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Quantitative measurements of the cytosolic Ca^{2+} activity within isolated guinea pig nerve-endings using entrapped arsenazo III and quin2

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

[a] *Åbo Akademi, Department of Biochemistry and Pharmacy, Porthansgatan 3, SF-20500 Turku,* [b] *University of Helsinki, Department of Physiology, SF-00170 Helsinki, and* [c] *University of Helsinki, Department of Zoology, Division of Physiology, SF-00100 Helsinki (Finland)*

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The absorbance changes of intrasynaptosomally entrapped arsenazo III have been converted into values of free Ca^{2+} concentration by correcting for the nonlinear response of arsenazo III at different concentrations of the dye as well as for changes in internal pH. An average resting value for free Ca^{2+} concentration around 0.4 μM is obtained. Depolarization with veratridine or gramicidin increases this value to around 3 μM. Measurements of cytosolic free Ca^{2+} with the quin2 method gives much lower values in similar conditions. The release of prelabelled [^{14}C]noradrenaline from the nerve-endings is maximally activated when the internal free Ca^{2+} concentration rises as measured with arsenazo III to about 4 μM when titrated with increasing concentrations of ionophore A23187.

Introduction

Several lines of evidence indicate that transmitter release as a response to electrical excitation is triggered by an increase in the cytosolic Ca^{2+} activity within nerve terminals [1,2]. Since the cytosolic free Ca^{2+} concentration in resting nerve cells is around 10^{-7} M [3] or lower [4], measurements of changes in this parameter in nerve-endings are technically difficult due to the small size of these structures.

In the squid giant synapse, the light output of intracellularly injected aequorin [5,6] or the absorbance of arsenazo III [7,8] have been used to monitor Ca^{2+} transients during activity. However, very little quantitative information concerning the magnitude of changes in internal Ca^{2+} have been obtained with these techniques. For studies of the role of Ca^{2+} in transmitter release in higher animals, fractions of isolated nerve-endings have often been used [2,9,10]. These pinched-off nerve terminals maintain a high membrane potential [11,12] and release transmitter substances when depolarized by various experimental means [9,13]. Since the trigger of transmission is an increase in the free cytosolic Ca^{2+} concentration, efforts have been made to measure this parameter using entrapped quin2 [14,15]. Meldolesi et al. [14] were unable to quantitate the increase in internal Ca^{2+} upon depolarization, while Ashley et al. [15] suggest that transmitter release is maximally activated at free Ca^{2+} concentrations around 0.4–0.8 μM. These values are in contradiction with previous indirect estimates using permeabilized adrenal medulla cells [16] or ionophore A23187-treated synaptosomes [17], suggesting that transmitter release is not activated until the free cytosolic Ca^{2+}

* To whom correspondence should be addressed.

Abbreviations: Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]amino}ethanesulfonic acid; H-EDTA, *N*-hydroxyethylethylenediaminetriacetic acid.

concentration rises above 0.5–1 μM. Quin2 has a high affinity for Ca^{2+} (K_d 115 nM) and hence the probe itself might buffer substantial amounts of Ca^{2+}. Furthermore, due to saturation of the probe response measurements of free Ca^{2+} concentrations higher than 1 μM may be difficult. We have recently described a method for entrapping the metallochromic indicator, arsenazo III, into isolated nerve-endings [18]. This probe has a relatively low affinity for Ca^{2+} ($K_d > 10^{-5}$ M) [19]. However, because the relative response of this probe varies with its concentration (see Ref. 20 for review) quantitation of the signals obtained is difficult.

The aim of the present study was to correct for the nonlinear arsenazo III response in order to obtain quantitative information concerning changes in free cytosolic Ca^{2+} in nerve-endings in relation to transmitter liberation.

Methods

Isolation of synaptosomes and experimental protocol. Synaptosomes were isolated from the cortical hemispheres (including corpus striatum and hypothalamus) of guinea pigs using discontinuous Ficoll density gradients as described previously [21]. 10 mM arsenazo III, purified as described by Scarpa [19], was added to the homogenization medium (0.32 M sucrose), in order to entrap the dye within subsequently isolated nerve-endings [18]. 0.5 mM EGTA was subsequently added to the homogenate. The synaptosomal fraction was removed from the Ficoll gradient. The material from the gradient was divided into separate centrifuge tubes, diluted 20-fold with the standard experimental Na^+-based medium (137 mM NaCl/ 5 mM KCl/ 1.2 mM Mg_2Cl_2/ 0.44 mM KH_2PO_4/ 4.2 mM $NaCHO_3$/ 20 mM Tes buffer, pH 7.4) and centrifuged at 15 000 × *g* for 15 min. The resulting pellets were covered by a small volume of fresh standard Na^+-based medium and stored on ice until use within 120 min. Each experiment was initiated by suspending the pellet into the Na^+-based medium containing 5 mM glucose. 1 mM $CaCl_2$ was subsequently added after a few minutes.

