value of n in the absence of K^+ and in low K^+ media is about 3.2, this being almost identical with that reported by Dodge & Rahamimoff (1967), despite the above-stated difference in both formulations from the

standpoint of physical meaning. As shown above, the inhibitory effect on synaptosomal Ca^{++} binding of raised external K^+ is noncompetitive; the Hill plot at no time demonstrated parallel shifts, both in n and K increase as K^+ concentration is increased. The change in θ and γ produced by raised external K^+ in the "linear" model of Cooke *et al.* (1973*b*) appeared in a general trend similar to, if not the same as, those described herein. If raised external K^+ concentration results in formation of the complex firmly bound with Ca-receptor leading to noncompetitive inhibition, the binding capacity for Ca^{++} per unit particulate protein should be reduced as in the presence of La^{+++} (manuscript in preparation). Such however is not the case. If n is the stoichiometric coefficient of receptors for Ca^{++}, it would be quite unreasonable that it significantly increases (double to treble) as inhibition due to raised K^+ concentration increases. Thus, it seems more plausible to postulate that the Hill exponent, n, is a parameter showing cooperativity of the Ca^{++} binding process, but not the number of Ca^{++} cooperating in quantal release of transmitter as already suggested by Dodge and Rahamimoff (1967).

According to Wyman's thermodynamic theory of the binding of ligands to macromolecules with interacting sites (1948, 1964, 1968), Hill's exponent as the "cooperativity index" or the "interaction coefficient" is closely related to the average free energy of interaction of the sites and the value of n greater than unity means that the interaction is a stabilizing one. For the synaptosomal Ca^{++} binding, therefore, it can be concluded that the higher the external K^+ concentration, the more stabilizing the interaction of receptive sites. As seen in Fig. 8, increases in n are marked when the K^+ concentration exceeds 30 mM, a fact suggesting that a strong interaction of binding sites occurs in such a high K^+ media. With respect to the external K^+ concentration, Ca^{++} release from synaptosomes in the presence of K^+ (Fig. 3) increases in the dissociation constant, K (Fig. 8) as well as K^+-induced swelling of synaptosomes reported earlier quite resemble this behavior of n. These findings indicate that changes in the state of synaptosomal membrane and/or binding sites markedly occur when the external K^+ concentration reaches over a certain critical level, possibly 30 to 40 mM.

The effect of K^+ recently reported on artificial and biological membranes appears to be consistent with our results. Concentration dependence of the effect of K^+ on the electrical capacitance of a model membrane system studied by Yoshida, Kobatake, Hashimoto and Morita (1971) is similar to that of synaptosomal Ca^{++}-binding which these investigators explained in terms of conformational changes in lipid structure. Based on extensive studies, Tasaki (1968) suggested that changes in membrane prop-

erties brought about by excitation could be explained by conformational changes in membrane macromolecular structures, the same of which are quite similar to those in a depolarized state induced by raised external K^+. On the basis of such a hypothesis the rapid effect of K^+ on m.e.p.p. and its inhibition and disinhibition by varying external Ca^{++} concentrations as studied by Cooke and Quastel (1973*a*) also appears to be feasible.

In the light of the above, our view that raised external K^+ induces a conformational change in synaptosomal membrane which results in a specific noncompetitive inhibition of its binding with Ca^{++} appears to be quite plausible.

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