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QUALITATIVE MEASUREMENTS OF CYTOSOLIC CALCIUM ION CONCENTRATION WITHIN ISOLATED GUINEA PIG NERVE ENDINGS USING ENTRAPPED ARSENAZO III

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If arsenazo III is present during homogenization of brain this metallochromic indicator is entrapped within subsequently isolated synaptosomes. A large proportion of the entrapped indicator is released upon addition of digitonin to disrupt the synaptosomal plasma membrane. A similar proportion of [^{3}H]sucrose is also trapped within synaptosomes if present in the homogenization medium, suggesting that homogenization causes a transient opening of the nerve ending as it is chopped off from the axon. Addition of the ionophore A23187 or depolarization of the plasma membrane by adding veratridine, gramicidin or increasing external K^+ changes the absorbance of the entrapped dye, with peaks of absorbance around 600 and 650 nm, typical of the arsenazo III-Ca^{2+} complex. The response to veratridine is inhibited by the Ca^{2+}-channel antagonist, verapamil, while that of A23187 is unaffected. The present method provides a sensitive technique for measurements of changes in cytosolic calcium ion concentrations within nerve endings.

Introduction

The role of an increase in intracellular free Ca^{2+} in initiating transmitter release is well known (for reviews see Refs. 1–3). Evidence for such a trigger mechanism comes mainly from studies on squid synapses. Thus injection of Ca^{2+} into squid nerve terminals triggers transmission [4] in the absence of electrical impulses. An inward current carried by Ca^{2+} and subsequently the release of transmitter [5,6] as well as an increase in cytosolic [Ca^{2+}] occur upon stimulation of nerve terminals [7]. Depolarization of the plasma membrane of nerve endings isolated from mammalian brain causes an increase in Ca^{2+} uptake by these structures [8] and transmitter release [9]. The increase in Ca^{2+} influx is insensitive to the Na^+-channel blocker tetrodotoxin [8,10], but is largely inhibited by a high concentration of Mg^{2+} [10] and totally blocked by a Ca^{2+}-channel antagonist, verapamil [11]. This suggests that, as in the case with squid axons [12] or cultured neurones [13], Ca^{2+} channels are activated as a response to depolarization. Flux studies using $^{45}Ca^{2+}$ give information concerning the total Ca^{2+} content of synaptosomes, while the actual trigger of transmission is a change in free Ca^{2+} within the nerve terminal. Changes in the cytosolic Ca^{2+} activity of squid axons [12,14], some smaller cells [15] and the squid synapse [7] have been monitored previously using the photoprotein aequerin [7,12,15] or the metallochromic indicator arsenazo III [14].

It is likely that during homogenization of brain the nerve terminals are chopped off from the axon and the cytosol would be in contact with the extracellular space for a while before closed synaptosomal structures are formed. Thus the aim of the present study was to incorporate arsenazo III at this stage in order to be able to measure changes

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Abbreviations: Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino)-ethanesulphonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*, *N'*-tetraacetic acid.

in intrasynaptosomal free [Ca^{2+}] induced by depolarization and calcium ionophore A23187.

Methods and Materials

Cortical synaptosomes were isolated from guinea pigs aged 4–8 weeks as described by Gripenberg et al. [10] except that 10 mM arsenazo III or 5 μCi/ml [^{3}H]sucrose was additionally present in the homogenization medium. For determination of the amount of entrapped arsenzao III the final synaptosomal pellet was resuspended into the basal experimental medium (137 mM NaCl/5.4 mM KCl/1.2 mM $MgCl_2$/0.44 mM KH_2PO_4/4.2 mM $NaHCO_3$/20 mM Tes, pH 7.4). Subsequently the synaptosomal suspension was diluted with the basal medium containing in addition digitonin and EGTA (2 mg/ml and 1 mM, respectively) in order to disrupt the plasma membrane [16]. Thereafter the disrupted synaptosomes were centrifuged through a mixture of butylphthalate and dinoylphthalate (2:1, v/v) as described previously and the arsenazo III content of the supernatant or pellet after solubilization in 0.1% sodium dodecyl sulphate was measured by estimating the difference in absorbance in the presence of 1 mM EGTA and 2 mM $CaCl_2$ at the wavelength pair 665–685 in an Aminco DW_2 spectrophotometer. Lactate dehydrogenase and glutamate dehydrogenase were measured from the supernatants as described previously [16,17] in order to estimate the extent of release of cytosolic and mitochondrial contents. Corrections were made for contents in the supernatant of undisrupted synaptosomes. Entrapment of [^{3}H]sucrose was measured by spinning the synaptosomes through oil. The pellets were extracted using 10% perchloric acid and were counted after subsequent neutralization using 0.5 M Tris base.

