Biochimica et Biophysica Acta 854 (1986) 207–212
Elsevier

BBA 72863

External calcium, intrasynaptosomal free calcium and neurotransmitter release

R.H. Ashley *

School of Biological Sciences, University of Sussex, Falmer, Brighton, BN1 9QG (U.K.)

(Received August 12th, 1985)

Key words: Synaptosomal membrane; Membrane potential; Ca^{2+}; Neurotransmitter release; (Guinea pig)

In a physiological medium the resting membrane potential of synaptosomes from guinea-pig cerebral cortex, estimated from rhodamine 6G fluorescence measurements, was nearly −50 mV. This agreed with calculations using the Goldman–Hodgkin–Katz equation. With external $[Ca^{2+}] \leqslant 3$ mM veratridine depolarisation (to −30 mV) was accompanied by increases in intrasynaptosomal freee calcium concentrations (monitored by entrapped quin2) and parallel increases in total acetylcholine release. With external $[Ca^{2+}] > 3$ mM both intrasynaptosomal free calcium concentrations and transmitter release were paradoxically reduced, providing further evidence for a close correlation between the two events. To support an explanation of these findings based on divalent cation screening of membrane surface charge (increasing the voltage gradient within the membrane and closing voltage-inactivated channels) surface potential measurements were made on synaptic lipid liposomes by using a fluorescent surface-bound pH indicator. These experiments provided evidence for the presence of screenable surface charge on synaptosomes, and it was further shown in depolarised synaptosomes themselves that total external $[Ca^{2+} + Mg^{2+}]$, and not $[Ca^{2+}]$ alone, set the observed peak in intrasynaptosomal free calcium.

Introduction

The calcium which couples nerve impulses to neurotransmitter release [1] accumulates by net influx across the presynaptic plasma membrane [2]. Specific (voltage-activated) Ca channels have been identified in mammalian brain synaptosomes [3] and Ca^{2+} may also enter through open Na channels [4]. The recent synthesis of fluorescent, intracellularly trappable Ca^{2+}-indicators [5,6] enables direct measurement of intrasynaptosomal free calcium concentrations ($[Ca^{2+}]_i$), including some of the changes following depolarisation [7]. The experiments I now report were undertaken to assess, within the limits of the technique, the relationship of $[Ca^{2+}]_i$ and acetylcholine release to external $[Ca^{2+}]$ ($[Ca^{2+}]_e$).

The apparent power dependence of quantal neurotransmitter release on $[Ca^{2+}]_e$ (e.g., Ref. 8) could not be subjected to an analogous study in synaptosomes where electrical depolarisation and the measurement of quantal transmitter release are impossible. Instead, the isolated nerve termianls were to be subjected to maintained depolarisation by veratridine, which holds Na channels open [9], to allow comparison of $[Ca^{2+}]_i$ and total transmitter release to $[Ca^{2+}]_e$.

Materials and Methods

Materials

Rhodamine 6G was obtained from BDH, Poole, Dorset, U.K. and 4-heptadecyl-7-hydroxycoumarin from Molecular Probes, Junction City, OR, U.S.A. Quin2 (for calibration) and quin2 ester were purchased from Lancaster Synthesis, Morecombe, Lancs, U.K. and Sigma, Poole, Dorset, U.K. Remaining materials were from normal commercial sources.

* Present address: Cardiothoracic Institute, 2, Beaumont Street, London, W1N 2DX, U.K.

Preparation of synaptosomes and liposomes

Synaptosomes were isolated from the cerebral cortices of one or two guinea-pigs by the methods of Gray and Whittaker [10] or Booth and Clark [11] as indicated. The final fractions were suspended in experimental medium containing 132 mM NaCl/5 mM KCl/2 mM $CaCl_2$/1.2 mM $MgCl_2$/1.2 mM NaH_2PO_4/10 mM glucose/20 mM Tris-HCl (pH 7.4 at room temperature). Variations will be indicated in the text. With additional $CaCl_2$ or $MgCl_2$ NaH_2PO_4 was omitted to avoid insoluble phosphate precipitation. Some preparations were subjected to lipid extraction as previously described [12]. Liposomes were formed from aqueous suspensions of these lipids or egg phosphatidylcholine by sonication [12].

