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RELEASE OF ACETYLCHOLINE FROM RAT BRAIN SYNAPTOSOMES BY VARIOUS AGENTS IN THE ABSENCE OF EXTERNAL CALCIUM IONS

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SUMMARY

1. The relationship between $^{86}Rb^+$ distribution across synaptosomal membrane and [^{14}C]acetylcholine (ACh) release have been studied in a rat brain cortex synaptosomal preparation using K^+, ouabain and veratridine depolarization.

2. Decrease in membrane potential, approximated from the $^{86}Rb^+$ distribution, is accompanied by an increase in [^{14}C]ACh release, but the extent of the increase at a certain depolarization is dependent on how the depolarization is induced. A substantial depolarization by K^+ is necessary to enhance ACh release, as compared to ouabain and veratridine where only a slight depolarization is accompanied by an increase in ACh release. In Ca^{2+}-free, EGTA-containing medium ouabain and veratridine can also increase [^{14}C]ACh release. The relationship between membrane potential and ACh release is very similar in the presence of ouabain and veratridine both in Ca^{2+}-containing and Ca^{2+}-free medium.

3. The effect of ouabain and veratridine on the Na–K exchange pump is different; ouabain can completely abolish Na–K-ATPase activity and $^{86}Rb^+$ uptake of synaptosomes, whereas veratridine does not seem to influence the activity of the pump.

4. *m*-Chloro-carbonylcianid phenyl hydrazon (50–500 nM) increases [^{14}C]ACh release in a concentration-dependent manner without a considerable change of membrane potential or Na–K pump activity.

5. The Ca^{2+} ionophore A 23187 induces a substantial increase in [^{14}C]ACh release in the absence of external Ca^{2+}. In this case neither Na–K pump activity nor membrane potential of synaptosomes is changed.

6. A possible role of intracellular Ca^{2+} mobilization as a consequence of increased intracellular Na^+ concentration in some depolarization-induced transmitter release is discussed.

INTRODUCTION

The pioneering electrophysiological studies of Katz & Miledi (1967*a*, *b*, *c*) revealed the central role of Ca^{2+} in the process of synaptic transmission both at the neuromuscular junction of the frog and in the giant synapse of the squid. According to the 'calcium hypothesis' (Katz & Miledi, 1967*a*, *b*, *c*) depolarization of nerve

terminals induces a sudden increase of permeability of the presynaptic plasma membrane for Ca^{2+} and the inward movement of the divalent cation brings about the release of transmitter substance. Indeed, voltage-dependent Ca^{2+} channels have been identified in the plasma membrane of various excitable tissues (see Kostyuk, 1980) and the intensity of presynaptic Ca^{2+} current was found to be in a linear relationship with the amplitude of the post-synaptic potential change (Llinas, Steinberg & Walton, 1976).

Techniques used in neurophysiology have proved useful for studying the events occurring during chemical neurotransmission. However, this approach also has some limitations. For example, in the papers cited above, release of a transmitter substance was assessed only indirectly, on the basis of the post-synaptic response. Furthermore, the small size and extreme complexity of some synaptic connexions render them inaccessible for electrophysiological studies. These problems have led to the application of different biochemical approaches, such as preparation of tissue slices or 'synaptosomes'. Although the use of synaptosomes also has limitations, it is suitable for direct investigations of transmitter release and of the possible participation of intracellular particles in the release mechanism. Release of transmitter substances from synaptosomes can be induced by chemical agents, e.g. high K^+ concentration, ouabain or veratridine. All these agents are known to depolarize the membrane; however, the effect of a prolonged depolarization either by chemical agents or by repetitive stimulation seems to differ in some respects from that of a brief nerve impulse. Tetanic stimulation increases the frequency of miniature end-plate potentials even in Ca^{2+}-free solutions (Miledi & Thies, 1971); similarly, ouabain (Vizi, 1972, 1977; Baker & Crawford, 1975; Meyer & Cooper, 1981; Vyas & Marchbanks, 1981) and veratridine (Sandoval, 1980; Meyer & Cooper, 1981; Vyas & Marchbanks, 1981; Schoffelmeer & Mulder, 1983) can enhance transmitter release in the absence of external Ca^{2+}, thus querying the exclusive role of external Ca^{2+} entry in the depolarization-induced transmitter release.

In the present work we have studied the dependence of neurotransmitter release on the change of membrane potential (as detected by change in $^{86}Rb^+$ distribution; Scott & Nicholls, 1980) under different depolarizing conditions (high K^+ concentration, ouabain, veratridine) both in the presence and absence of Ca^{2+}. As far as we know this is the first attempt to relate depolarization to transmitter release in the case of isolated nerve endings of the mammalian central nervous system.

In addition, enhanced transmitter release is evoked by a mitochondrial uncoupler and by a Ca^{2+} ionophore, without intervention of membrane depolarization or extracellular Ca^{2+}, a case in which Ca^{2+} originating from intracellular pools is probably involved.

METHODS

Preparation of synaptosomes

Preparation was carried out as described by Hajós (1975) from the cerebral cortices of CFY rats weighing 120–150 g. Synaptosomes yielded in 0·8 M-sucrose were diluted with an equal volume of ice-cold (4 °C) 3K + Ca medium containing (mM): NaCl, 126; KCl, 3; $MgCl_2$, 2; Na phosphate buffer, 10, pH 7·2; glucose, 10; $CaCl_2$, 2 and centrifuged for 20 min at 16000 *g*. (In 3K − Ca + EGTA medium there is no Ca^{2+} and 1 mM-EGTA is present.) The pellet was used for further manipulation.

Release of [^{14}C]acetylcholine ([^{14}C]ACh) from synaptosomes

The release of [^{14}C]ACh synthesized from externally added [^{14}C]choline ([^{14}C]Ch) and endogenous acetyl-CoA was measured as described by Wonnacott & Marchbanks (1976) with few modifications. The synaptosomal pellet was resuspended in 3K + Ca medium to give a final concentration of 4–6 mg protein/ml and was incubated with 2 μM-[^{14}C]Ch (0·1 μCi/ml; 3·7 kBg/ml) for 30 min at 37 °C. After

TABLE 1. $^{86}Rb^+$ distribution and [^{14}C]ACh release in rat brain cortex synaptosomes under resting conditions

Incubation conditions	Internal Rb^+/ external Rb^+	Membrane potential (mV)	^{14}C activity in supernatants (ct/min . mg)		[^{14}C]ACh release (ct/min . mg)
			ACh	Ch	
3K + Ca	11·9 ± 1·1 (n = 16)	63·4 ± 2·3 (n = 16)	2986 ± 1999 (n = 15)	1777 ± 117 (n = 15)	1410 ± 223 (n = 15)
3K − Ca + EGTA	10·5 ± 0·8 (n = 9)	60·2 ± 1·9 (n = 9)	2190 ± 115 (n = 12)	2190 ± 120 (n = 12)	614 ± 175 (n = 12)
3K − Ca + EGTA (0 min)	N.d.	N.d.	1576 ± 259 (n = 15)	2151 ± 273 (n = 15)	