Veratridine and arsenazo III were obtained from Sigma Chemical Co., St. Louis, MO. Arsenazo III was purified as described by Scarpa [18] before use. Verapamil was a gift from Knoll AG, Ludwigshafen, F.R.G. All the other reagents were commercial products of the highest grade available.

Results

Determination of the amount of entrapped arsenazo III

If arsenazo III is present in the homogenization medium the dye is entrapped within the synaptosomes, as judged from their blue-red colour. The dye associated with synaptosomes cannot be removed by repeated washings.

In order to find out the amount of entrapped indicator the synaptosomes were disrupted using digitonin at a concentration (2 mg/ml) which releases 65% of cytosolic lactate dehydrogenase and 11% of the intramitochondrial glutamate dehydrogenase. This treatment results in the release of 1.6 nmol of arsenazo III per mg of synaptosomal protein (Table I). With a value of 3 μl per mg of synaptosomal protein for intracellular H_2O [19] the concentration of entrapped indicator would be about 0.5 mM, i.e., about 5% of the concentration of arsenazo III in the homogenization medium. A similar percent of entrapped [^{3}H]sucrose is also obtained (Table I). Prolonged incubation (for 1 h)

TABLE I

CONTENTS OF ARSENAZO III AND [^{3}H]SUCROSE WITHIN SYNAPTOSOMES

See Methods section for experimental details. Contents are given as nmol/mg protein, ± S.E.

	Digitonin treatment		Total content	% released	% of initial concentration entrapped
	supernatant	pellet			
Arsenazo III	1.6 ± 0.1	1.0 ± 0.04	2.6 ± 0.14	58	5.0
Lactate dehydrogenase	—	—	—	65	
Glutamate dehydrogenase	—	—	—	11	
[^{3}H]Sucrose	—	—	14.4 ± 4	—	4.5

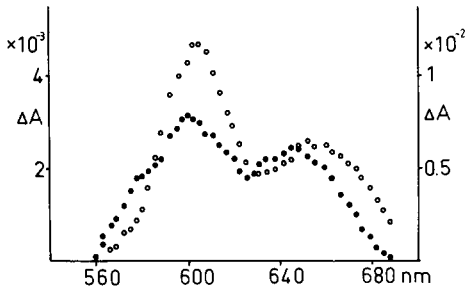

Fig. 1. Differential spectrum of the effect of veratridine and A23187 on intrasynaptosomally entrapped arsenazo III. Synaptosomes (4.5 mg protein/ml) were suspended into the basal experimental medium at 34°C. 10 mM glucose and 1 mM $CaCl_2$ were added subsequently and a baseline was drawn. Addition of 80 μM veratridine (●, scale on the left) and 4 μM A23187 subsequently (○, scale on the right); both were added from stock solutions in ethanol. The same concentration of ethanol (0.2%) had no effect on the absorbance spectrum.

of arsenazo III containing synaptosomes at 37°C causes a slow release of the indicator (not shown). The mitochondrial and plasma membrane potentials of synaptosomes loaded with arsenazo III are not significantly different from synaptosomes prepared in the absence of arsenazo III as measured

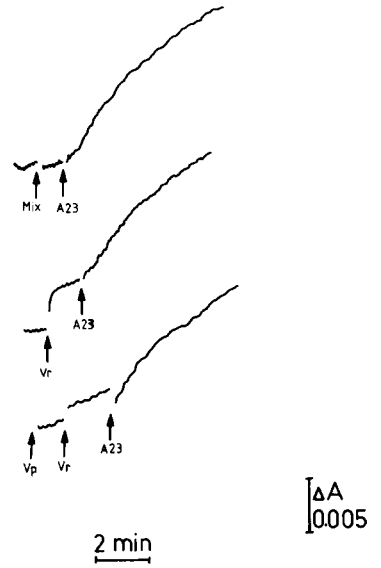

Fig. 2. Effect of veratridine and A23187 on the absorbance of entrapped arsenazo III. Synaptosomes (6.5 mg protein per ml) were suspended in the basal experimental medium. 1 mM $CaCl_2$ and 10 mM glucose were subsequently added and changes in absorbance at the wavelength pair 660–685 nm were monitored. 10 μM A23187 (A 23), 80 μM veratridine (Vr) or 100 μM verpamil (Vp) were added as indicated.

