Presynaptic Calcium Channels in Rat Cortical Synaptosomes: Fast-Kinetics of Phasic Calcium Influx, Channel Inactivation, and Relationship to Nitrendipine Receptors

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Fast-mixing and rapid-filtration techniques were used to analyze the kinetics of potassium-depolarization-dependent ($\Delta K^+ = 47.5$ mM) influx of ^{45}Ca into synaptosomes, in the time range from 50 msec to 5 sec. The results are consistent with the presence in synaptosomes of a homogenous population of voltage-sensitive Ca channels. With 1 mM Ca_o in the medium, the ΔK^+ -dependent Ca influx has a single-exponential time course with the half-life, $t_{1/2} \sim 0.5$ -0.7 sec. Ca influx, measured between 0.1 and 10 mM Ca_o , shows half-saturation (K_{Ca}) at 1.5 mM Ca_o and has the limiting value (J_{Ca}^{max}) of 5.9 nmol/sec/mg protein, or a current of approximately 0.06 pA/ μm^2 surface area. The estimated density of functional Ca channels is 0.6-6 μm^{-2} .

Voltage- and time-dependent inactivation of Ca channels was measured in synaptosomes prepolarized in 52.5 mM K_o^+ with Ca omitted from the medium. Channel inactivation is a single-exponential process with a half-life of $t_{1/2} \sim 2.3$ sec. Channel recovery in 5 mM K_o^+ media is likewise a single-exponential process with a half-life of $t_{1/2} \sim 4.3$ sec. The slower rate of voltage-dependent channel inactivation than of decay of Ca influx suggests that Ca entry into synaptosomes terminates by a mechanism that depends on Ca influx itself.

Synaptosomes contain 200 fmol/mg protein, or $\sim 6 \mu\text{m}^{-2}$ high-affinity ($K_D = 0.12$ nM) ^3H -nitrendipine binding sites; however, nitrendipine at concentrations $>10^4 \times K_D$ is without effect on the phasic influx of Ca measured at 215 msec with either 1.0 or 0.1 mM Ca_o . This suggests that Ca channels characterized in this study belong to a class of dihydropyridine-insensitive channels.

In view of the importance of voltage-gated Ca influx in the release of neurotransmitters (Katz, 1969; Llinás and Heuser, 1977), it is of considerable interest to characterize the biophysical and pharmacological properties of Ca channels in nerve terminals and elucidate the factors that regulate the functional state of the channels. Investigation of presynaptic voltage-sensitive Ca channels in brain has been hindered by the inaccessibility of presynaptic terminals to direct electrophysiological recording techniques; however, it is possible to characterize properties of these channels from measurements of K^+ -depolarization-dependent ^{45}Ca influx in isolated nerve terminals *in vitro*, i.e., synaptosomes.

In synaptosomes, the K^+ -depolarization-stimulated Ca entry is biphasic and consists of "fast" and "slow" components (Nachshen and Blaustein, 1980, 1982). There is evidence suggesting that the initial, rapidly decaying phase of Ca influx is associated with the opening and subsequent inactivation of Ca channels

(Nachshen, 1985; Nachshen and Blaustein, 1980, 1982) and that these channels mediate brisk, transient release of transmitters from synaptosomes (Drapeau and Blaustein, 1983; Suszkiw and O'Leary, 1983).

Inactivation and reactivation of Ca channels in nerve terminals is of considerable interest as a potential mechanism of transmitter-release modulation. Reversible voltage and/or Ca_i -dependent channel inactivation has been described in a number of different systems (for review, see Hagiwara and Byerly, 1981; Kostyuk, 1981; Tsien, 1983). Nachshen (1985) suggested that the rapid decay of Ca influx, measured in the millisecond range, reflects primarily a voltage-dependent inactivation of the synaptosomal Ca channels; however, it is not entirely clear if, and to what extent, Ca entry itself may contribute to the observed decay of Ca influx in synaptosomes. A Ca-dependent channel inactivation has been demonstrated in the squid giant axon terminals (Augustine and Eckert, 1984).

