Biochimica et Biophysica Acta 815 (1985) 203–208
Elsevier

BBA 72568

Dependence of cytoplasmic calcium transients on the membrane potential in isolated nerve endings of the guinea pig

Erkki Heinonen [a,*], Karl E.O. Åkerman [b], Kai Kaila [c] and Ian G. Scott [b]

[a] *Department of Physiology, University of Helsinki, Siltavuorenpenger 20J, SF-00170 Helsinki 17,* [b] *Department of Biochemistry and Pharmacy, Åbo Akademi and* [c] *Department of Zoology, Division of Physiology, University of Helsinki, Helsinki (Finland)*

(Received November 20th, 1984)

Key words: Synaptosome; Ca^{2+} transport; Membrane potential; Arsenazo III; (Guinea pig nerve)

The relation of changes in internal, free Ca^{2+}, measured with arsenazo III, to the membrane potential, measured with the cyanine dye di-S-C_2(5) or $^{86}Rb^+$ distribution ratio, was studied in isolated guinea pig cortical nerve endings. Depolarization of the plasma membrane with veratridine or gramicidin as well as addition of ionophore A23187 led to an increase in cytosolic Ca^{2+}. Only the response to veratridine was inhibited by tetrodotoxin. The dependence of the depolarization-induced increase in intraterminal, free Ca^{2+} on the membrane potential between about −50 to 0 mV was sigmoidal. A maximal increase in cytosolic Ca^{2+} was reached when the membrane potential was depolarized from the resting level, about −64 mV, to about −40 mV. These results show that in isolated nerve endings the activation of voltage-sensitive Ca^{2+} channels concomitantly leads to an increase in cytosolic, free Ca^{2+}. Comparison of the results of the present study with the previous electrophysiological observations indicate that Ca^{2+} channels in synaptosomes, presynaptic nerve terminals of the squid giant synapse and cardiac cells have essentially similar voltage dependency.

Introduction

Several lines of evidence indicate that transmitter release as a response to electrical excitation is triggered by an increase in cytosolic Ca^{2+} activity [1], which occurs as a result of the opening of voltage-sensitive Ca^{2+} channels in the plasma membrane of nerve endings [2,3]. Since the cytosolic free Ca^{2+} in resting nerve cells is about 10^{-7} M [4] or even lower [5], accurate measurements of this parameter are difficult in small nerve endings. Most of the current information available is derived from studies on the squid giant synapse. In this synapse, the light output of intracellularly injected aequorin has been monitored and a rise in cytosolic Ca^{2+} during single-action potentials prolonged by tetraethylammonium [6] or trains of spikes [7] has been demonstrated. In more recent studies, arsenazo III was used to monitor Ca^{2+} transients during single and trains of action potentials [8,9]. A strict dependence between the change in intracellular Ca^{2+} and transmitter release, quantified by the excitatory postsynaptic potential, was observed. The properties of voltage-sensitive Ca^{2+} channels in the presynaptic nerve endings of the squid giant synapse have been extensively studied by using electrophysiological techniques [10,11]. For studies on the role of Ca^{2+} in transmitter release in mammalian brain, fractions of isolated nerve endings, synaptosomes, have been used to a large extent [12,13]. These isolated pinched-off

* To whom correspodnence should be addressed.

Abbreviations: Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; di-S-C_2(5), 2,2′-diethylthiodicarbocyanine.

nerve terminals maintain a high membrane potential [14] in physiological conditions and may be depolarized by various experimental means [15,16]. As a response to depolarization, an increase in Ca^{2+} influx [15,17–19] and transmitter release [20–22] occurs. We have recently demonstrated [23] that when arsenazo III is simply added to the homogenisation medium this Ca^{2+} indicator is entrapped within isolated nerve endings and subsequently changes in the internal free Ca^{2+} can be monitored.

The aim of the present study was to characterize further the mechanisms of Ca^{2+} regulation in mammalian nerve endings using entrapped arsenazo III. Particular attention was paid to the relation between internal Ca^{2+} and membrane potential.

Materials and Methods

Isolation of synaptosomes. Synaptosomes were isolated from the cortical hemispheres of guinea pigs using discontinuous Ficoll density gradients as described previously [18]. 10 mM arsenazo III, purified as described by Scarpa [24], was added to the homogenisation medium in order to entrap the dye within subsequently isolated synaptosomes [23]. The synaptosomal fraction was removed from the Ficoll gradient. It was divided in separate centrifuge tubes, diluted 20-fold with the standard experimental Na^+-based medium and centrifuged at $15\,000 \times g$ for 15 min. The resulting pellets, covered by a small volume of fresh standard Na^+-based medium, were stored on ice until use within 120 min.