Differential spectra and dual wavelength measurements were carried out with an Aminco DW2 or a Schimadzu UV3000 spectrophotometer with thermostatically controlled cuvette-holder keeping a temperature of 35°C in the cuvette.

The quin2 loading into synaptosomes, fluorescence measurements and calibration of the response were performed as described by Ashley et al. [15]. The fluorescence was recorded in a Perkin Elmer 512 double-beam spectrofluorimeter using 339 nm as excitation and 492 nm as emission wavelength at 35°C.

Calibration of the arsenazo III response. Several problems are encountered when trying to obtain quantitative information concerning the relation of internal absorbance changes to changes in free Ca^{2+}. The synaptosomes have to be permeabilized and the spectral changes calibrated with known free Ca^{2+} concentration in conditions prevailing in the cytosol. We have previously shown that treatment of synaptosomes with digitonin disrupts the plasma membrane, as indicated by a release of more than 90% of the intrasynaptosomal lactate dehydrogenase content, used as a marker of cytosol, with little release or intramitochondrial contents [22,23]. Since arsenazo III also reacts to changes in pH [24] and Mg^{2+} concentration [19], any changes in these parameters ought to be accounted for. Intracellular pH is of the order of 7 in various cells [25]. With isolated nerve-endings incubated at pH 7.4, a value around 7.2 has been obtained by measuring the distribution of [^{14}C]methylamine [26]. Intracellular Mg^{2+} is usually of the same magnitude as the extracellular concentration of this cation [27], and arsenazo III is much less sensitive to changes in Mg^{2+} as compared to Ca^{2+} [19]. Furthermore, no changes in the absorbance of arsenazo III in response to either depolarization or ionophore A23187 are observed when extracellular Ca^{2+} is chelated with EGTA [18]. Therefore, it appears valid to assume with some reservation that the calibration procedure can be performed in conditions prevailing in the external medium.

The free Ca^{2+} in the calibration experiments was varied using an H-EDTA (1 mM)/Ca^{2+} (10–1970 μM) buffer system. The free Ca^{2+} was calculated by the reiterative method of Portzehl et al. [28]. Since stability constants reported in the literature usually are higher than those measured with Ca^{2+} electrodes in physiological solutions

[29], the dissociation constant of the H-EDTA-Ca^{2+} complex was measured using a Radiometer F2112 Ca^{2+} electrode as described by Bers [30]. We obtained a value of $3.8 \cdot 10^{-6}$ and of $6.1 \cdot 10^{-6}$ M at pH 7.4 and 7.2, respectively. The literature values for the stability constant of the H-EDTA-Mg^{2+} complex vary considerably [31]. Hence, an average value was taken and values for the calculated dissociation constants used were $2.8 \cdot 10^{-4}$ and $4.5 \cdot 10^{-4}$ M at pH 7.4 and 7.2, respectively. As calculated using these dissociation constants, a variation in the free Mg^{2+} between 0.5 and 1 mM will occur in the buffer solution during calibration. Such a change in free Mg^{2+} gives an increment of 2.6% of the change due to Ca^{2+}. Since such a small change is within experimental error, it was disregarded. Corrections were in all conditions made for the binding of Ca^{2+} to arsenazo III using dissociation constants (around 40 μM) measured as described by Bers [30]. Although the response of arsenazo III varies with concentration (see below), no significant variation was observed with respect to the dissociation constant at different concentrations of arsenazo III at pH 7.4 and 7.2. The Ca^{2+} contaminating the standard experimental medium was assayed using the Ca^{2+} electrode or by titrating back the arsenazo III signal with 1-μM pulses of EGTA. This contamination was usually about 10 μM and the individual values were taken into account in the calibrations.

The change in absorbance induced by Ca^{2+} is not a simple function of the arsenazo III concentration [20]. Fig. 1 shows that especially at the Ca^{2+} concentrations of interest (0.3–3 μM) there is a relative increase in absorbance with the arsenazo III concentration up to 0.3 mM followed by a drop around 1 mM arsenazo III. The intrasynaptosomal arsenazo III concentration is usually around 0.3 mM [18] and the dye concentration is reduced to a few micromolar after release with digitonin. Thus, corrections have to be made in order to allow for this difference. The following procedure adopted from that of Yingst and Hoffman [32] was thus used for the calibration of the response of internal dye to free Ca^{2+} concentrations.