In parallel samples internal Rb^+/external Rb^+ ratio in the pellet and [^{14}C]Ch and [^{14}C]ACh (ct/min . mg) in the supernatants have been determined after incubating the synaptosomes for 15 min in the appropriate medium. Samples in 3K − Ca + EGTA medium where indicated (0 min) have been sedimented immediately after adding synaptosomal aliquots. In these samples $^{86}Rb^+$ distribution has not been determined (N.d.). [^{14}C]ACh release is expressed by subtracting [^{14}C]ACh activity measured in the supernatants of samples sedimented at 0 min from that measured after 15 min incubation (± S.E. of mean).

sedimentation at 10000 g the pellet was washed twice with ice-cold 0·32 M-sucrose, then suspended in 0·32 M-sucrose to give a final protein concentration of 20 mg/ml. 50 μl aliquots were added to Eppendorf tubes containing 0·5 ml 3K ± Ca + 20 μM-eserine and pre-incubated for 5 min at 37 °C. Different substances (veratridine, ouabain, etc.) were added after 5 min and incubated for an additional 10 min. The release was terminated by spinning in a Janetzky TH 12 microcentrifuge at 12000 g for 30 s and the supernatants were kept for extraction. [^{14}C]Ch and [^{14}C]ACh were extracted and separated as described by Nemeth & Cooper (1979). This method gave the same results as that of Marchbanks & Israel (1971) and Wonnacott & Marchbanks (1976). Radioactivities were counted in a Beckman LS 250 liquid scintillation spectrometer. Table 1 illustrates the radioactivities measured in the supernatants of control medium. In 3K − Ca medium at 0 min (immediately after adding 50 μl synaptosomal aliquot the samples have been centrifuged) there is both [^{14}C]ACh and [^{14}C]Ch in the supernatant. This is due partly to contamination, remaining after the two washings, and partly to leakage from the synaptosomes kept in cold 0·32 M-sucrose. [^{14}C]ACh release (ct/min . mg) is expressed as a difference between the values measured in the supernantants after 15 min incubation and at 0 min. During the incubation period in 3K ± Ca medium [^{14}C]Ch is not released or leaked out from synaptosomes, whereas [^{14}C]ACh release is increased slightly in Ca^{2+}-free but substantially in Ca^{2+}-containing medium (Table 1).

$^{86}Rb^+$ distribution in synaptosomes

Theoretical aspects. Keen & White (1971) showed that synaptosomal plasma membrane is similar to other excitable membranes in that the permeability for K^+ is in great excess to that for Na^+. As mitochondria are highly impermeable for both K^+ and Rb^+ neither intrasynaptosomal nor contaminating free mitochondria accumulate these ions. Thus, the distribution of K^+ or Rb^+ between the intra- and extracellular compartment can be used for a rough estimation of the plasma membrane potential on the basis of the Nernst equation (Scott & Nicholls, 1980; Akerman & Nicholls, 1981*b*).

In our experiments transmitter release was induced by either high K^+ concentrations, ouabain or veratridine. In all of these cases a new ('lower') equilibrium of $^{86}Rb^+$ distribution could be measured within 2 min following the addition of the drugs and this value remained unchanged for the next 30 min after any manipulation. It was also checked that the value of internal Rb^+/external Rb^+ was independent of the ratio of $^{86}Rb^+$ to cold K^+ in the medium; thus alterations of the extracellular K^+ concentrations (3–40 mM) did not give rise to experimental error due to changes of the 'specific activity' ($^{86}Rb^+$:$^{40}K^+$).

Blaustein & Goldring (1975) estimated that 75 μM-veratridine caused a fivefold increase in Na^+ permeability. The increase induced in our experiments by 5–20 μM-veratridine was probably less. The large surface-to-volume ratio of the synaptosomes and the long incubation time (10 min) allow K^+ (and Rb^+) to reach a new equilibrium. This fact provides an argument for regarding $^{86}Rb^+$ distribution in the presence of these concentrations of veratridine as an information about membrane potential.

Determination of $^{86}Rb^+$ distribution. In order to establish identical conditions in measurement of $^{86}Rb^+$ distribution and [^{14}C]ACh release, synaptosomes suspended in 3K + Ca medium (protein concentration, 4–6 mg/ml) were incubated for 30 min at 37 °C and washed twice with ice-cold 0·32 M-sucrose. After the second washing synaptosomes were suspended in ice-cold 0·32 M-sucrose at a protein concentration of 20 mg/ml. An aliquot of 50 μl was then added to 0·5 ml 3K ± Ca + eserine medium supplemented with $^{86}Rb^+$ (0·1 μCi/ml; 3·7 kBq/ml) (specific activity, 6·15 GBq/g) and ^3H_2O (1 μCi/ml; 37 kBq/ml). Synaptosomes were incubated for 5 min in a shaking bath (Tecam SB 4) at 37 °C. Different releasing agents were then added in small volumes (5–20 μl) and incubated for further 10 min.

Incubation was terminated by rapid separation of synaptosomes from the suspending medium in a Janetzky TH 12 microcentrifuge at 12000 *g*. Supernatants were discarded and pellets were resuspended in 100 μl 3K + Ca medium and transferred to scintillation cuvettes. Radioactivity was counted as described above. Distribution ratio of $^{86}Rb^+$ was calculated as detailed by Scott & Nicholls (1980). Corrections of the extrasynaptosomal $^{86}Rb^+$ content of the pellet were done by calculating the contaminating external space. The latter is given by the difference of the total water space of the pellet and the intrasynaptosomal volume (see below). Data of $^{86}Rb^+$ distribution determined under resting conditions can be seen in Table 1. The approximate values of resting membrane potentials in the presence and absence of extracellular Ca^{2+}, calculated by the Nernst equation, are 63·4 ± 2·3 and 60·2 ± 1·9 mV, respectively, which are very close to those measured in mammalian central neurones by micro-electrodes (Li, 1959). Similar values were estimated for synaptosomes by fluorescence dye technique (Blaustein & Goldring, 1975), by measuring [^{3}H]triphenylmethylphosphonium distribution (Creveling, McNeal, McCulloh & Daly, 1980) or $^{86}Rb^+$ distribution (Scott & Nicholls, 1980).

Determination of intrasynaptosomal volume

Synaptosomes were incubated for 15 min at 37 °C in 0·5 ml 3K + Ca medium supplemented with ^3H_2O (1 μCi/ml) and [^{14}C]sucrose (0·1 μCi/ml). Incubation was terminated by rapid centrifugation. The supernatants were discarded, and the pellets resuspended in 100 μl 3K + Ca medium and transferred to scintillation vials. Counting was carried out as described above. The difference between water space and sucrose space was regarded as intrasynaptosomal space. Twelve determinations gave an average value of 2·4 ± 0·04 (S.E. of mean) μl/mg protein, this being in agreement with the data of Marchbanks (1967) and Scott & Nicholls (1980).

$^{86}Rb^+$ uptake of synaptosomes as a measure of Na–K-pump activity

The synaptosomal pellet was suspended in ice-cold 0·32 M-sucrose to give a final protein concentration of 20 mg/ml. Aliquots of 50 μl were added to Eppendorf tubes containing 3K ± Ca medium plus the drugs studied and incubated for 5 min at 37 °C then $^{86}Rb^+$ (0·1 μCi/ml) was added. After incubating for a definite time the samples were centrifuged and the pellet was counted for $^{86}Rb^+$.