Several reports indicate that synaptosomal Ca channels are not sensitive to the myocardial and smooth muscle Ca channel antagonists such as nitrendipine and related dihydropyridines (Daniell et al., 1983; Nachshen and Blaustein, 1979; Rampe et al., 1984); however, this has recently been questioned (Turner and Goldin, 1985). Thus, although synaptosomes contain specific high-affinity receptors for nitrendipine and related dihydropyridines (Rampe et al., 1984; Yamamura et al., 1982), the relation of these receptors to the presynaptic Ca channels remains conjectural (Miller, 1985; Miller and Friedman, 1984).

In the present study we attempted (1) to characterize the kinetics of Ca channels in synaptosomes and estimate the density of functional channels from the Ca flux measurements in the millisecond time range, (2) to characterize the time course of channels inactivation and recovery, and determine whether the decay of Ca influx in synaptosomes is associated with a voltage- or Ca_i -dependent channel inactivation, and (3) to compare the density of ^3H -nitrendipine binding sites with the estimated number of functional Ca channels in synaptosomes, and examine the effect of nitrendipine on the initial rates of K^+ -stimulated Ca influx in the millisecond time range. Preliminary accounts of this work have appeared in abstract form (O'Leary and Suszkiw, 1985; Wang et al., 1985).

Materials and Methods

Materials

$^{45}\text{CaCl}_2$ (13-47 mCi/mg), 5-methyl- ^3H -nitrendipine (81.3 Ci/mmol, lot no. 2175-088), ^{14}C (U)-sucrose (273 mCi/mmol), and $^3\text{H}_2\text{O}$ (25 mCi/gm), were obtained from New England Nuclear (Boston). Nitrendipine was obtained from Miles Laboratories, Inc. (New Haven, NJ). TTX, quin-2, and its acetoxymethyl tetraester, quin-2AM, were obtained from Sigma Chemical Co. (St. Louis). All other chemicals were of reagent grade.

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Figure 1. Schematic of quench-flow (QF) procedure. Syringe (Syr 1) suspension of synaptosomes in HKR; Syr 2, low- or high- K^+ -HKR and ^{45}Ca ; Syr 3, 30 mM EGTA-quench solution. Activation of stepping motor drives syringe pistons and initiates the flow and mixing of solutions in 1:1:1 volume ratios at precisely controlled times. Berger ball mixers provide rapid mixing and allow measurements of reaction rates with half-lives of less than 5 msec. The uptake time is determined by the transit time of reaction medium through the aging coil. Quenched reaction medium is collected in a test tube and synaptosomes are immediately separated from the medium by filtration. For detailed description of QF apparatus, see Froehlich et al. (1976).

Media

Standard suspension medium was a HEPES-buffered Krebs-Ringer (HKR) solution composed of 140 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 10 mM glucose, and 5 mM Na-HEPES, pH 7.4. Na^+ -deficient solutions were made by isosmotic substitution of NaCl by choline chloride (Ch-HKR). The depolarizing media were made by isosmotically substituting KCl for NaCl (or for ChCl in Na^+ -deficient media), in proportions such as to yield after mixing with synaptosomes, 52.5 mM K^+ and 92.5 mM Na^+ in the case of Na^+ -containing media, and 52.5 mM K^+ and 92.5 mM Ch $^+$ in the case of Na^+ -deficient media. The nondepolarizing, low K^+ -HKR media were modified by substituting equivalent concentrations of LiCl or ChCl for NaCl so that after mixing with synaptosomes the final concentrations in the basal conditions were 5 mM K^+ , 92.5 mM Na^+ , and 47.5 mM Ch $^+$ or Li $^+$. In the case of nondepolarizing, Na^+ -deficient solutions, either LiCl substitution was used to obtain 5 mM K^+ , 92.5 mM Ch $^+$, and 47.5 mM Li $^+$, or Ch-HKR was used directly without further modifications. EGTA-quench solutions were made in HKR or Ch-HKR. Depending on the procedure, these solutions contained either 20 or 30 mM EGTA, with NaCl or ChCl appropriately reduced to maintain isosmolality. The EGTA-wash solutions contained 1 mM EGTA in standard HKR. All solutions were oxygenated prior to experiments and maintained at pH 7.4.