One synaptosomal pellet was divided into several parts. With one part, the actual experiment was performed followed by an addition of a maximally effective concentration of ionophore A23187 (10 μM, Ref. 18). It should be noted that ionophore A23187 will equilibrate the pH difference and increase the internal pH from 7.17 ± 0.02 ($\pm$S.D., $n = 6$) to 7.4 ± 0.06 ($\pm$S.D., $n = 6$), measured as described by Åkerman and Nicholls [26]. Another part of the pellet was spun-down at $12000 \times g$ for 2 min and the arsenazo III concentration in the supernatant was measured at the wavelength pair 600–555 nm by measuring the difference in absorbance in the presence of 1 mM H-EDTA (or 1 mM EGTA) and a saturating Ca^{2+} concentration (1.97 mM), respectively. A third part was treated with 2 mg/ml digitonin for 10 min and the total amount of arsenazo III was measured as described above. The internal dye

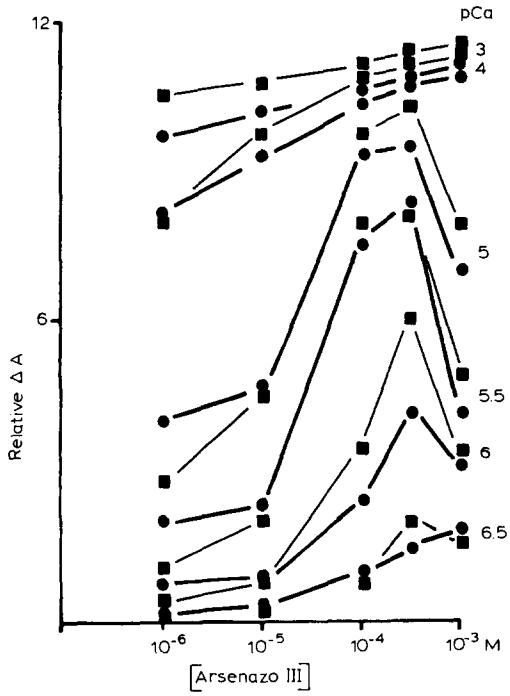

Fig. 1. Changes in absorbance of arsenazo III at varying concentrations of the dye in response to changes in free Ca^{2+} concentration at pH 7.4 (●) and 7.2 (■). The free Ca^{2+} concentration was varied using a H-EDTA (1 mM)/Ca^{2+} buffer system in the Na^+-based experimental medium and the absorbance was recorded at 600–570 nm. The lightpath was 1 cm (1–10 μM arsenazo III) or 1 mm (100 μM arsenazo III and above). The free Ca^{2+} concentration after binding to arsenazo III was measured using a Ca^{2+} electrode as described in Methods. Results are expressed as change in absorbance at 600–570 nm·(arsenazo III)$\cdot l^{-1}$ ($A \cdot \mu M^{-1} \cdot cm^{-1}$).

concentration was then calculated as described previously [18] and corrections to approximate the response of internal dye were made according to the data in Fig. 1.

Measurements of [^{14}C]noradrenaline release. Uptake and release of noradrenaline was measured essentially as described previously [17]. Briefly, the synaptosomes were incubated for 25 min at 35°C in a shaking water-bath containing the standard Na^+-based medium with 1 mM $CaCl_2$, 5 mM D-glucose, 1 μM (0.1 μCi/ml) [^{14}C]noradrenaline, 1 μCi/ml ^3H_2O (as a measure of synaptosomal H_2O), 10 μM nialamide and 1 mM sodium ascorbate, whereafter 20 μM imipramine was added to block further uptake of noradrenaline and prevent any release through reversal of the uptake mechanism [17]. After 5 min further incubation, Ca^{2+} influx was induced by addition of ionophore A23187 (0.1–10 μM), samples (400-μl) were taken after 2 min and spun through a 2:1 (v/v) mixture of butylphthalate and dinonylphthalate. The aqueous supernatant was sucked off, the walls of the tubes washed with 500 μl H_2O, the oil layer sucked off and the isotopes extracted from the pellet with 10% perchloric acid, neutralized with Tris-base and transferred into scintillation vials. The synaptosomal internal H_2O was measured using 2 μCi/ml ^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-isotope determination separating ^3H from ^{14}C.

Materials

Arsenazo III, veratridine and digitonin were obtained from Sigma (Poole, Dorset, U.K.). Gramicidin from Fluka A.G. (Buchs, Switzerland), ionophore A23187 from Boehringer (Mannheim, F.R.G.). The radiochemicals were obtained from Amersham International (U.K.).