Measurements of intracellular Ca^{2+} and membrane potential. Differential spectra and dual wavelength measurements were carried out with an Aminco DW2 spectrophotometer equipped with a thermostated cuvette holder, keeping the temperature at 35°C in the cuvette. Arsenazo III absorbance was measured using the wavelength pair 600–555 nm, which is optimal in the present conditions [23]. Changes in membrane potential were monitored by measuring spectral changes of the cyanine dye [25] di-S-C_2(5) at the wavelength pair 645–590 nm.

Alternatively, the membrane potential was measured from the $^{86}Rb^+$ distribution ratio as described in Ref. 16 using 0.5 μCi/ml $^{86}Rb^+$ and 0.2 μCi/ml [^{14}C]sucrose. The intrasynaptosomal H_2O was measured using 2 μCi ^3H_2O and 0.2 μCi/ml [^{14}C]sucrose. Otherwise, conditions were as above. The samples were counted in an LKB/Wallac Rackbeta scintillation counter using simultaneous gate settings computed for double or triple isotope determination separating ^{3}H from ^{14}C and ^{14}C from $^{86}Rb^+$.

Experimental media. Standard Na^+-based medium: 137 mM NaCl/5 mM KCl/1.2 mM $MgCl_2$/0.44 mM KH_2PO_4/4.2 mM $NaHCO_3$/20 mM Tes buffer (pH 7.4). K^+-based medium: 142 mM KCl/1.2 mM $MgCl_2$/0.44 mM KH_2PO_4/4.2 mM $KHCO_3$/20 mM Tes buffer (pH 7.4).

Materials. Arsenazo III, veratridine and tetrodotoxin were obtained from Sigma Chemicals Co. (Poole, Dorset, U.K.), gramicidin from Fluka A.G. (Buchs, Switzerland), ionophore A23187 from Boehringer (Mannheim, F.R.G.). The cyanine dye di-S-C_2(5) was kindly donated by Professor A. Waggoner (Amherst College, MA, U.S.A.).

Results

Depolarization-induced increase in intracellular Ca^{2+}

Some typical absorbance recordings of intrasynaptosomally entrapped arsenazo III are shown in Fig. 1. Depolarization induced by addition of 100 μM veratridine, which enhances Na^+ influx by opening Na^+ channels, increased the absorbance of entrapped dye, indicating an increase in cytosolic Ca^{2+}. The spectral change was composed of a fast phase, which was followed by a steady level. It should be noted that the rate of the response is limited by the mixing. Since the dye concentration is in the order of 10^{-3} M within the nerve endings [23], a large proportion of Ca^{2+} flowing into the endings was buffered by the probe. This, together with intraterminal Ca^{2+} buffers, mitochondria, explains the relatively slow response. The steady level observed, was probably due to a compensating increase in Ca^{2+} efflux from the nerve ending balancing the increase in influx [16,26]. A subsequent addition of 4 μM ionophore A23187 gave the maximal absorbance change due to saturation of the dye response around 50–100 μM [23,24]. Tetrodotoxin (0.2 μM)

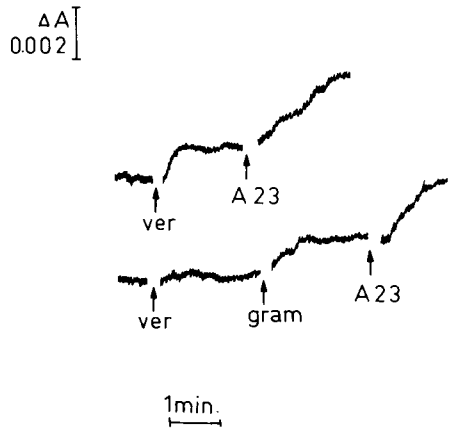

Fig. 1. Effect of tetrodotoxin on depolarization-induced increase in cytosolic Ca^{2+}. Synaptosomes were suspended at a concentration of 1.5 mg protein per ml in the standard incubation medium containing 1 mM $CaCl_2$ and 5 mM D-glucose. Additions were made of 100 μM veratridine (ver), 4 μM ionophore A23187 (A23) or 1 μM gramicidin (gram). In the lower trace, 0.2 μM tetrodotoxin was additionally present.