Na–K-ATPase activity

The synaptosomal pellet was suspended in distilled water (2 ml/g original cortex). For the ATPase assay the medium contained: Tris HCl, 50 mM, pH 7·4; $MgCl_2$, 5 mM; NaCl, 100 mM; KCl, 20 mM; ATP, 5 mM. The final volume was 2 ml. After pre-incubation of the medium for 1 min at 37 °C the

reaction was started by addition of synaptosomes (0·2 mg protein). The incubation was carried out at 37 °C for 10 min and was stopped by 1 ml 20% trichloroacetic acid. The phosphate content of the protein-free solution was determined by the method of Fiske & Subbarow (1925). Na–K-ATPase activity was calculated from the total ATPase activity by subtracting those measured in the absence of Na^+ and K^+ and expressed in nmol P_i/mg.min.

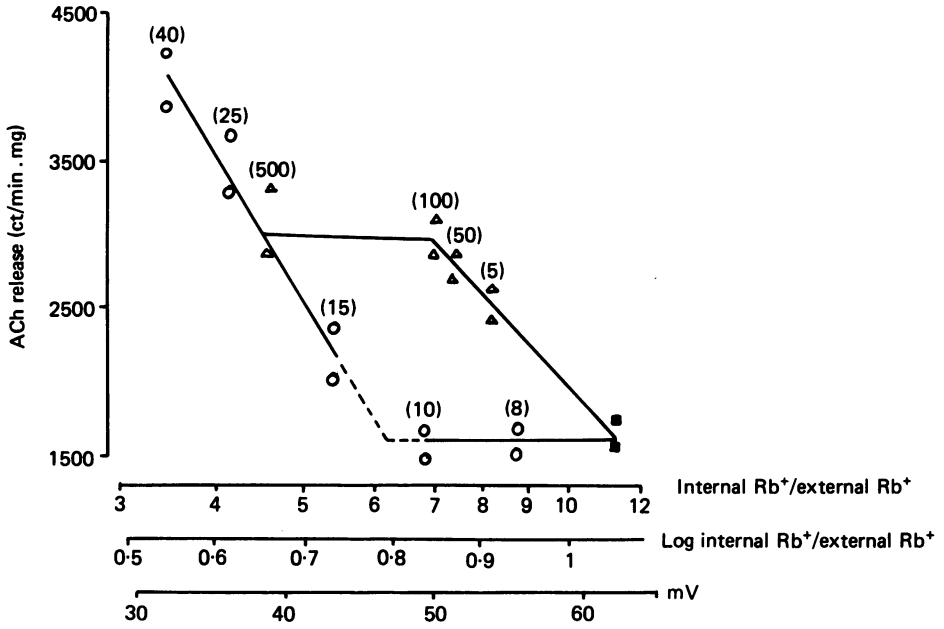

Fig. 1. Effect of K^+ and ouabain on $^{86}Rb^+$ distribution and [^{14}C]ACh release of synaptosomes in the presence of 2 mM-Ca^{2+}. Points represent the results of one typical experiment with parallel determinations. The concentrations of K^+ (mM) and ouabain (μM) are in parentheses. ■, control; ○, K^+ (8–40 mM); △, ouabain (5–500 μM).

Protein determination

The protein content was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovin serum albumin as standard.

Materials

A 23187, *m*-chloro-carbonylcianid phenyl hydrazon (CCCP) and eserine sulphate were purchased from Calbiochem Behring Co. Ouabain was obtained from Serva Feinbiochemical GmbH, veratridine from EGA-Chemical Co. and EGTA from Sigma Chemical Co. [^{14}C]choline was purchased from The Radiochemical Centre, Amersham, ^{86}Rb and ^3H_2O from Institute of Isotopes of Hungarian Academy of Sciences.

RESULTS

Dependence of [^{14}C]ACh release on membrane depolarization in the presence of extracellular Ca^{2+}

Synaptosomes have been exposed to K^+, ouabain and veratridine, respectively, and [^{14}C]ACh release was detected parallel to $^{86}Rb^+$ distribution. In Fig. 1 the effect of different concentrations of K^+ and ouabain are compared illustrating [^{14}C]ACh release

as a function of $^{86}Rb^+$ distribution. Under resting conditions (measured in 3K + Ca medium) the internal Rb^+/external Rb^+ was 11·5.

In the presence of 8 mM-K^+ this value was reduced to 8·6 indicating a depolarization of the membrane due to the increased external K^+ concentration. Addition of 5 μM-ouabain had approximately the same effect as 8 mM-K^+: internal Rb^+/external Rb^+ was depressed to 8·2. In spite of the similar values of $^{86}Rb^+$ distribution [^{14}C]ACh release was very different under the two conditions: 8 mM-K^+ did not induce any transmitter release whereas 5 μM-ouabain stimulated the release by approximately 900 ct/min . mg. Higher concentrations of ouabain brought about a proportional decrease of internal Rb^+/external Rb^+ and an increase of [^{14}C]ACh release. However, a very high concentration of the drug (500 μM) affected the two parameters differently: $^{86}Rb^+$ distribution was further depressed whereas transmitter release was not further enhanced.

Increasing the concentration of K^+ above 10 mM induced the release of [^{14}C]ACh but even at an internal Rb^+/external Rb^+ value of 5·5 the amount of liberated transmitter was less than the value observed in the presence of ouabain at an internal Rb^+/external Rb^+ value of 7.

However, while our attention was focused on the difference observed at small depolarizations, it is also remarkable that more [^{14}C]ACh could be released by high K^+ concentrations (25 and 40 mM) than by high concentration of ouabain (500 μM).

The difference between ACh release induced by increasing the concentration of K^+ or by ouabain detailed above was observed in every experiment although the absolute values of both $^{86}Rb^+$ distribution and transmitter release were fairly variable. The consistency of the distinct effect of K^+ and ouabain can be demonstrated if the control values of both log internal Rb^+/external Rb^+ and [^{14}C]ACh release are subtracted from the respective experimental data. Fig. 2*A* summarizes the results of eight different experiments 'normalized' in this way. It can be seen that the points representing the effect of ouabain or high K^+ concentration are clearly distinguishable: they form two distinct straight lines with similar slopes but different intercepts, both lines having high correlation coefficients. (In the case of ouabain the points obtained in the presence of very high concentrations (above 100 μM), i.e. where Rb^+ distribution and ACh release were not proportional, were disregarded.) The fact that the K^+ line does not intercept the abscissa at or near to the origin but at a positive value means that in this case internal Rb^+/external Rb^+ has to be considerably decreased in order to induce transmitter release. If the data on $^{86}Rb^+$ distribution are used to estimate the changes of the membrane potential, approximately 10 mV seems to be the minimum depolarization necessary for induction of ACh release. Using veratridine (2–20 μM) as a depolarizing agent we could see a very similar relationship between $^{86}Rb^+$ distribution and ACh release as in the case of ouabain (Fig. 2*B*).