Results

A typical difference spectrum of the response of intrasynaptosomally entrapped arsenazo III to depolarization with gramicidin and to addition of

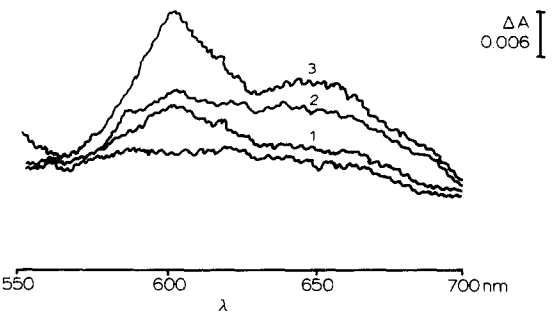

Fig. 2. Difference spectrum of the effect of gramicidin on entrapped arsenazo III. Synaptosomes were resuspended at a concentration of 1.7 mg protein/ml into the basal experimental medium containing 5 mM glucose and 1 mM $CaCl_2$ at 35°C. Additions were made of 0.5 mg/ml gramicidin and the difference spectrum was recorded within 1 min (1) and after 5 min (2), when a stable spectrum was obtained during subsequent scannings. 4 μM ionophore A23187 was subsequently added (3).

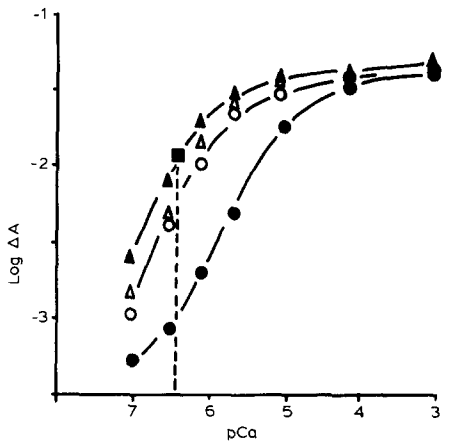

Fig. 3. Calibration of the arsenazo III response in synaptosomes. See Methods for details. Calibration with varying free Ca^{2+} concentrations were performed after addition of 2 mg/ml digitonin (●) or 10 μM ionophore A23187 (○). The synaptosomes were treated with digitonin, whereafter 1 mM H-EDTA was added and the response to increase Ca^{2+} concentrations were measured in the supernatant. The results with ionophore A23187 were obtained by adding 10 μM ionophore A23187 to the synaptosomal suspension, followed by 1 mM H-EDTA and increasing Ca^{2+} concentrations. The response of the arsenazo III contaminating the external medium was measured in the supernatant after centrifugation of the synaptosomal suspension. Corrections of results obtained with the digitonin supernatant were made after calculation of entrapped dye concentration (△) and for a pH of 7.2 (▲) in resting conditions. The absorbance of three synaptosomal suspensions in resting conditions in the presence of 1 mM $CaCl_2$ (■) is also shown. The S.D. values do not exceed the diameter of the symbol.

ionophore A23187 is shown in Fig. 2. There is an increase in absorbance at 600 nm and around 650 nm, typical of the arsenazo III-Ca^{2+} complex [19].

Fig. 3 shows the Ca^{2+} dependence of the spectral change at the wavelength pair 600–570 nm in digitonin (2 mg/ml) or ionophore A23187 (10 μM) treated synaptosomes. Digitonin releases the entrapped dye, while ionophore A23187 is expected to equilibrate the Ca^{2+} gradient across the plasma membrane. The relative change in absorbance at low free Ca^{2+} concentrations is significantly smaller in synaptosomes treated with digitonin as compared to those treated with ionophore A23187. If corrections are made according to data like that in Fig. 1 in order to approximate the response of intrasynaptosomally entrapped dye to Ca^{2+}, the difference between digitonin- and ionophore A23187-treated synaptosomes is almost totally abolished, suggesting that the correction is valid. The internal pH of synaptosomes is 7.2 in resting conditions and increases to 7.4 (see Methods) in the presence of ionophore A23187. A further correction to allow for the difference in pH shifts the calibration plot further to the left, giving a value of 0.4 ± 0.02 μM ($\pm$S.D., $n = 3$) for the resting cytosolic Ca^{2+} concentration. The average value for nine preparations isolated on different days was 0.4 ± 0.3 μM ($\pm$S.D.). The changes in absorbance of entrapped arsenazo III in the presence of ionophore A23187 are fully reversible and may be titrated back with EGTA (not shown).

The response of entrapped arsenazo III to veratridine-induced depolarization is shown in Fig. 4. In this case, the resting value of free Ca^{2+} was 0.6 μM and veratridine increased this value to around 3 μM. The average value of free Ca^{2+} after veratridine-induced depolarization in six synaptosomal preparations isolated on different days is 3.1–1.3 μM ($\pm$S.D.). Subsequent addition of 4 μM ionophore A23187 (Fig. 4) caused a maximal change in absorbance with no apparent further change upon further additions of the ionophore. Digitonin (2 mg/ml) when added in similar conditions caused an increase in the absorbance to about the same extent as ionophore A23187 (not shown). However, this treatment frequently caused disturbances due to nonspecific light-scattering and thus the synaptosomes have to be spun-down and calibration performed with the supernatant.