Fluorescence measurements

Quin2 loading of synaptosomes and the measurement of $[Ca^{2+}]_i$ have been described in detail [7]. Intrasynaptosomal quin2 concentrations (measured by comparison to free-acid standards) rarely exceeded 1.5 mM. The remaining probes were incorporated by simple mixing, in the case of liposomes adding the charged hydroxycoumarin (in ethanol) to lipid solutions at a probe/lipid molar ratio of 1 : 1500 prior to solvent evaporation and sonication. Membrane suspensions were stirred (either continuously or intermittently) when settling was noticeable or to mix any additions. Measurements were made at 37°C for synaptosomes and at room temperature for liposomes.

Other methods

After labelling internal acetylcholine by incubating synaptosomes with [^{14}C]choline chloride transmitter release was assessed as previously described [7]. As generally noted when the bases are extracted and separated [13] both choline and acetylcholine were released together, but most of the released radioactivity originated from acetylcholine, especially under depolarising conditions (when release was complete within 10 min). Accordingly total dpm per mg protein was found to be a reliable index of transmitter release. Protein was estimated by the method of Miller [14] and lipid phosphorus by the method of Bartlett [15] after digesting the phospholipids in boiling 72% (w/v) perchloric acid. Intrasynaptosomal ion concentrations were measured by flame photometry [16].

Results

Synaptic depolarisation

The plasma membrane diffusion potential ($\Delta\psi_p$) of non-depolarised synaptosomes was calculated from the Goldman–Hodgkin–Katz (GHK) constant-field equation [17,18] expressed in the form:

$$\Delta\psi_p = \frac{RT}{zF} \ln \frac{[K^+]_e + \alpha[Na^+]_e}{[K^+]_i + \alpha[Na^+]_i} \quad (1)$$

α, the permeability ratio of Na^+ to K^+, is near 0.05 under these conditions [19]. R, T, F and z have their usual thermodynamic significance. From several preparations in which K^+ and Na^+ were measured the corresponding intrasynaptosomal cation concentrations (given a cytoplasmic volume of 3 μl per mg protein [20]) were 73 ± 3 and 23 ± 3 mM, respectively (both means ± S.E., $n = 5$). On this basis the resting membrane diffusion potential ($[K^+]_e = 5$ mM) was −48 ± 1 mV (mean ± S.E., $n = 5$).

Rhodamine 6G fluorescence was examined in preparations from an isosmotic Ficoll-based technique [11]. These synaptosomes were reportedly purer (ibid) than those obtained from sucrose gradients (e.g., Ref. 10). It was hoped thereby to minimise interactions of the dye with non-synaptosomal material. For potential measurements the protocol described by Aiuchi et al [21] was followed and the authors' expression describing the voltage dependent phase-partitioning of the dye in synaptosomal membranes may be written in the form:

$$\lambda/\lambda' = \frac{RT}{F\Delta\psi_p}(1 - e^{-F\Delta\psi_p/RT}) \quad (2)$$

λ/λ' is the ratio of phase-partition coefficients (each representing bound/free probe) in test medium and high-K^+ medium (primed). In the latter typically $[K^+]_e \geqslant 137$ mM (i.e., fully depolarising, Eqn. 1). The equation was solved numerically for $\Delta\psi_p$ for the various conditions examined. A resting membrane potential of −49 ± 2 mV (mean ± S.E., $n = 9$ preparations) decreased after 10 min of depolarisation with 150

μM veratridine to -30 ± 5 mV (mean $\pm$ S.E., $n = 3$). 1.5% (v/v) ethanol (present at up to about 1% (v/v) after adding ethanolic veratridine) did not itself reduce $\Delta\psi_p$ (-53 ± 4 mV, mean $\pm$ S.E., $n = 3$).