Blaustein (1975) revealed that synaptosomes have a voltage-sensitive Ca^{2+} entry mechanism which starts to operate when depolarization exceeds a certain amount. He estimated the relationship between membrane potential and Ca^{2+} conductance during K^+ depolarization. From his calculations it appears that displacement of the membrane potential from the resting value by up to 10 mV does not cause any increase in Ca^{2+} conductance, but above 10 mV it gradually increases. Our calculations to estimate the smallest amount of depolarization resulting in increased transmitter

release gave the same value; after a depolarization of more than about 10 mV the further drop of the membrane potential can be correlated with the increase in [^{14}C]ACh release. Thus, in the case of K^+ depolarization also the quantitative data are in accordance with the suggestion of the calcium hypothesis, i.e. with the following sequence of events: membrane depolarization, voltage dependent Ca^{2+} entry,

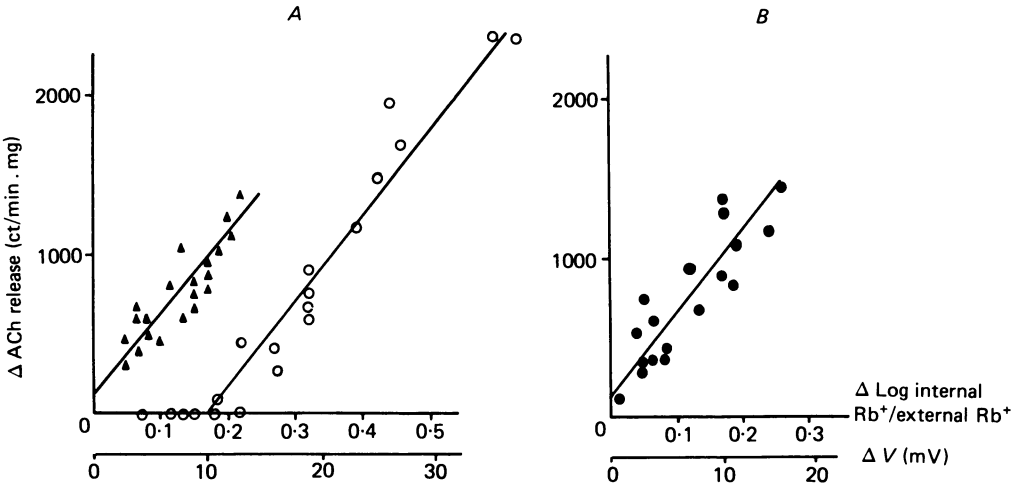

Fig. 2. *A*, the change in ACh release induced by 8–40 mM-K^+ (○) and 5–100 μM-ouabain (▲) is replotted against the change of log internal Rb^+/external Rb^+. *B*, the same in the presence of 2–20 μM-veratridine (●). ΔACh release is calculated by subtracting the corresponding control (3K + Ca) ACh release (ct/min . mg) from ACh release measured in depolarized samples. ΔLog internal Rb^+/external Rb^+ (or Δ *V*) is the displacement of log internal Rb^+/external Rb^+ (or membrane potential, *V*) in depolarized samples from the resting value. Points are derived from eight (ouabain, veratridine) or seven (K^+) different experiments and each point represents the average of two determinations. Straight lines are drawn according to equations calculated from the corresponding points by the method of least-squares. These equations are: $y = 92{\cdot}2x - 902$, $r = 0{\cdot}966$ for K^+; $y = 83{\cdot}8x + 136$, $r = 0{\cdot}919$ for ouabain; and $y = 86{\cdot}8x + 144$, $r = 0{\cdot}916$ for veratridine, if x is expressed in mV.

transmitter release. However, something else should occur when depolarization is brought about by ouabain or veratridine, as in these cases a minor depolarization was associated with the release of a considerable amount of ACh. Regarding the increase of intracellular Na^+ concentration upon treatment with ouabain or veratridine one can suppose an increased Ca^{2+} influx by a Na^+–Ca^{2+} exchange mechanism operating in synaptosomes (Blaustein & Oborn, 1975) as well as in squid giant axons (see Baker, 1972). This mechanism might be activated by the increase of internal Na^+ accompanying an already small depolarization and therefore might be responsible for the increased transmitter release observed. This possibility was tested in experiments using Ca^{2+}-free medium.

Dependence of [^{14}C]ACh release on membrane depolarization in the absence of extracellular Ca^{2+}

In these experiments Ca^{2+} was omitted from the medium and 1 mM-EGTA was added. It has already been shown that ouabain (Vizi, 1972, 1977; Baker & Crawford, 1975; Sandoval, 1980; Meyer & Cooper, 1981; Vyas & Marchbanks, 1981) and

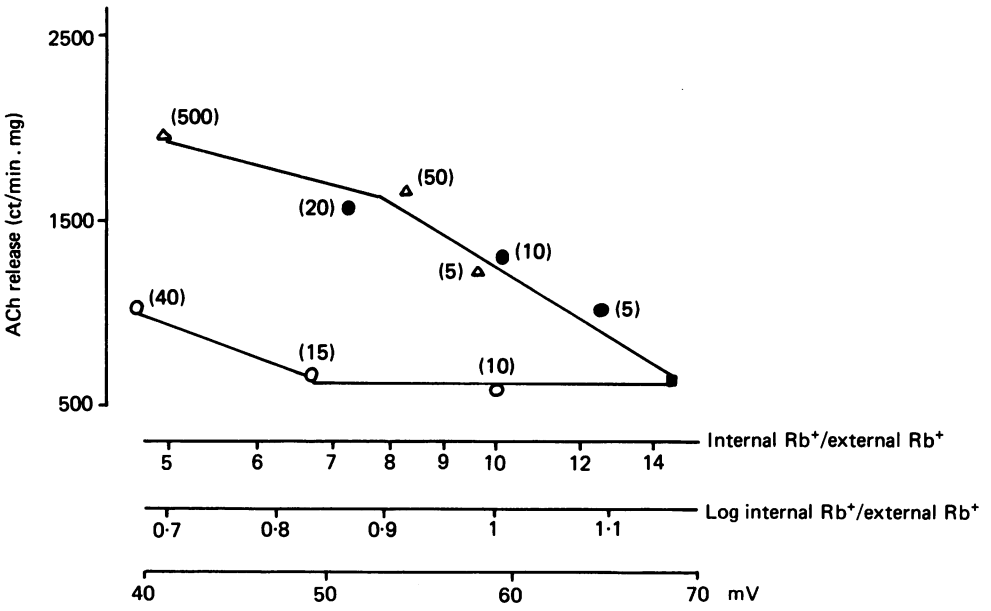

Fig. 3. Effect of ouabain (△), veratridine (●) and K^+ (○) on $^{86}Rb^+$ distribution and [^{14}C]ACh release in the absence of extracellular Ca^{2+} (and in the presence of 1 mM-EGTA). Points represent the average of three determinations (S.E. of mean values are less than 5% of the averaged values at each point). The concentrations of ouabain and veratridine (μM) and K^+ (mM) are given in parentheses. ■ represents the control value.

veratridine (Meyer & Cooper, 1981, Schoffelmeer & Mulder, 1983) are also able to increase transmitter release from different tissues under these conditions. Our aim was to follow not only the change in ACh release but also $^{86}Rb^+$ distribution.

In the absence of Ca^{2+}, K^+ could only slightly increase ACh release in spite of the considerable depolarization (Fig. 3): a fall of internal Rb^+/external Rb^+ from 14·7 to 4·6 is accompanied by a marginal increment of ACh release.

However, the effects of ouabain and veratridine are very similar to those observed in the presence of Ca^{2+}, i.e. small decrease in internal Rb^+/external Rb^+ is already associated with an increase of ACh release (Fig. 3). All of the basic characteristics of the curve showing the relationship between membrane potential and ACh release are also similar to that of Fig. 1, although in the absence of Ca^{2+} the absolute values of ACh release in ct/min . mg are lower and the slope of the curve is slightly reduced.