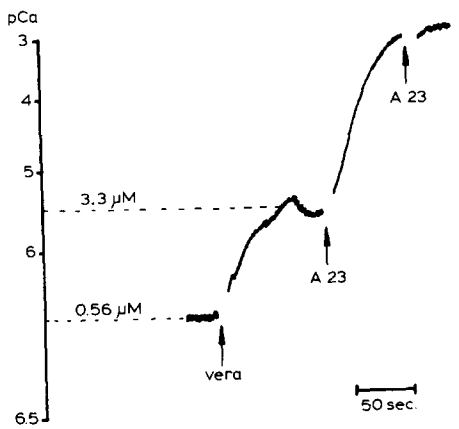

Fig. 4. Depolarization-induced increase in cytosolic free Ca^{2+} concentration. Synaptosomes were suspended at a concentration of 2.5 mg protein/ml in the basal experimental medium containing 5 mM glucose and 1 mM $CaCl_2$ at 35°C. The absorbance was recorded at 600–555 nm. Additions were made of 40 μM veratridine (vera) or 4 μM ionophore A23187 (A23), respectively. Calibration was performed as described in Methods. The numbers denoted at the ordinate by the intersecting dotted lines indicate values of free Ca^{2+} concentration (μM). Note that the scale below pCa < 5 is corrected for an internal pH of 7.2 while at pCa > 5 the scale is for a pH of 7.4.

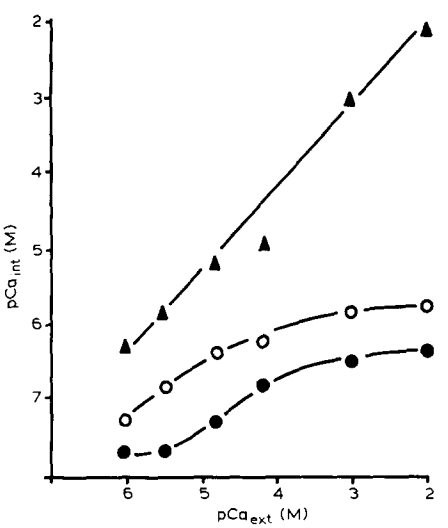

Fig. 5. Effect of external free Ca^{2+} concentration on the internal free Ca^{2+} concentration. Conditions were as in Fig. 4. The external Ca^{2+} concentration was varied using an H-EDTA/Ca^{2+} buffer system in control conditions (●). Additions were made of 40 μM veratridine (○) or 10 μM ionophore A23187 (▲).

The response of intrasynaptosomally entrapped arsenazo III to increasing external free Ca^{2+} concentrations in the presence of veratridine or ionophore A23187 is shown in Fig. 5. In resting conditions, an increase in cytosolic Ca^{2+} is apparent around 10–100 μM Ca^{2+} followed by a saturation. The veratridine-induced change in absorbance is apparent already at low external Ca^{2+} concentrations and is also saturable. As expected from the data in Fig. 3, the response to ionophore A23187 is nonsaturable and indicates an almost total equilibration of the Ca^{2+} gradient. The equilibration of the Ca^{2+} gradient seems to be more apparent at high Ca^{2+} concentrations, at which concentrations, ionophore A23187 and digitonin treatment give the same values of absorbance. At low Ca^{2+} concentrations, there seems to be a Ca^{2+} gradient across the plasma membrane.

In order to be able to relate internal free Ca^{2+} concentration to transmitter release, the difference spectrum of internal arsenazo III at increasing concentrations of ionophore A23187 was recorded (Fig. 6) and the internal free Ca^{2+} concentration was calculated. There is an apparent linear relation between p*Ca* and the ionophore A23187 concentration with a fairly good correlation between six synaptosomal preparations. In Fig. 7, transmitter liberation induced by ionophore A23187 is measured in parallel in similar conditions and the values for [^{14}C]noradrenaline release are plotted against the p*Ca*. The results suggest that the half-maximal increase in [^{14}C]noradrenaline release occurs around 1–2 μM free Ca^{2+} with a maximal change above 3 μM. The effect of 100 μM veratridine is also shown in Fig. 7. The value obtained with this alkaloid fits very well with those obtained with ionophore A23187.