Ca^{2+}-entry and neurotransmitter release

Following veratridine-depolarisation entrapped quin2 initially reported a phase of net Ca^{2+} influx (but will have buffered fast transients). This was followed by a period of maintained $[Ca^{2+}]_i$elevated (Fig. 1) which was the value measured. Membrane depolarisation, by bringing $\Delta\psi_p$ closer to the Ca^{2+} equilibrium potential (+120 mV in these experiments) should favour active extrusion with eventual reduction of $[Ca^{2+}]_i$ to below resting levels. Several reasons may be advanced for the changes actually seen [7], including incomplete closure of Ca channels. In these experiments Ca^{2+} entry through open Na channels may also occur. Addition of the 'calcium' channel blocker verapamil [22] did in fact reduce $[Ca^{2+}]_i$ (Fig. 1).

Several lines of evidence converge to suggest that $[Ca^{2+}]_i$ determines the rate of synaptic neurotransmitter release and further experimental support is provided in Fig. 2. The expected in-

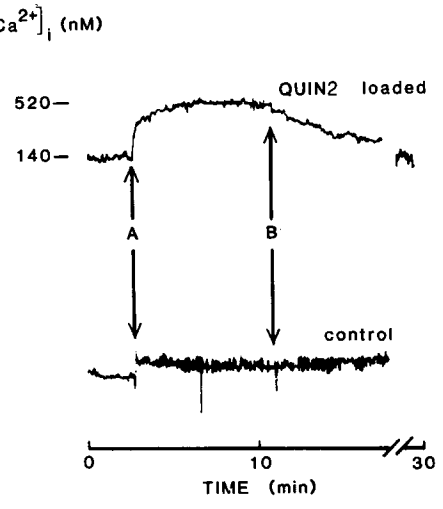

Fig. 1. $[Ca^{2+}]_i$ and veratridine-depolarisation. Superimposed traces of the fluorescence of quin2 loaded and, immediately afterwards, unloaded control synaptosomes, continuously stirred in physiological medium containing 1 mM $CaCl_2$. 150 μM veratridine added at A, 200 μM verapamil at B. $[Ca^{2+}]_i$ was calculated from calibrating traces [6].

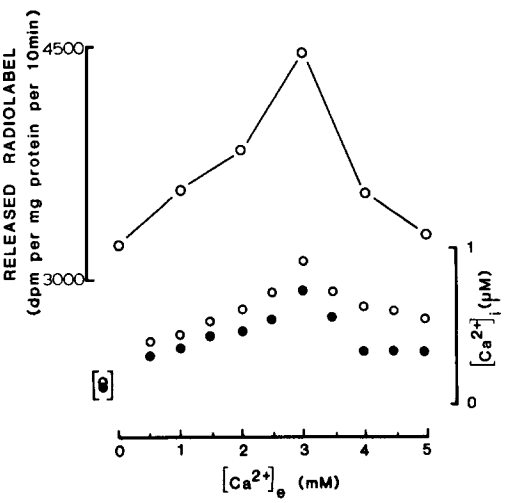

Fig. 2. External Ca^{2+}, $[Ca^{2+}]_i$ and transmitter release, in medium containing 150 μM veratridine. For 'transmitter release' (see Materials and Methods) each point represents the mean of three experiments for which the S.E. was normally less than 15% and in each of which the trend with increasing $[Ca^{2+}]_i$ was the same. The lines are drawn for guidance only. For $[Ca^{2+}]_i$ results for two experiments are shown. The points in parentheses represent predepolarisation values.

crease in transmitter release as $[Ca^{2+}]_e$ was raised was succeeded by a definite decline with still higher $[Ca^{2+}]_e$. Similar findings have been reported from more physiological preparations [23–25]. The noteable finding here (illustrated in Fig. 2) is the demonstration of a corresponding peak in $[Ca^{2+}]_i$ in each of several preparations examined. The close correspondence between these two rather unexpected maxima contributes further support for a close connection between $[Ca^{2+}]_i$ and transmitter release. A plausible mechanism for these observations [23,25] was now examined.