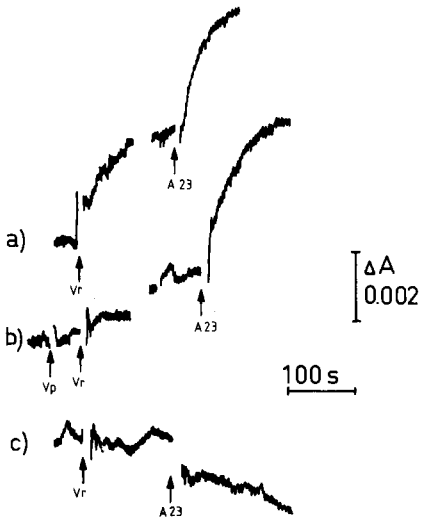

Fig. 3. Effect of veratridine, A23187, EGTA and verapamil on the absorbance of entrapped arsenazo III. Conditions as in Fig. 2 except that the wavelength pair was 600–555 nm. 200 μM veratridine (Vr), 4 μM A23187 (A 23) or 40 μM verapamil (Vp) were added as indicated. In (c) 5 mM EGTA was added 2 min after $CaCl_2$. Synaptosomal protein was 2 mg/ml. The discontinuation of traces in the absence of arrows represents intervals of 200 s.

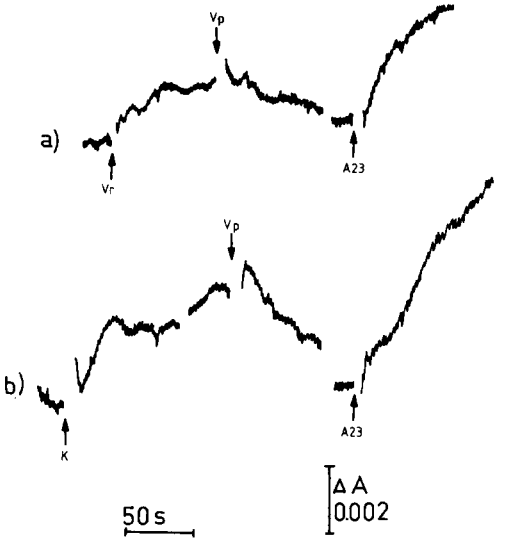

Fig. 4. Effect of veratridine, high K concentration, verapamil and A23187 on the absorbance of entrapped arsenazo III. Conditions as in Fig. 3. In a) synaptosomal protein was 1.5 mg/ml, and in b) 3 mg/ml. Additions were made of 200 μM veratridine (Vr), 200 μM verapamil (Vp), 10 mM KCl (K) or 4 μM A23187 (A 23) as indicated. Discontinuation of traces indicates intervals of about 200 s.

as described in Ref. 19. This suggests that the dye has no adverse effects on the function of synaptosomes (not shown).

Differential spectrum of intrasynaptosomal arsenazo III in the presence of veratridine and ionophore A23187

Addition of veratridine, an alkaloid which depolarizes synaptosomes by opening Na^+ channels in the plasma membrane [19–21], causes an increase in the absorbance of the synaptosomal suspension with peaks of absorbance around 600 and 650 nm (Fig. 1). This spectral change is typical of the arsenazo III-Ca^{2+} complex [18]. The change in absorbance is far more pronounced at 600 nm. A further addition of A23187 considerably enhances the spectral change.

Time course of changes in intrasynaptosomal [Ca^{2+}] as measured by dual-wavelength spectrophotometry

The increase in the absorbance of arsenazo III around 650 nm is specific for changes in free [Ca^{2+}], while Mg^{2+} is also sensed around 600 nm [18]. By using the wavelength pair 650–685 nm very variable results concerning effects of veratridine are observed with different synaptosomal preparations. This is partially due to the smaller sensitivity of the peak of absorbance at 650 nm (see Fig. 1). Furthermore, nonspecific changes in light transmission are usually observed in turbulent suspensions at wavelengths above 650 nm, further attenuating the response. An increase in absorbance at 650–685 nm is always observed to a variable extent by addition of A23187 (Fig. 2). Fig. 2 shows a typical experiment at this wavelength pair. Addition of veratridine causes a fast increase in the absorbance of the entrapped dye, suggesting an increase in intrasynaptosomal [Ca^{2+}]. This response is completely abolished by the Ca^{2+}-channel antagonist verapamil.

Because the absorbance changers are larger at 600 nm (Fig. 1) measurements were made using the wavelength pair 600–555 nm. At this wavelength pair a very reproducible increase in absorbance upon veratridine addition is observed (Fig. 3). A low concentration of verapamil (40 μM) considerably reduces this response, while this compound does not affect the effect of A23187 (Figs. 2 and 3). No significant changes in any of these conditions are observed in the presence of EGTA, suggesting that also at this wavelength pair changes in the free [Ca^{2+}] within synaptosomes are measured. It should be noted that quite large changes in free [Mg^{2+}] are required for any measurable response of arsenazo III [18].