In order to compare results obtained with arsenazo III to those obtained with quin2, experiments were performed using the same batch of synaptosomes under similar conditions with both techniques. Fig. 8 demonstrates that with quin2 a

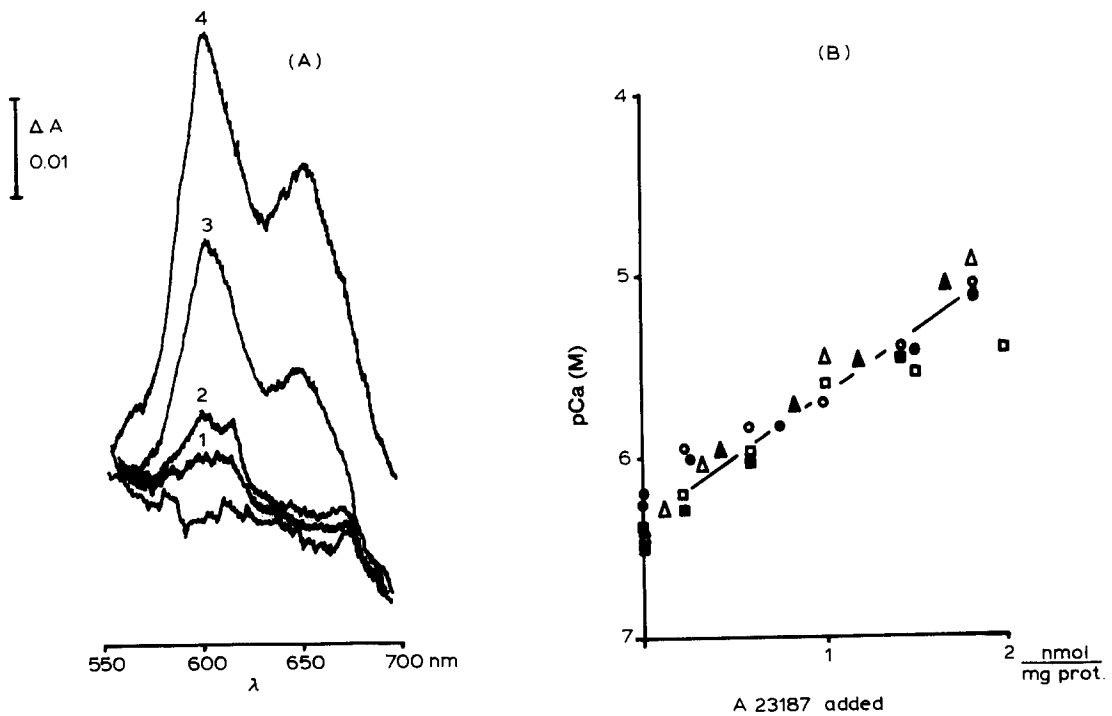

Fig. 6. Effect of ionophore A23187 on the absorbance of entrapped arsenazo III. (A) Conditions were as in Fig. 2. Additions were made of 0.1 μM (1), 0.8 μM (2), 2.4 μM (3) or 6.4 μM (4) ionophore A23187. Each spectrum is drawn after stabilization of the trace upon three subsequent scannings. (B) The data from six batches of synaptosomes are plotted as a function of the ionophore A23187 concentration. Calibration was performed as described in Methods. Synaptosomal protein concentration was 2 mg/ml.

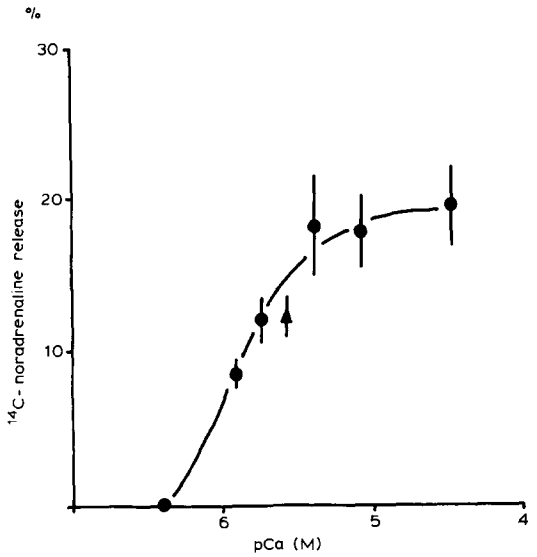

Fig. 7. Relation of [^{14}C]noradrenaline release to internal free Ca^{2+} concentration. The internal Ca^{2+} concentration of arsenazo III-containing synaptosomes was varied with different concentrations of ionophore A23187 (●) like in Fig. 6 and [^{14}C]noradrenaline release was measured in parallel in identical conditions as described in Methods (± S.D., $n = 4$). The effect of a maximally effective concentration (100 μM) of veratridine is also shown (▲).