These observations in the Ca^{2+}-free, EGTA-containing medium rule out a possible role of Ca^{2+} entry in the transmitter release induced by ouabain or veratridine. On the other hand it is conspicuous that the effects of these two drugs are similar both

in the presence (Fig. 2) and the absence (Fig. 3) of extracellular Ca^{2+}: a particular amount of depolarization by either of them is accompanied by the release of the same amount of ACh. This suggests that there may be common steps in the intracellular events by which ouabain and veratridine induce transmitter release.

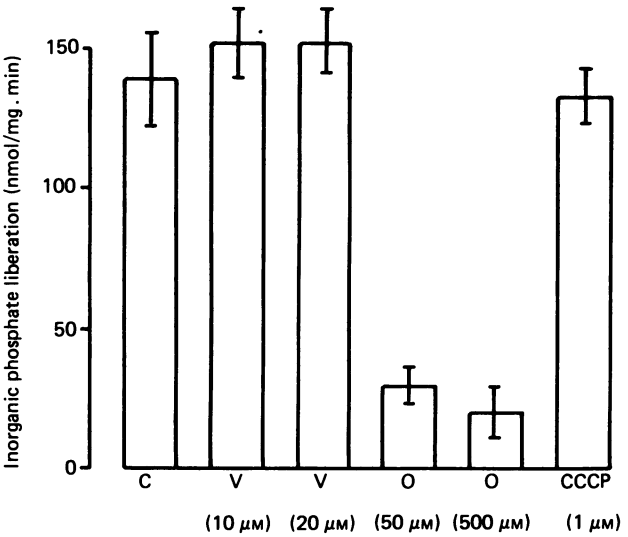

Fig. 4. Na–K-ATPase activity of disrupted synaptosomes measured by P_i liberation. The columns represent the average of three determinations ±S.E. of mean. Abbreviations: C, control; O, Ouabain; V, veratridine.

The effect of ouabain and veratridine on Na–K-pump of synaptosomes

Ouabain is known to be a specific inhibitor of Na–K-ATPase located in the plasma membrane. The inhibition of this enzyme has been suggested to be directly responsible for the increase of transmitter release (cf. Vizi, 1978), and the transmitter-releasing effect of ouabain in Auerbach's plexus and cortex slices has been attributed to the inhibition of Na–K-ATPase (Vizi, 1977). Meyer & Cooper (1981) also found a correlation between Na–K-ATPase inhibition and ACh release in synaptosomal preparations. Because of the similarity between the two drugs presented in Figs. 2*A* and *B* and 3, we also compared their effects on the Na–K-ATPase activity of synaptosomes. Measuring the P_i liberation of disrupted synaptosomes, it has been found that in contrast to ouabain, veratridine has no direct effect on the enzyme (Fig. 4). This does not necessarily mean that in a more normal system such as intact synaptosomes the pump cannot be affected. The activity of the pump can be assessed by measuring $^{86}Rb^+$ uptake of synaptosomes. However, only data from the very early period following $^{86}Rb^+$ introduction can be used, as $^{86}Rb^+$ distributes across the membrane in compliance with the membrane potential. Accordingly one has to be cautious when studying the effect of a depolarizing drug such as veratridine which produces a new $^{86}Rb^+$ equilibrium within a few minutes. With this complication in mind we measured $^{86}Rb^+$ uptake of synaptosomes after an exposure time of 15–60 s, in which range the uptake was linear (data not shown). To demonstrate the effect

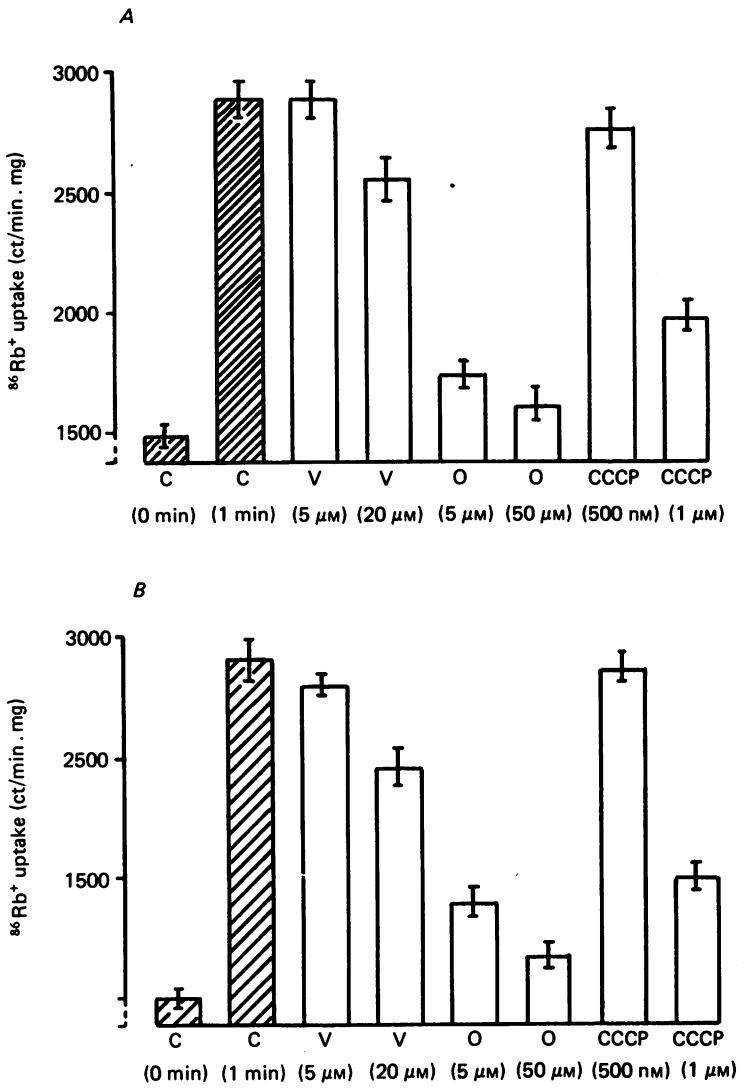

Fig. 5. $^{86}Rb^+$ uptake of synaptosomes measured after 60 s exposure to $^{86}Rb^+$ in the presence (*A*) or absence (*B*) of extracellular Ca^{2+}. The data are the average of three determinations ±S.E. of mean. Abbreviations: C, control; O, ouabain; V, veratridine.

of different conditions an exposure time of 60 s has been chosen where the data from different experiments were comparable and most reproducible. Ouabain substantially inhibits $^{86}Rb^+$ uptake, whereas in the presence of veratridine $^{86}Rb^+$ found in synaptosomes is only slightly lower (2540±81 ct/min. mg) than the control value (2825±82) and the effect is not dependent on the presence or absence of Ca^{2+} (Fig. 5). In this respect our results do not agree with those of Meyer & Cooper (1981) who showed a considerable decrease of ^{86}Rb uptake by veratridine which was Ca^{2+} dependent. As an explanation they suggested that in the presence of extracellular

Ca^{2+} the depolarization by veratridine could be followed by Ca^{2+}-entry, which could then inhibit the Na–K-ATPase. However, the reduced $^{86}Rb^+$ uptake in their experiments is only appreciable after an exposure to $^{86}Rb^+$ for 2 min or even longer, when the depolarization-induced new $^{86}Rb^+$ equilibrium can already mask the $^{86}Rb^+$