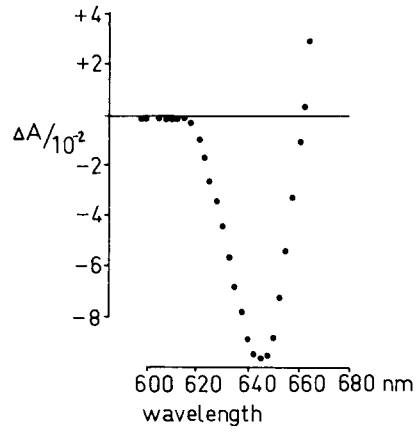

Fig. 2. Differential absorbance spectrum of polarized versus depolarized synaptosomes in the presence of a cyanine dye di-S-C_2(5). Synaptosomes were suspended into a small volume of the standard Na^+-based experimental medium containing 1 mM $CaCl_2$ and 5 mM D-glucose at room temperature (22°C). 30 μl of this suspension (giving a final protein concentration of 1 mg/ml) was added into cuvettes containing 2.5 ml of either K^+- or Na^+-based standard medium with 1 mM $CaCl_2$, 5 mM D-glucose and 1 μM di-S-C_2(5) and a difference spectrum was recorded. The values shown represent the difference between absorbance values in the absence and presence of synaptosomes in the medium.

completely abolished the response to veratridine but did not affect that of gramicidin, a monovalent-cation selective channel forming ionophore, or that of ionophore A23187 (Fig. 1, lower trace). Note that tetrodotoxin did not affect the total absorbance change obtained.

Relation of internal Ca^{2+} to the membrane potential

Cyanine dyes have been successfully used to detect changes in membrane potentials in isolated nerve endings [15] as well as in various small cells and organelles [25,27,28]. Fig. 2 shows the absorbance difference spectrum of control versus depolarized (high K^+) synaptosomes in the presence of the cyanine dye di-S-C_2(5). There is a decrease in absorbance around 646 nm and an extensive increase above 670 nm with no significant change around 590 nm. We chose to monitor changes at 646–590 nm since at higher wavelengths (above 670 nm) disturbances due to nonspecific light scattering are extensive. The measured changes in absorbance of the dye are in agreement with the observations of Sims et al. [25] who used the same probe for detecting K^+ diffusion potentials in liposomes. Since in some conditions cyanine dye responses are nonlinearily related to membrane potential [29], the dye response was calibrated by varying the external K^+ and Na^+ concentrations of the incubation medium. Fig. 3 shows the dye response at 646–590 nm on addition of synaptosomes to either Na^+ or K^+ (maximal depolarization) based experimental medium. There was a decrease in absorbance at the respective wavelength pair also in the K^+-based medium. This was probably due to the association of the probe with the membrane, because a similar phenomenon is observed with nonpolarized liposomes [25]. Optical probes of membrane potential also distribute into intracellular mitochondria [30–32]. Valinomycin, which depolarizes intracellular mitochondria [30], was added subsequently to estimate the contribution of the membrane potential of intrasynaptosomal mitochondria to the spectral change. Fig. 4 shows the effect of different Na^+ and K^+ concentrations on the absorbance of 1 μM di-S-C_2(5); synaptosomal protein was 1 mg/ml. It should be noted that both dye concentration and the amount of synaptosomes affect the linearity of the dye response. The probe response in the present conditions was fairly lin-

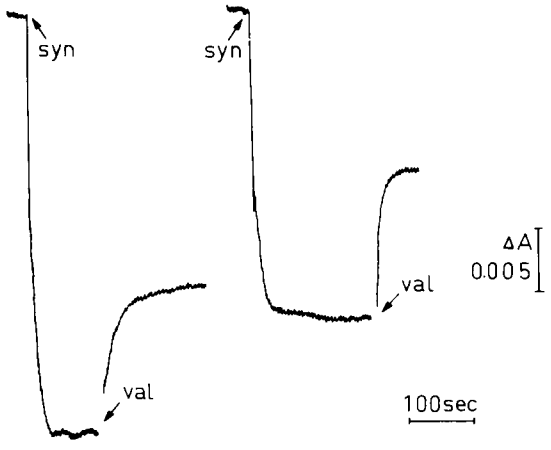

Fig. 3. Changes in the absorbance of di-S-C_2(5) on addition of synaptosomes to Na^+- and K^+-based standard media. Conditions were as in Fig. 2, except that the absorbance was measured using the wavelength pair of 646–590 nm. Additions were made of synaptosomes (syn) to a final concentration of 1 mg/ml protein to the Na^+-based (left-hand trace) or K^+-based (right-hand trace) standard medium containing 1 mM $CaCl_2$ and 5 mM D-glucose, followed by an addition of 100 ng/ml valinomycin (val).

earily related to the apparent K^+ diffusion potential, assuming a constant intrasynaptosomal K^+ concentration of 100 mM. The slope of the plot was identical in the presence of valinomycin, suggesting that the mitochondrial contribution to the probe response does not alter its relation to the plasma membrane potential.