Membrane surface potential in synaptic lipid liposomes

Divalent cation screening of surface charge will reduce the outer membrne surface potential (ψ_0) and $[K^+]$adjacent ot the membrane (to an extent predicted by the Boltzmann distribution). This accordingly increases the K^+-component of the membrane diffusion potential [23,25]. The resulting increase in the voltage gradient within the membrane promotes closure of voltage-inactivated channels. Further raising external divalent cation concentrations will increasingly reduce Ca^{2+} entry

and transmitter release. The membrane potential, measured across both electrical double-layers, remains constant. This interpretation could explain the present findings if synaptosomes have surface potentials screenable by divalent cations.

These essential preliminary questions were examined after incorporating the surface bound pH indicator 4-heptadecyl-7-hydroxycoumarin into synaptosomal lipid liposomes. Because dye fluorescence (377 nm excitation, 452 nm emission, uncorrected wavelengths) is proportional to the degree of dissociation of the hydroxyl group [26] the Henderson–Hasselbach equation predicts half-maximal dissociation (and half-maximal fluorescence) at a medium pH equal to the pK of the membrane bound dye [26]. The pK in phosphatidylcholine liposomes increased in charged synaptosomal lipid liposomes following the attraction

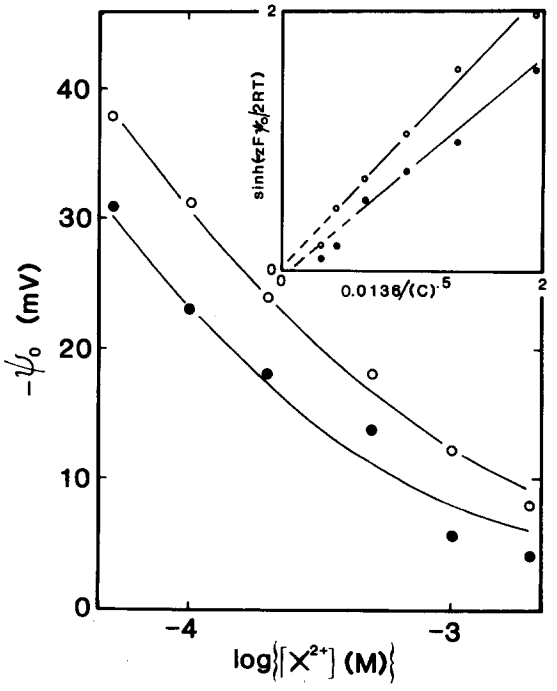

Fig. 3. Divalent cations and surface potentials in synaptic lipid liposomes. In the main figure each point represents the averaged results from two sets of lipid extracts. Ψ_0 was measured from shifts in the pK of a surface-bound hydroxycoumarin in medium containing only Ca^{2+} (closed circles) or Mg^{2+} (open circles). σ was found from the slopes of the inset graphs to allow construction of the ideal screening curves in the main diagram.

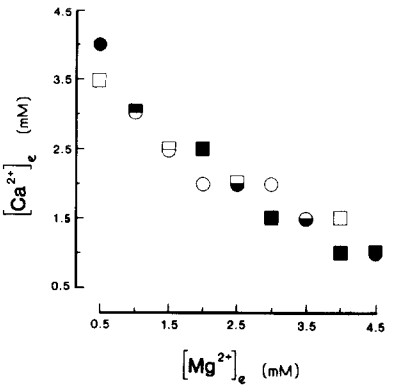

Fig. 4. Total $[Ca^{2+}]_e$ requirement for the $[Ca^{2+}]_i$ peak in veratridine-depolarised synaptosomes suspended (with continuous stirring) in media with various $[Mg^{2+}]_e$. The four preparations (separate symbols) had been quin2 loaded in normal medium containing 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$.

of protons (from the dissociation of water molecules) towards the membrane/solution interface. From the Boltzmann distribution,

$$pK' = pK - \frac{\psi_0 F}{2.3RT} \tag{3}$$

where pK' and pK refer to charged and uncharged interfaces. The equation was solved for ψ_0.