TABLE 2. Effect of various concentrations of CCCP on $^{86}Rb^+$ distribution and [^{14}C]ACh release in rat brain cortex synaptosomes in the absence of extracellular Ca^{2+}

	Internal Rb^+/ external Rb^+	V (mV)	[^{14}C]ACh release (ct/min . mg)
Control (3K − Ca + EGTA)	8·2 ± 0·9	54 ± 2·5	460 ± 48
CCCP (50 nM)	7·2 ± 1·1	51 ± 3·2	703 ± 50
CCCP (100 nM)	7·1 ± 0·8	50 ± 1·9	948 ± 23
CCCP (500 nM)	7·2 ± 0·8	51 ± 2·2	1159 ± 80
CCCP (1 μM)	5·9 ± 0·5	45 ± 2·1	1368 ± 64

Data represent the average of three determinations ± S.E. of mean. CCCP has been added after pre-incubating synaptosomal samples in 3K − Ca + EGTA medium for 5 min and has been incubated for further 10 min.

uptake driven by the Na–K pump. Even in our experiments the slight decrease of $^{86}Rb^+$ content measured at after only 60 s in the presence of veratridine might be a consequence of depolarization.

Whatever the explanation for the slight decrease of $^{86}Rb^+$ uptake by veratridine, our experiments revealed a clear difference between the effect of ouabain and veratridine on the plasma membrane Na–K-ATPase whereas their effects on membrane potential and ACh release are similar.

Manipulation of intracellular Ca^{2+}

One thing is certainly a concomitant of both ouabain and veratridine depolarization but by different mechanisms; an increase of intracellular Na^+ concentration. Baker & Crawford (1975) raised the possibility of an intracellular Ca^{2+} mobilization due to the elevated internal Na^+ concentration as an explanation of the increase in the miniature end-plate potential frequency caused by ouabain in frog neuromuscular junction. The same mechanism was proposed for [^{3}H]γ-aminobutyric acid (GABA; Sandoval, 1980) and [^{3}H]noradrenaline (Shoffelmeer & Mulder, 1983) release from synaptosomes observed in the absence of extracellular Ca^{2+}. Akerman & Nicholls (1981*a*) have shown that FCCP (*p*-trifluoro-metoxi carbonyl cianid phenylhydrazon), a mitochondrial uncoupler is able to produce Ca^{2+} efflux from isolated brain mitochondria as well as from mitochondria occluded within synaptosomes. This observation led us to the idea of investigating the effect of CCCP on ACh release under Ca^{2+}-free conditions. We have found that in a very low concentration range (50–500 nM) CCCP is able to increase ACh release in a concentration-dependent manner (Table 2). However, CCCP as an uncoupler of mitochondrial oxidation and phosphorylation inhibits ATP synthesis and as a consequence could lead to depolarization of the plasma membrane. Therefore, we tested the effect of CCCP on both $^{86}Rb^+$ distribution (Table 2) and $^{86}Rb^+$ uptake (Fig. 5). Table 2 shows that the

ratio internal Rb^+/external Rb^+ is slightly decreased by 50 nM-CCCP but shows no further changes up to 500 nM. Thus, in this range ACh release is increased in proportion to the increase of CCCP concentration, without any measurable change of the membrane potential which might indicate mobilization of intracellular calcium from mitochondria.

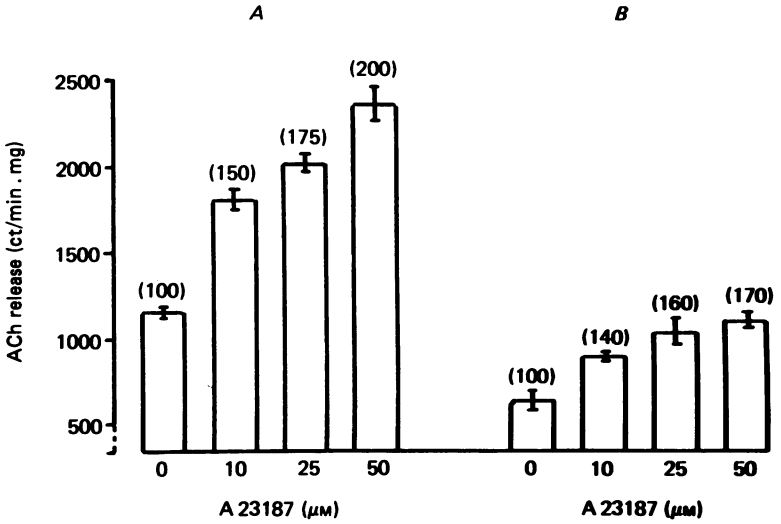

Fig. 6. Effect of various concentrations of A 23187 on ACh release in the presence (*A*) and in the absence (*B*) of extracellular Ca^{2+}. Columns represent the average of three determinations ±S.E. of mean, the numbers in parentheses express the effects as percentage change.

Higher concentrations of CCCP (1 μM and above) markedly decreased both the internal Rb^+/external Rb^+ ratio (Table 2) and Na–K-ATPase activity of intact synaptosomes measured on the basis of $^{86}Rb^+$ uptake (Fig. 5), although it had no effect on the enzyme activity of disrupted synaptosomes (Fig. 4). Under these conditions intracellular ATP content probably reached a low value where a limitation on the ATPase activity became evident.

The effect of the Ca^{2+} ionophore, A 23187 is more selective, allowing the free movement of Ca^{2+} across various biological membranes. In synaptosomes in the presence of 1–2 mM-external Ca^{2+} A 23187 was found to decrease both the mitochondrial and plasma membrane potential, to induce the uptake of Ca^{2+} (Akerman & Nicholls, 1981*b*) and to stimulate at the same time the release of noradrenaline (Akerman & Nicholls, 1981*b*), ACh (Meyer & Cooper, 1981) and GABA (Asakura, Hoshino & Kobayashi, 1982). However, in the case of low external Ca^{2+} concentration A 23187 should mobilize Ca^{2+} from intrasynaptosomal stores just as it was shown to induce the efflux of this cation both from isolated mitochondria (Pfeiffer, Hutson, Kauffman & Lardy, 1976; Nakashima, Dordick & Garlid, 1982) and from intact hepatocytes (Blackmore, Brumley, Marks & Exton, 1978; Chen, Babcock & Lardy, 1978). Following these observations we studied the effect of A 23187 in Ca^{2+}-free medium. Data obtained in the presence of Ca^{2+} serve as controls.

Fig. 6 shows that ACh release was stimulated by A 23187 in a concentration-dependent manner both in the presence and in the absence of external Ca^{2+}. The absolute values of ACh release were much lower in Ca^{2+}-free medium but the proportion of transmitter release was similar in the two cases.