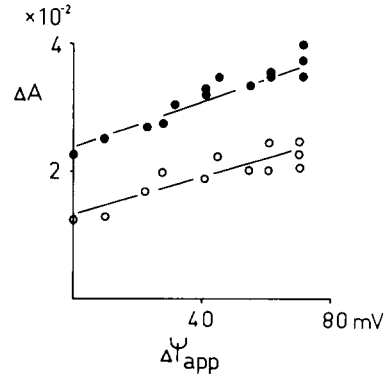

Fig. 4. Decrease in the absorbance of di-S-C_2(5) as a function of the apparent K^+ equilibrium potential. Experiments similar to those in Fig. 3 were carried out by varying the proportion of Na^+- and K^+-based standard media in the cuvette. The apparent membrane potential was calculated from the Nernst equation, assuming a constant internal K^+ concentration of 100 mM (●). Values obtained after subsequent addition of 100 ng/ml valinomycin are also shown (○).

In order to relate changes in cytosolic free Ca^{2+} to the membrane potential, the cyanine and arsenazo III responses were recorded in parallel during the addition of small pulses of veratridine, as shown in Fig. 5. There was a graded depolarization and a further addition of gramicidin caused an extensive spectral change due to a complete depolarizatoin [15,33]. In order to see whether the increase in internal Ca^{2+} may interfere with the response of the cyanine dye [34] similar experiments as those above were performed in the absence of Ca^{2+} and presence of 0.1 mM EGTA. Cyanine dye responses similar to those in Fig. 5 were observed (not shown).

The intrasynaptosomal Ca^{2+} was plotted as a function of membrane depolarization in Fig. 6 from results similar to those in Fig. 5. The plot in

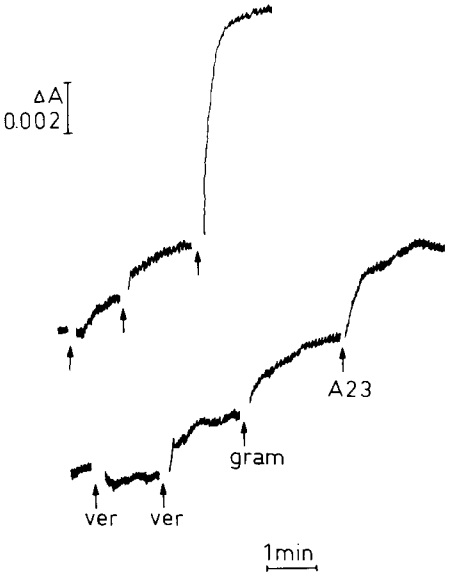

Fig. 5. Effect of veratridine and gramicidin on the absorbance of di-S-C_2(5) (upper trace) and entrapped arsenazo III (lower trace). Synaptosomes were suspended in the Na^+-based standard medium at a concentration of 1.3 mg protein/ml. The conditions were similar to those of Fig. 1, except that in the upper trace 1 μM di-S-C_2(5) was additionally present. In both recordings, two additions of 1 μM veratridine (ver) were made and thereafter 1 μM gramicidin (gram), as indicated. In the lower trace, the maximal Ca^{2+} response was obtained with 4 μM ionophore A23187 (A23).

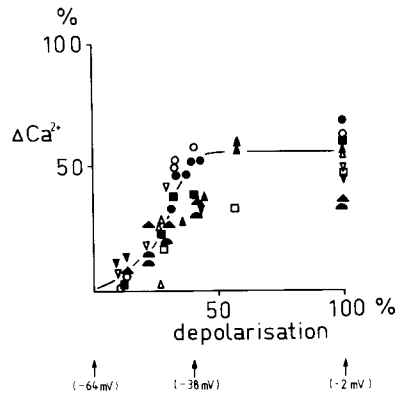

Fig. 6. Effect of membrane depolarization on the absorbance of entrapped arsenazo III. The relation of change in the absorbance of entrapped arsenazo III to change in the absorbance of di-S-$C_2(5)$ was obtained by addition of varying concentrations of veratridine (1–100 μM) and 1 μM gramicidin in conditions similar to those in Fig. 5. ΔCa^{2+}, expressed as percent of maximal change obtained with a saturating concentration of ionophore A23187 is plotted against percent of maximal change of depolarization obtained with gramicidin. The values of membrane potential shown in brackets are derived from the $^{86}Rb^+$ distribution ratio in control conditions, or in the presence of maximally effective concentrations of veratridine and gramicidin. The various symbols represent values obtained from different batches of synaptosomes ($n = 10$).