To investigate divalent cation screening, the liposomes were suspended in a simplified medium containing only the cation of interest so that the Gouy expression could be applied:

$$\sinh\frac{-zF\psi_0}{2RT} = \frac{136\,\sigma}{\sqrt{C}} \tag{4}$$

where σ is the surface charge density (e^- per 0.01 nm^2) and C is the total cation concentration in M. $[X^{2+}]$, with X = Ca or Mg, was varied between 50 μM and 5 mM to obtain the data summarised in Fig. 3. The inset graphs confirm the validity of the Gouy expression for this analysis and their slopes yield surface charges of e^- per 120 nm^2 and per 90 nm^2 in $CaCl_2$ and $MgCl_2$, respectively. The values are not unreasonable, and the lower charge in Ca medium reflects the fact that more Ca^{2+} than Mg^{2+} actually binds to specific anionic phospholipid head groups [27]. As ψ_0 exceeds RT/F (24.4 at 20°C), the sinh function approximates to half the exponent, and ψ_0 tends to change by up

to -29 mV for a 10-fold change in $[X^{2+}]_e$. Because of the (expectedly) low values for σ in these lipid-only preparations, this occurred only when $[X^{2+}]_e \leqslant 10^{-4}$ M. Similar screening may be assumed to occur in the original synaptosomes (which cannot be so directly investigated).

External calcium and magnesium and $[Ca^{2+}]_i$

If increases in the concentration of external Ca^{2+} act essentially to reduce $[Ca^{2+}]_i$ in depolarised synaptosomes by a screening mechanism a similar effect should be produced by any external (divalent) cation. This prediction was now tested by varying $[Mg^{2+}]_e$. Synaptosomes were loaded with quin2 in normal medium containing 0.5 mM $CaCl_2$ and 1.0 mM $MgCl_2$ and were resuspended in appropriate media for the experiments described in Fig. 4.

It is clear that $[Ca^{2+} + Mg^{2+}]_e$, and not $[Ca^{2+}]_e$ alone, led to the paradoxical peak in $[Ca^{2+}]_i$ as $[Ca^{2+}]_e$ was increased, and this strongly supports an interpretation of the effect in terms of the screening of surface membrane charge. Slightly higher total external divalent cation concentrations were required at high $[Mg^{2+}]_e$, consistent with an effect of Mg^{2+} (or $[Mg^{2+}]_i$) on Ca^{2+}-conductance, or possibly attributable to more Ca^{2+} than Mg^{2+} binding to specific charged groups (as mentioned earlier).

Discussion

Membrane potentials obtained from the constant-field equation and from rhodamine 6G fluorescence measurements agree well (-48 and -49 mV). Rhodamine 6G fluorescence also corresponded closely to K^+-induced variations in membrane potentials measured electrically in cultured neuroblastoma cells [28]. The extent of veratridine depolarisation reported here (some 20 mV) is therefore likely to be reasonably accurate. The findings in this study (Fig. 2) concerning depolarisation-induced Ca^{2+} entry offer further support for a correlation between $[Ca^{2+}]_i$ and transmitter release, most persuasively advanced by Katz and Miledi [2] who demonstrated that presynaptic depolarisation at the neuromuscular junction beyond the Ca^{2+} equilibrium potential actually decreased release.

When seeking an explanation in terms of a screening effect on membrane surface charge [23,25] for the paradoxical reduction in $[Ca^{2+}]_i$ as $[Ca^{2+}]_e$ was increased, surface potential measurements were made on liposomes [26] rather than on synaptosomes themselves, which could have been damaged by the large pH changes (up to 6 Units) required. The results showed that such an effect was quite tenable, but the experiments where external $[Ca^{2+}]$ and $[Mg^{2+}]$ were varied together offered more compelling evidence in its favour in that the peak in $[Ca^{2+}]_i$ depended on the total external divalent cation concentration rather than on $[Ca^{2+}]_e$ alone.

Investigations of ion permeabilities in excitable tissues have come to rely heavily on sophisticated electrophysiological techniques such as noise analysis and single-channel recording [29] and the measurement of gating currents [30]. These methods together with the use of ion-sensitive microelectrodes cannot, however, be applied to mammalian nerve terminals because of their small size (typically 0.5 μm diameter). One important approach involves the use of biochemical isolation techniques, in particular the incorporation of membrane vesicles into voltage-clamped planar lipid bilayers for single-channel recording (e.g., Ref. 31).