Table 3 summarizes the effects of A 23187 on the Na–K-ATPase and $^{86}Rb^+$ distribution. The ionophore did not decrease the production of P_i from ATP in

TABLE 3. Effect of different concentrations of A 23187 on Na−K-ATPase and on $^{86}Rb^+$ distribution in the presence and absence of extracellular Ca^{2+}

		In the presence of Ca^{2+}		In the absence of Ca^{2+}	
	Na−K-ATPase (nmol/mg.min)	Internal Rb^+/external Rb^+ (membrane potential)	$^{86}Rb^+$ uptake (Na−K pump)	Internal Rb^+/external Rb^+ (membrane potential	$^{86}Rb^+$ uptake (Na−K pump)
A 23187					
(0 μM)	143±18	17·9	2825±82	16·5	2825±75
(25 μM)	172±25	6·1	1696±157	17·6	2969±45
(50 μM)	146±23	5·8	1441±91	17·0	2915±36

Data represent the average of three determinations ±S.E. of mean.

disrupted synaptosomal membranes, showing that the ionophore had no direct effect on the Na–K-ATPase enzyme. However, uptake of $^{86}Rb^+$ into synaptosomes was strongly inhibited in the presence of external Ca^{2+} and the ratio of internal Rb^+/external Rb^+ was correspondingly decreased. Both effects have to be attributed to Ca^{2+} entry, as in the absence of this cation A 23187 does not inhibit $^{86}Rb^+$ uptake or influence $^{86}Rb^+$ distribution. Thus, in the presence of Ca^{2+} the sequence of events can be reconstructed as follows: A 23187 increases Ca^{2+} permeability and the entering Ca^{2+} induces transmitter release and at the same time inhibits Na–K-ATPase. Whether this inhibition is a direct effect of the increase in intracellular Ca^{2+} concentration (as suggested by Meyer & Cooper, 1981) or the consequence of a decrease of mitochondrial ATP synthesis due to the drop of the mitochondrial membrane potential (Akerman & Nicholls, 1981*b*) has to be clarified in separate experiments. Anyhow, inhibition of the Na–K pump explains the depolarization of the plasma membrane observed in the presence of Ca^{2+} (Table 3; see also Akerman & Nicholls, 1981*b*).

The case is completely different in the absence of extracellular Ca^{2+}. In these experiments ACh release was observed under conditions where the activity of Na–K-ATPase enzyme remained unchanged, no depolarization of the plasma membrane occurred and no Ca^{2+} could enter from the extracellular space (Table 3). Under these conditions the release of ACh could very likely be ascribed to liberation of Ca^{2+} from intracellular stores.

DISCUSSION

In the Introduction the advantages of synaptosomes in neurochemical and neurophysiological studies have been outlined. However, the shortcomings of synaptosomes from the mammalian central nervous system also have to be mentioned,

especially the most serious one: the heterogeneity. The synaptosomal preparation from the mammalian cortex contains nerve endings with different kinds of transmitters which can modulate the release of ACh. There is no information available concerning the fine, distinct characteristics of the different synaptosomal subpopulations. Thus, for evaluating our results we have to assume that K^+ is equally able to depolarize different kinds of synaptosomes and that Na–K-ATPase as well as veratridine-sensitive Na^+ channels are evenly distributed in synaptosomes working with different neurotransmitters. In this case a conceivable modulation of ACh release by other neurotransmitters can alter only the absolute values of the release by K^+, ouabain or veratridine but the differences between their effects should not be changed. The other problem with our method is its relatively low temporal resolution. Transmitter release during a 10 min incubation period was followed, whereas under physiological conditions chemical transmission occurs within milliseconds. It cannot be excluded that the effect of the prolonged depolarization induced by different chemical agents or tetanic stimulation is different from that of a brief nerve impulse. Keeping this possible objection in mind we can summarize our results as follows.

K^+ depolarization

One of the aims of the present study was to reveal whether the response of isolated nerve terminals to depolarization manifested as transmitter release is uniform regardless of how depolarization was induced. It has been found that in the presence of extracellular Ca^{2+}, application of high K^+ concentration can lead to transmitter release in a different way from that of ouabain and veratridine. Our calculated data for the minimal depolarization by K^+ necessary for the increase of transmitter release agree with that estimated by Blaustein (1975) for increase in Ca^{2+} conductance of synaptosomes. This fact and the Ca^{2+} dependence of the K^+-evoked release are in good agreement with the 'calcium hypothesis' (Katz & Miledi, 1976*a*, *b*, *c*) and the suggestion of Blaustein (1975): a certain extent of depolarization by K^+ can induce Ca^{2+} influx which results in an increase of transmitter release in synaptosomes.

Ouabain and veratridine: intracellular Ca^{2+}

In the case of ouabain and veratridine, extracellular Ca^{2+} seems to play only a minor role in the increased ACh release. Vyas & Marchbanks (1981) suggested that in the presence of extracellular Ca^{2+} a 'normal', depolarization-induced, Ca^{2+}-dependent release process is produced in synaptosomes by ouabain whereas in the absence of external Ca^{2+} an unspecific change in membrane permeability independent of depolarization leads to increased transmitter release. Our finding, the clearly similar character of the relationship between membrane potential and ACh release in the presence and absence of external Ca^{2+}, would suggest one principal mechanism operating in both cases. This is suggested to be similar to that of veratridine as the effects of ouabain and veratridine are strikingly similar with and without Ca^{2+}. This common mechanism is, very likely, not the inhibition of Na–K-ATPase as in this respect their effects were clearly different (Figs. 4 and 5). This observation also indicates that the inhibition of the Na–K ATP pump might not be a necessary prerequisite for the transmitter release. There is a common event accompanying the effect of both ouabain and veratridine: the increase of intracellular Na^+ concentration.

Na^+ itself was shown to induce Ca^{2+} efflux from isolated (Crompton, Moser, Ludi & Carafoli, 1978) as well as from intrasynaptosomal mitochondria (Silbergeld, 1977) and Na–Ca exchange was also found in endoplasmic reticulum (Carafoli & Crompton, 1978). In the case of ouabain and veratridine the elevated intracellular Na^+ concentration might also lead to an increase in the free intracellular Ca^{2+} concentration, resulting in an enhanced transmitter release.

The possible participation of internal Ca^{2+} stores in the transmitter release is strongly supported by our findings with CCCP (Table 2) and A 23187 (Fig. 6 and Table 3) which could increase ACh release in the absence of external Ca^{2+}. On the basis of data concerning mitochondria (Pfeiffer *et al.* 1976; Nakashima *et al.* 1982) and isolated hepatocytes (Chen *et al.* 1978; Blackmore *et al.* 1982) it can be suggested that in the case of both CCCP and A 23187 the most likely factor responsible for the transmitter release is the elevated intracellular Ca^{2+} coming from intrasynaptosomal particle(s) without membrane depolarization or Na–K-ATPase inhibition.

This suggestion is in accord with the finding of Miledi (1973) who succeeded in raising intracellular Ca^{2+} concentration by injecting Ca^{2+} into the nerve terminal and could detect transmitter release without any depolarization.

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REFERENCES

AKERMAN, K. E. O. & NICHOLLS, D. G. (1981*a*). Intrasynaptosomal compartmentation of calcium during depolarization-induced calcium uptake across the plasma membrane. *Biochim. biophys. Acta* **645**, 41–48.

AKERMAN, K. E. O. & NICHOLLS, D. G. (1981*b*). Influence of ionophore A 23187 on plasma-membrane potential, plasma-membrane calcium transport, mitochondrial membrane potential, respiration, cytosolic free-calcium concentration and noradrenaline release. *Eur. Jnl. Biochem.* **115**, 67–73.