resting cytosolic Ca^{2+} activity around 0.1 μM is obtained, when the dye response is calibrated exactly as described by Ashley et al. [15]. In similar conditions, arsenazo III gives a resting value of 0.6 μM. Depolarization using veratridine increases the cytosolic free Ca^{2+} concentration to around 0.3 μM in the quin2-loaded synaptosomes and to around 2 μM, as measured using entrapped arsenazo III. Thus, there is a difference of almost one order of magnitude between values obtained by these two techniques. The release of [^{14}C]noradrenaline induced by 1 μM ionophore A23187 was also considerably attenuated in quin2-loaded synaptosomes when compared to synaptosomes incubated in exactly the same way in the absence of quin2. The release decreased from 13 ± 5% (± S.D., $n = 4$) to 0.2 ± 3 ($n = 4$) in control and quin2-loaded synaptosomes, respectively.

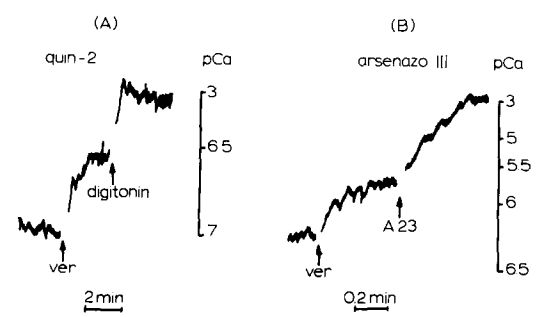

Fig. 8. Effect of veratridine on the intrasynaptosomal free Ca^{2+} concentration as measured with entrapped arsenazo III or quin2. Conditions were as in Fig. 5. In (A) synaptosomes had been loaded with 50 μM quin2/acetoxymethylester. The fluorescence was recorded at 35°C. Additions were made of 25 μM veratridine (ver) and 2 mg/ml digitonin as indicated. In (B) the absorbance of entrapped arsenazo III was measured. Additions were made of 25 μM veratridine (ver) and 4 μM ionophore A21387 (A23) as indicated. Calibration of dye responses were performed as described in Methods.

Discussion

The results of the present study describe a method for the conversion of changes in the absorbance of intrasynaptosomally entrapped arsenazo III to free Ca^{2+} concentrations. Because arsenazo III has a low affinity for Ca^{2+} ($K_d \approx 40$ μM) compared to Ca^{2+} concentrations existing in the cytosol, this probe will not buffer the cytosolic Ca^{2+} to the same extent as probes like, for instance, quin2 (see below). The disadvantage in using arsenazo III is the very nonlinear response as a function of dye concentration. At the Ca^{2+} concentrations of interest (around 1–10 μM), the nonlinearity is most apparent. The relative absorbance increases up to dye concentrations around 300 μM followed by a fall in the signal. In the present conditions for loading the dye into synaptosomes, the internal concentration was usually around 300 μM (see also Ref. 18), which concentration, according to the present data, appears to be optimal for the detection of Ca^{2+} transients.

A resting free cytosolic Ca^{2+} concentration around 0.4 μM, but often as high as 0.6 μM, was obtained after corrections were made for internal pH and dye concentration. This value is higher than those reported earlier for various cell types [20]. The reason for this might be due to at least two factors. Firstly, the stability constants for Ca^{2+} buffer systems, reported in the literature, are considerably higher than those determined by

using Ca^{2+} electrodes in physiological solutions [29]. By using a dissociation constant of 10^{-6} μM for the H-EDTA-Ca^{2+} complex calculated from stability constants reported by Sillen and Martell [31], values around 0.1–0.2 μM for the resting free Ca^{2+} would be obtained also in our conditions. Our values are, however, based upon measured values for the Ca^{2+}-H-EDTA dissociation constant in our experimental conditions. The other possible reason for high values of resting free Ca^{2+} may be entrapment of arsenazo III within nonfunctional vesicular material having a high level of free internal Ca^{2+} thus giving apparent high levels of resting absorbance. This background absorbance is not expected to affect values obtained for changes in cytosolic free Ca^{2+} in response, for instance, to depolarization. The very small sensitivity of arsenazo III at low free Ca^{2+} concentrations also makes exact measurement of resting values more unreliable. The same is the case concerning exact values at high Ca^{2+} concentrations (above 100 μM) due to partial saturation of the signal, although relative changes in absorbance at these levels are readily measurable. It should also be noted that with the present method for calibration of the dye response the maximal absorbance values obtained will significantly influence the apparent values at low free Ca^{2+} concentrations.