The normal function of Ca channels does, however, appear to rely heavily on cellular metabolism, especially systems for cyclic nucleotide generation [32]. It is therefore likely that synaptosomes will continue to be widely employed to furnish parallel physiological models for reconstituted systems. For example, the implication here that Ca channel inactivation in mammalian brain synaptosomes is voltage-dependent (rather than $[Ca^{2+}]_i$-dependent) is consistent with the results of the isotope flux studies of Nachshen and Blaustein [33], and precise details of the inactivation mechanism in reconstituted systems [31] will be of interest.

Acknowledgements

I thank Dr. R.M. Marchbanks and Dr. M.J. Brammer for helpful discussions. Some of this work was carried out in the Biochemistry Department of the Institute of Psychiatry (British Postgraduate Medical Federation) with financial sup-

port from the British MRC by way of a Studentship to the author and part of grant No. G8125170N to Dr. Marchbanks.

References

1 Reichardt, C.F. and Kelly, R.B. (1983) Annu. Rev. Biochem. 52, 871–926
2 Katz, B. and Miledi, R. (1967) J. Physiol. 192, 407–436
3 Turner, T.J. and Goldin, S.M. (1985) J. Neurosci. 5, 841–9
4 Baker, P.F., Hodgkin, A.L. and Ridgway, E.B. (1971) J. Physiol. 218, 709–755
5 Tsien, R.Y. (1980) Biochemistry 19, 2396–2404
6 Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) J. Cell Biol. 94, 325–334
7 Ashley, R.H., Brammer, M.J. and Marchbanks, R.M. (1984) Biochem. J. 219, 149–158
8 Dodge, F.A. and Rahamimoff, R. (1967) J. Physiol. 193, 419–432
9 Ohta, M., Narashi, T. and Keeler, R.F. (1973) J. Pharmac. Exp. Therap. 184, 143–154
10 Gray, E.G. and Whittaker, V.P. (1962) J. Anat. 96, 79–87
11 Booth, R.F.G. and Clark, J.B. (1978) Biochem. J. 176, 365–370
12 Ashley, R.H. and Brammer, M.J. (1984) Biochim. Biophys. Acta 769, 363–369
13 Marchbanks, R.M. and Israel, M. (1971) J. Neurochem. 18, 439–448
14 Miller, G.L. (1959) Anal. Biochem. 31, 964
15 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
16 Goldfarb, P.S.G. and Rodnight, R. (1970) Biochem. J. 120, 15–24
17 Goldman, D.E. (1943) J. Gen. Physiol. 27, 37–60
18 Hodgkin, A.L. and Katz, B. (1949) J. Physiol. 108, 37–77
19 Keene, D. and White, T.D. (1971) J. Neurochem. 18, 1097–1103
20 Scott, I.D. and Nicholls, D.G. (1980) Biochem. J. 186, 21–33
21 Aiuchi, T., Daimatsu, T., Nakaya, T. and Nakawa, Y. (1982) Biochim. Biophys. Acta 685, 289–296
22 Reuter, H. (1983) Nature 301, 569–574
23 Matthews, G. and Wickelgren, W.O. (1977) J. Physiol. 266, 91–101
24 Beume, R. and Pott, L. (1978) Pflügers Arch. 376, 21–26
25 Madden, K.S. and Van der Kloot, W. (1978) J. Physiol. 276, 227–232
26 Fernández, M.S. (1981) Biochim. Biophys. Acta 646, 23–26
27 Ohki, S. (1972) Biochim. Biophys. Acta 282, 55–71
28 Miyake, M., Nekomiya, A. and Kurihara, K. (1984) Brain Res. 301, 73–81
29 Conti, F. (1984) Curr. Top. Memb. Trans. 22, 371–405
30 Armstrong, C.M. and Bezanilla, F. (1973) Nature 242, 459–461
31 Nelson, M.T., Rounda, M. and Bamberg, E. (1983) Am. J. Physiol. 245, C151–C156
32 Kostyuk, P.G. (1981) Biochim. Biophys. Acta 650, 128–150
33 Nachshen, D.A. and Blaustein, M.P. (1980) J. Gen. Physiol. 76, 709–728