ASAKURA, T., HOSHINO, M. & KOBAYASHI, T. (1982). Effect of calcium ions on the release of γ-aminobutyric acid from synaptosomal fraction. *J. Biochem.* **92**, 1919–1923.

BAKER, P. F. (1972). Transport and metabolism of calcium ions in nerve. *Prog. Biophys. & mol. Biol.* **24**, 179–223.

BAKER, P. F. & CRAWFORD, A. C. (1975). A note on the mechanism by which inhibitors of the sodium pump accelerate spontaneous release of transmitter from motor nerve terminals. *J. Physiol.* **247**, 209–226.

BLACKMORE, P. F., BRUMLEY, F. T., MARKS, J. L. & EXTON, J. H. (1978). Studies on α-adrenergic activation of hepatic glucose output. *J. biol. Chem.* **253**, 4851–4858.

BLAUSTEIN, M. P. (1975). Effects of potassium, veratridine and scorpion venom on calcium accumulation and transmitter release by nerve terminals in vitro. *J. Physiol.* **247**, 617–665.

BLAUSTEIN, M. P. & GOLDRING, J. M. (1975). Membrane potentials in pinched-off presynaptic nerve terminals monitored with a fluorescent probe: evidence that synaptosomes have potassium diffusion potentials. *J. Physiol.* **247**, 589–615.

BLAUSTEIN, M. P. & OBORN, C. J. (1975). The influence of sodium on calcium fluxes in pinched-off nerve terminals *in vitro*. *J. Physiol.* **247**, 657–686

CARAFOLI, E. & CROMPTON, M. (1978). The regulation of intracellular calcium by mitochondria. *Ann. N.Y. Acad. Sci.* **307**, 269–284.

CHEN, J. L. J., BABCOCK, D. F. & LARDY, H. A. (1978). Norepinephrine, vasopressin, glucagon and A 23187 induce efflux of calcium from an exchangeable pool in isolated rat hepatocytes. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2234–2238.

CREVELING, C. R., MCNEAL, E. T., MCCULLOH, D. A. & DALY, J. W. (1980). Membrane potentials in cell-free preparations from guinea-pig cerebral cortex: effect of depolarizing agents and cyclic nucleotides. *J. Neurochem.* **35**, 922–932.

CROMPTON, M., MOSER, R., LUDI, M. & CARAFOLI, E. (1978). The interrelations between the transport of sodium and calcium in mitochondria of various mammalian tissues. *Eur. Jnl. Biochem.* **82**, 25–31.

FISKE, C. H. & SUBBAROW, Y. (1925). The colorimetric determination of phosphores. *J. biol. Chem.* **66**, 375–400.

HAJÓS, F. (1975). An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res.* **93**, 485–499.

KATZ, B. & MILEDI, R. (1967*a*). The release of acetylcholine from nerve endings by graded electric pulses. *Proc. R. Soc.* B **167**, 23–38.

KATZ, B. & MILEDI, R. 1967*b*). On the timing of calcium action during neuromuscular transmission. *J. Physiol.* **189**, 533–544.

KATZ, B. & MILEDI, R. (1967*c*). A study of synaptic transmission in the absence of nerve impulses. *J. Physiol.* **192**, 407–436.

KEEN, P. & WHITE, T. D. (1971). The permeability of pinched-off nerve endings to sodium, potassium and chloride and the effect of gramicidin. *J. Neurochem.* **18**, 1097–1103.

KOSTYUK, P. G. (1980). Calcium ionic channels in electrically excitable membranes. *Neuroscience* **5**, 945–959.

LI, C. L. (1959). Cortical intracellular potentials and their responses to strychnine. *J. Neurophysiol* **22**, 436–450.

LLINAS, R., STEINBERG, I. Z. & WALTON, K. (1976). Presynaptic calcium currents and their relation to synaptic transmission: voltage clamp study in squid giant synapse and theoretical model for the calcium gate. *Proc. natn. Acad. Sci., U.S.A.* **73**, 2918–2922.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

MARCHBANKS, R. M. (1967). The osmotically sensitive potassium and sodium compartments of synaptosomes. *Biochem. J.* **104**, 148–157.

MARCHBANKS, R. M. & ISRAEL, M. (1971). Aspects of acetylcholine metabolism in the electric organ of Torpedo marmorata. *J. Neurochem.* **18**, 439–448.

MEYER, E. M. & COOPER, J. R. (1981). Correlations between Na^+–K^+-ATPase activity and acetylcholine release in rat cortical synaptosomes. *J. Neurochem.* **36**, 467–475.

MILEDI, R. & THIES, R. (1971). Tetanic and post-tetanic rise in frequency of miniature end-plate potentials in low-calcium solutions. *J. Physiol.* **212**, 245–257.

MILEDI, R. (1973). Transmitter release induced by injection of calcium ions into nerve terminals. *Proc. R. Soc.* B **183**, 421–425.

NAKASHIMA, R. A., DORDICK, R. S. & GARLID, K. D. (1982). On the relative role of Ca^{2+} and Mg^{2+} in regulating the endogenous K^+/H^+ exchanger of rat liver mitochondria. *J. biol. Chem.* **257**, 12540–12545.

NEMETH, E. F. & COOPER, J. R. (1979). Effect of somatostatin on acetylcholine release from rat hippocampal synaptosomes. *Brain Res.* **165**, 166–170.

PFEIFFER, D. R., HUTSON, S. M., KAUFFMAN, R. F. & LARDY, H. A. (1976). Same effects of ionophore A 23187 on energy utilization and the distribution of cations and anions in mitochondria. *Biochemistry, N.Y.* **15**, 2690–2697.

SANDOVAL, M. E. (1980). Sodium-dependent efflux of ^{3}H-GABA from synaptosomes probably related to mitochondrial calcium mobilization. *J. Neurochem.* **35**, 915–921.

SCHOFFELMEER, A. N. M. & MULDER, A. H. (1983). ^{3}H-noradrenaline release from brain slices induced by an increase in the intracellular sodium concentration: role of intracellular calcium stores. *J. Neurochem.* **40**, 615–621.

SCOTT, I. D. & NICHOLLS, D. G. (1980). Energy transduction in intact synaptosomes. *Biochem. J.* **186**, 21–33.

SILBERGELD, E. K. (1977). Na^+ regulates release of Ca^{2+} sequestered in synaptosomal mitochondria. *Biochem. biophys. Res. Comm.* **77**, 464–469.

VIZI, E. S. (1972). Stimulation, by inhibition of (Na^+- K^+- Mg^{2+})-activated ATP-ase, of acetylcholine release in cortical slices from rat brain. *J. Physiol.* **226**, 95–117.

VIZI, E. S. (1977). Termination of transmitter release by stimulation of sodium–potassium-activated ATPase. *J. Physiol.* **267**, 261–280.

VIZI, E. S. (1978). Na^+–K^+-activated adenosinetriphosphatase as a trigger in transmitter release. *Neuroscience* **3**, 367–384.

VYAS, S. & MARCHBANKS, R. M. (1981). The effect of ouabain on the release of (^{14}C) acetylcholine and other substances from synaptosomes. *J. Neurochem* **37**, 1467–1474.

WONNACOTT, S. & MARCHBANKS, R. M. (1976). Inhibition by botulinum toxin of depolarization-evoked release of (^{14}C) acetylcholine from synaptosomes in vitro. *Biochem. J.* **156**, 701–712.