Fig. 6 shows that intrasynaptosomal Ca^{2+} started to rise when the resting membrane potential, which was -64 ± 1 mV (mean $\pm$ S.D., $n = 8$) calculated from $^{86}Rb^+$ distribution ratio, was reduced by 20% or more. A plateau of this Ca^{2+} increase was reached after a depolarization of 40% from the resting level, resulting in a membrane potential of -38 ± 5 mV (mean $\pm$ S.D., $n = 4$) calculated from $^{86}Rb^+$ distribution. Subsequent gramicidin-induced depolarization to -2 ± 7 mV (mean $\pm$ S.D., $n = 11$), calculated from $^{86}Rb^+$ distribution, only slightly increased internal Ca^{2+}. Thus, the maximal depolarization-induced increase in cytosolic Ca^{2+} occurred around the membrane potential of -30 to -40 mV.

Discussion

The results of the present study indicate that depolarization of the plasma membrane of isolated mammalian nerve endings causes an increase in cytosolic Ca^{2+}, as measured using entrapped arsenazo III. This occurs by a mechanism which is unaffected by tetrodotoxin (this study) but blocked by a Ca^{2+} channel blocker, verapamil [23]. Since no change in the arsenazo III absorbance took place upon depolarization when the extrasynaptosomal Ca^{2+} was chelated with EGTA [23], it is likely that the increase in intrasynaptosomal Ca^{2+} occurs mainly as a response to the opening of voltage-dependent Ca^{2+} channels in the plasma membrane [35].

In heart and squid giant synapse as well as in sevreal other excitable tissues (for reviews see Refs. 35 and 36), the onset of the activation curve of voltage-sensitive Ca^{2+} channels is sigmoidally related to the change in membrane potential. The depolarization-induced Ca^{2+} inward current activates around the membrane potential of -50 to -40 mV and reaches a plateau around the membrane potential of 0 mV. The results of the present study are in qualitative agreement with these observations. Thus, it appears that the activation of Ca^{2+} channels is closely followed by an increase in the free Ca^{2+} within nerve terminals.

Recently, a fluorescent Ca^{2+} indicator quin-2 has been used to detect intrasynaptosomal free Ca^{2+} [37,38]. In both studies, a resting level of free Ca^{2+} around 100 nM was obtained. In their study Meldolesi et al. [37] had technical difficulties in efforts to measure depolarization-induced Ca^{2+} transients in synaptosomes. However, Ashley et al. [38] reported a linear relation between membrane potential and cytoplasmic Ca^{2+}. Two methodological differences exist between this and our study. Firstly, Ashley et al. used KCl-induced depolarizations and long-term, i.e., 10-min equilibrium levels of internal Ca^{2+} were measured but the change in membrane potential was not measured. Instead, the membrane potential was calculated from the reduced Goldman equation. Secondly, since the K_d of Ca^{2+} binding to quin-2 is in the order of 100 nM and the internal concentration of quin-2 around 1 mM, a large proportion of Ca^{2+} flowing into the synaptosomes will be buffered by the probe. Hence, quin-2 may limit the rise in cytosolic, free Ca^{2+}. The K_d of the arsenazo III-Ca^{2+} complex is around 10–50 μM [24]. Thus, the buffering of intrasynaptosomal free Ca^{2+} by this probe is far less.

In the present study, the half-maximal change in the arsenazo III absorbance was obtained using gramicidin-induced depolarizations, as also shown previously [23]. Therefore, taking into account the K_d of arsenazo III, it is plausible that when compared with the results of Ashley et al. essentially higher, i.e., few micromolar free Ca^{2+} levels are measured in our study. We have been unable so far to obtain a reliable method for quantitation of the arsenazo III response within synaptosomes due to the nonlinear response of arsenazo III at various concentrations of the dye and to the small responses observed at low Ca^{2+} concentrations. Our results are in agreement with those of Blaustein and co-workers who showed a sigmoid dependence between depolarization-induced ^{45}Ca influx [12] or transmitter release [20] and membrane potential.

In conclusion, our results with isolated guinea pig cortical nerve endings indicate that Ca^{2+} channels, similar to those of various excitable cells, are the main pathway for increasing cytosolic free Ca^{2+} in a voltage-dependent manner. Intrasynaptosomally entrapped arsenazo III offers a simple method for direct measurements of intracellular Ca^{2+} in relation to membrane potential.

Acknowledgements

This study was aided by grants from the Academy of Finland and the Sigrid Juselius Foundation. We are grateful to Professors Nils-Erik Saris and Mårten Wikström from the Department of Medical Chemistry for placing the facilities of their laboratories at our disposal.

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