Preparation of synaptosomes

Synaptosomes were isolated from cerebral cortices of Sprague-Dawley rats (175–200 gm body weight) by the method of Gray and Whittaker (1962) as described by us previously (Suszkiw and O'Leary, 1983). After purification in sucrose density gradients, synaptosomes were slowly diluted with 10 vol of HKR or Ch-HKR solutions, pelleted at $10,000 \times g$ for 20 min and resuspended in the respective medium. All preparative

steps were performed at 4°C. All subsequent measurements were done at 30°C after preincubating synaptosomes at this temperature for 10–20 min. Synaptosomal protein was determined by the procedure of Lowry et al. (1951) with crystalline bovine albumin as the standard.

Measurements of ^{45}Ca uptake

A Froehlich-Berger Chemical Quench Flow (QF) apparatus (Commonwealth Technologies Inc., VA) was used for measurements of ^{45}Ca uptake between 0.05 and 1 sec reaction time. In this procedure (Fig. 1), uptake reaction was initiated by mixing synaptosomes (syringe 1) with high- or low- K^+ medium, which contained various concentrations of $CaCl_2$ and ^{45}Ca tracer (syringe 2). After variable reaction times, determined by the transit time through the "aging coil," uptake was terminated with 30 mM EGTA-quench solution (10 mM EGTA after mixing; delivered from syringe 3). Aliquots (0.8 ml) of the quenched samples were rapidly filtered through 0.65 μm Millipore (type DAWP 025) filters held under suction in a Swinex filter holder modified to allow easy access to the filter. The filters were rapidly rinsed $3 \times$ each with 5 ml of EGTA-wash solution, air-dried, and placed in scintillation vials containing 1 ml of 1% Triton X-100 in 0.1 N HCl. After about 20 min elution, 10 ml of scintillation fluor in toluene-Triton X-100 [2:1, vol/vol; 4 gm 2,5-diphenyloxazole, and 0.05 gm 1,4-bis-(5-phenyloxazolyl) benzene/L-toluene] was added, and the radioactivity was measured in a Beckman LS9000 scintillation spectrometer in the quench correction mode. In a typical experiment, 14–16 measurements could be obtained with synaptosomes prepared from three rat brains. The concentration of synaptosome protein employed was about 1 mg/ml of suspension. Depending on the total concentration of Ca, 1–10 μCi ^{45}Ca tracer/ml of reaction medium was used.

Manual procedure was used in the measurements at times 1 sec or longer. In this procedure, 450 μl of either low- or high- K^+ -HKR medium, containing the desired concentration of $CaCl_2$ and ^{45}Ca tracer (1–10 μCi /ml) was rapidly injected into a test tube containing 50 μl of synaptosome suspension (0.2–0.5 mg protein), and after various time intervals the uptake reaction was stopped by injecting 0.5 ml of 20 mM EGTA-quench solution. Aliquots (0.8 ml) of the samples were immediately filtered, and the filters were processed exactly as described for the QF experiments. The concentrations of Na^+ and K^+ in low- and high- K^+ media were adjusted to provide after mixing with synaptosomes exactly the same ionic composition as in QF experiments. The injections of media were timed with electronic timer calibrated in 1/100th sec divisions (A.W. Haydon Co.) and were made with 1 ml tuberculin syringes fitted with 7.5 cm long, 20 gauge stainless steel needles. The needles were secured to stoppers in 15×100 mm polystyrene test tubes. A rapid depression of syringe plunger delivered a jet of liquid causing a turbulent mixing of the reaction media. We have confirmed that this manual method for measuring Ca uptake yielded results that were comparable to within 10% of those obtained by the quench-flow procedure.

Net ΔK^+ -stimulated influx of Ca was calculated after subtracting ^{45}Ca uptake by synaptosomes in 5 mM K^+ from uptake in 52.5 mM K^+ . The results were expressed as nmol Ca/mg protein, on the basis of specific radioactivity of ^{45}Ca in the medium.