Depolarization of synaptosomes either with veratridine or gramicidin increases the cytosolic Ca^{2+} to around 2–3 μM. Similar values have been measured during activation in various excitable cells using optical probes or Ca^{2+}-selective microelectrodes [20]. The low values obtained with the quin2 method (0.3 μM) in similar conditions [15] and in this study could be explained by a damping of changes in cytosolic Ca^{2+} by the probe itself. It should be noted that a change in cytosolic Ca^{2+} from 0.1 μM to 0.3 μM would hardly be detected by arsenazo III in our conditions, since it would, for instance, represent only a 5% change or less of the total absorbance of entrapped arsenazo III to ionophore A23187 (see Fig. 5) and would be within experimental error. On the other hand, the most optimal range of Ca^{2+} concentrations possible to detect with arsenazo III is between 1 and 10 μM and hence the values obtained after depolarization may be considered far more reliable. Thus, although the resting values for free Ca^{2+} appear uncertain, it appears that during depolarization cytosolic Ca^{2+} rises to values above 1 μM.

Several lines of evidence suggest that the increase in cytosolic Ca^{2+} upon depolarization is a result of the opening of Ca^{2+} channels in the plasma membrane of the nerve-ending. Thus, Ca^{2+} influx or the depolarization-induced increase in cytosolic Ca^{2+} are inhibited by Ca^{2+} channel blockers [18,33–35]. Furthermore, the voltage dependence of the depolarization-induced increase in cytosolic Ca^{2+} is similar to that of Ca^{2+} channels in various tissues [36]. The apparent saturation of the depolarization-induced increase in cytosolic Ca^{2+} observed in this study, which has previously been observed also in $^{45}Ca^{2+}$ influx studies [21], could be due to a saturation of Ca^{2+}-binding sites in the channel, a counteracting efflux, or Ca^{2+}-dependent inactivation of the channel [37]. These possibilities are currently being investigated. The present data do not permit distinction between these possibilities.

Estimates of the relation of transmitter release to internal Ca^{2+} have been obtained with permeabilized adrenal medullary cells by varying external Ca^{2+} using an EGTA/Ca^{2+} buffer system [16], indicating that there is a steep increase in transmitter liberation when the cytosolic free Ca^{2+} is raised above 0.5 μM, with a maximal change around 10 μM Ca^{2+}. These values are in good agreement with the known values for the average dissociation constant of the Ca^{2+}-calmodulin complex [38]. With isolated nerve-endings, we have previously shown a graded increase in [^{3}H]noradrenaline liberation when increasing concentrations of ionophore A23187 were added [17]. The increase in transmitter liberation correlated well with the increase in Ca^{2+} flow into the nerve-endings induced by the ionophore [17]. By measuring simultaneously the Ca^{2+}-dependent increase in synaptosomal respiration in the presence of ionophore A23187, indirect estimates of the dependency of transmitter release is activated once the cytosolic Ca^{2+} rises above 1 μM, with a maximal change around 30 μM [17,38]. The results of the present study, where intracellular free Ca^{2+} has been measured using entrapped arsenazo III, are in good general agreement with these values. it should be noted, however, that noradrenaline re-

lease was measured in this study from whole brain synaptosomes. Hence, the changes in internal Ca^{2+} are an average of the changes in all nerve-endings present, while transmitter liberation occurred only in a small fraction of catecholaminergic endings in the preparation. However, similar changes in internal free Ca^{2+} concentration have been observed in hypothalamic and striatal synaptosomes which are enriched in catecholaminergic endings (unpublished data), although the small amount of available material has not permitted studies on the relation of release to changes in internal Ca^{2+}. The quin2 method has recently been used in attempts to relate cytosolic free Ca^{2+} of synaptosomes [15] to transmitter liberation. These results suggested that [^{14}C]acetylcholine liberation is fully activated around 0.4–0.8 μM free Ca^{2+}. However, transmitter release was not measured in quin2-loaded synaptosomes and hence the buffering of cytosolic free Ca^{2+} by the probe was not accounted for. Our results obtained when comparing these two methods (Fig. 7) and the lack of release in quin2-loaded synaptosomes are in agreement with this. The buffering of internal Ca^{2+} by quin2 has recently been observed also in various cells [39–41] and quin2 incorporation inhibits a Ca^{2+}-dependent activation of some processes [39,40].

It is concluded that quantitative information concerning changes in intrasynaptosomal free Ca^{2+} levels during Ca^{2+} transients may be obtained with the arsenazo III method. The advantage of the arsenazo III method is the low affinity of the probe for Ca^{2+}. Thus, arsenazo III is expected to buffer the cytosolic Ca^{2+} far less than probes with higher affinity for Ca^{2+}, like quin2. Our results suggest that transmitter release is maximally activated when cytosolic Ca^{2+} rises to about 3 μM. Our results also suggest that, if quantitative information is required, considerable care should be taken to exclude the buffering of cytosolic Ca^{2+} by optical probes, which have a high affinity for Ca^{2+}, like quin2.

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