Verapamil causes a slow efflux of Ca^{2+} from depolarized synaptosomes when added after the depolarization-induced uptake has been completed [11]. Fig. 4 demonstrates that also the change in absorbance of entrapped arsenazo III is slowly reversed upon addition of a high concentration of verapamil (200 μM). As also shown in Fig. 4, increasing the external [K^+] to 15 mM causes an increase in the absorbance of the entrapped dye. Also this effect is reversed by verapamil.

Fig. 5 compares the effect of varying concentrations of A23187 to that of veratridine. It is seen that 200 μM veratridine causes an absorbance change corresponding to about 0.5 nmol of A-23187/mg of protein. It should be noted that the saturation of the A23187 response is probably not due to a total equilibration of the Ca^{2+} gradient but due to a saturation of the dye response to Ca^{2+} which occurs at a [Ca^{2+}] of about 20–50 μM [18]. The nonspecific cation-selective channel-forming ionophore gramicidin, which depolarizes a variety of membranes including synaptosomes [20,21] causes a more extensive increase in the absorbance of entrapped arsenazo III.

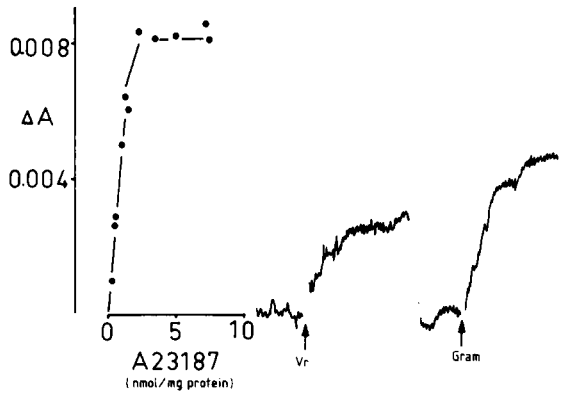

Fig. 5. Comparison of the effects of various A23187 concentrations with those of veratridine and gramicidin. Conditions as in Fig. 3. Additions were made of various A23187 concentrations (0.25–7.5 nmol/mg protein) 200 μM veratridine (Vr) or 0.5 μM gramicidin (Gram) as indicated. Protein content 1.5 mg/ml.

Discussion

The results of the present study show that if arsenazo III is present in the homogenization medium the dye is entrapped within subsequently isolated synaptosomes. This indicates that there is a transient contact of the homogenization medium with the cytosol when the nerve endings are chopped off from the axon. Since the concentration of arsenazo III and [^{3}H]sucrose entrapped within synaptosomes is only about 5% of their initial concentrations in the homogenization medium the contact with the intracellular space probably is very brief not allowing a significant depletion of intrasynaptosomal material. By measuring changes in the absorbance of this compound information concerning changes in the free Ca^{2+} concentration within isolated nerve endings is obtained. We cannot exclude the possibility that arsenazo III is entrapped within other structures contaminating the synaptosomal preparation. However, the large responses to depolarization suggest that the signals measured are derived from particles, i.e., synaptosomes being able to maintain a membrane potential and Ca^{2+} gradient at 1 mM external Ca^{2+}.

It has previously been shown that depolarization of the synaptosomal plasma membrane by various means leads to an increase in Ca^{2+} uptake by these structures. The present results indicate that the increase in Ca^{2+} uptake is linked to an increase in cytosolic [Ca^{2+}]. So far we have been unable to quantitate the magnitude of changes in free [Ca^{2+}] observed. Indirect estimates indicate that A23187-induced transmitter release is activated when the cytosolic [Ca^{2+}] increases to 10^{-6}–10^{-5} M [22,23]. This occurs at A23187 concentrations above 0.1 nmol per mg of synaptosomal protein. Similar Ca^{2+} concentrations are required for catecholamine release from adrenal medullary cells [24,25]. The K_d of the arsenazo III-Ca^{2+} complex is around 10 μM at neutral pH [18] and the half-maximal change in the arsenazo III absorbance as a response to A23187 occurs at concentrations around 0.8 nmol/mg protein of the ionophore. This correlates well with our previous indirect estimates [22] of cytosolic [Ca^{2+}] within synaptosomes at this ionophore concentration, which would give a value around 6–10 μM Ca^{2+}. The large spectral responses observed with veratridine, gramicidin and high [K] as compared to those occurring in the presence of A23187 indicate that our method may well be sensitive enough to measure such depolarization-induced increases of cytosolic free Ca^{2+} that are required for transmitter liberation.

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