Time course of Ca channel inactivation and recovery from inactivation

Synaptosomes were "predepolarized" by mixing 50 μl of synaptosome suspension with 450 μl of high K^+ -HKR as described for uptake experiments, except that in this case Ca was omitted from the high- K^+ medium. After 1–30 sec of predepolarization, 1 ml of 52.5 mM K^+ -HKR containing ^{45}Ca was injected into the reaction medium to initiate the uptake reaction. Uptake was terminated after 1 sec by quenching the reaction with 1.5 ml of 20 mM EGTA-quench solution. Aliquots of quenched samples were immediately filtered and processed as described in Ca uptake experiments. Reactivation of the channels was measured as follows. In order to inactivate Ca channels, synaptosomes were first predepolarized for 10 sec in 52.5 mM K^+ -HKR by mixing 25 μl aliquots of synaptosomes with 25 μl of 100 mM K^+ -HKR solution. At the end of predepolarization, the mixture was rapidly diluted 10.5-fold by injecting 475 μl of appropriately modified K^+ -free HKR to reduce K^+ concentration back to 5 mM, without otherwise altering the composition of the reaction medium. After various periods of recovery in 5 mM K^+ -HKR, the channel activity was assayed by injecting into the suspension 1 ml of high- K^+ -HKR- ^{45}Ca medium, so that after mixing, the concentration of K^+ was restored to 52.5 mM and the concentrations of other

ions remained unchanged. After 1 sec incubation, uptake of ^{45}Ca was stopped by injecting 1.5 ml of 20 mM EGTA-quench solution. Aliquots of quenched reaction mixtures were rapidly filtered through the Millipore filters, which were then handled in the usual way. The background uptake was measured in parallel by exposing synaptosomes to low (5 mM K^+) solutions in place of 52.5 mM K^+ . After subtracting the background uptake, the ΔK^+ -dependent ^{45}Ca uptake was expressed as a fraction of the control uptake in synaptosomes at zero-time of the predepolarization–repolarization sequence.

Equilibrium binding of ^3H -nitrendipine

Equilibrium binding of ^3H -nitrendipine (NTD) was performed essentially as described by Rampe et al. (1984). Synaptosomes (approx. 200–400 μg protein) were incubated 60 min at 4°C in 5 ml Ch-HKR containing 0.02–1.5 nM ^3H -NTD. Nonspecific binding was assayed in parallel, in the presence of excess (1 μM) unlabeled NTD. The drug was made in absolute ethanol, and the final concentration of ethanol in the incubation mixtures was 0.1%. All manipulations were done under dark-room (dim yellow light) conditions; the incubations were carried out in total darkness. After incubation, 4.5 ml aliquots of suspensions were filtered through fibreglass, Whatman GF/F filters under gentle suction. The filters were washed with 15 ml (3×5 ml) of cold Ch-HKR and counted in toluene–Triton X-100 fluor as described for Ca uptake experiments. The remaining 0.5 ml portions of the incubation media were centrifuged 20 sec at $12,000 \times g$ in a Fisher microfuge, and aliquots of the supernatants were counted to determine the equilibrium concentrations of free ^3H -NTD in the media.

To determine effects of NTD on ΔK^+ -stimulated Ca entry, synaptosomes were preincubated at 0 – 4°C for 60 min with a total of 10 μM nitrendipine added, exactly as described for the equilibrium binding experiments. Control preparations were processed identically and contained 0.1% ethanol vehicle. After 60 min equilibration, synaptosomes were prewarmed for 10–20 min at 30°C , and ^{45}Ca uptake was measured by means of the quench-flow apparatus.

Determination of Na^+ and K^+

The Na^+ and K^+ content of synaptosomes was determined in a Perkin-Elmer 3030 Atomic Absorption Spectrophotometer. Aliquots (250 μl , approx. 1 mg protein) of synaptosome suspensions preequilibrated in HKR or Ch-HKR were filtered through 0.65 μm Millipore filters. The filters were washed with 25 ml of Na^+ - and K^+ -free, isosmotic Ch-HKR buffered with 10 mM HEPES–Tris, pH 7.4. After air-drying, the filters were extracted with 1 ml of 0.1 N HCl, and aliquots of the extracts were assayed for Na^+ and K^+ against standard solutions of NaCl and KCl in 0.1 N HCl. Filter blanks were subtracted from the readings.

Determination of intrasynaptosomal water space

Suspensions of synaptosomes in HKR or Ch-HKR (200 μl , approx. 0.4 mg protein) were incubated with approximately 0.4 μCi $^3\text{H}_2\text{O}$ and 0.1 μCi of ^{14}C (U) sucrose. After a 10–30 min equilibration, synaptosomes were pelleted for 2 min at $12,000 \times g$ in a Fisher microfuge. The pellets were separated from the supernatants and lysed overnight in 0.5 ml of 1% Triton X-100/0.1 N HCl. The ^3H and ^{14}C counts in the pellets and supernatants were measured in Beckman LS 9000 scintillation spectrometer optimized for double-label counting. The water space of synaptosomes was calculated after subtracting the $^3\text{H}_2\text{O}$ associated with the ^{14}C sucrose space from total $^3\text{H}_2\text{O}$ counts in the pellets.

Determinations of free Ca , with fluorescent calcium indicator Quin 2

The procedure was based on the method of Tsien et al. (1982) essentially as described for synaptosomes by Ashley et al. (1984).

Data analysis

A nonlinear least-squares regression program BMDP P3R (Jennrich, 1983) was used for curve fitting.

Results

Synaptosomes have a number of Ca permeability pathways that could interfere with the measurement of Ca flux through the specific Ca channels. The influx of Ca through voltage-sensitive Na channels in K^+ -depolarized synaptosomes can be excluded, since these channels inactivate in a matter of a few milliseconds

and we have confirmed that TTX (1 $\mu\text{g}/\text{ml}$) was without effect on the measured influx of ^{45}Ca (Nachshen and Blaustein, 1980). More problematic is the entry of Ca via the Na/Ca exchange carrier. Since synaptosomes tend to accumulate significant amounts of Na^+ (Campbell, 1976; Coutinho et al., 1984; Marchbanks and Campbell, 1976), this may lead to the reversal of the Na/Ca exchange that normally serves to extrude Ca from nerve terminals (Blaustein and Oborn, 1975; Mullins, 1976). Additional factors that must be considered in connection with high internal Na^+ in synaptosomes are the likelihood of partial depolarization of synaptosomes and increase in steady-state concentrations of free Ca_i in synaptoplasm due to enhanced entry of Ca from outside, as well as displacement of Ca from the intraterminal mitochondria (Carafoli and Crompton, 1978). Since inactivation of Ca channels may have both voltage- and Ca_i -dependent components, partial depolarization and elevation in Ca_i may lead to progressive inactivation of channels in synaptosomes.

In order to assess the potential complications arising from accumulation of Na^+ in synaptosomes, we measured cation distribution in synaptosomes. Also, in some experiments we employed synaptosomes with low internal Na^+ concentration, obtained by suspending them in Na^+ -deficient Ch-HKR. Furthermore, to prevent unpredictable alterations in the levels of free Ca_i in synaptosomes, we elected to omit Ca from the preincubation media. Finally, to minimize time-dependent variables among preparations, we routinely carried out all experiments within 45–60 min after suspension of synaptosomes in salt solutions and limited preincubations to 10–20 min.

Concentrations of intrasynaptosomal Na^+ , K^+ , and Ca^{2+} in Na^+ -loaded and Na^+ -deficient preparations

Table 1 compares the concentrations of Na^+ and K^+ ions in synaptosomes suspended in standard, Na^+ -rich media (Na-syn) and in Na^+ -deficient, choline media (Ch-syn). The indicated redistribution of ions in synaptosomes apparently occurred during transfer of synaptosomes from sucrose to saline media at 0°C . There was relatively small additional change in Na^+ and K^+ contents during subsequent 30 min incubation at 30°C , indicating that at least with respect to Na^+ and K^+ distribution, synaptosomes were in steady state during this period of time. Essentially, all Na^+ and K^+ were associated with the osmotically sensitive compartment in synaptosomes, as osmotic lysis of synaptosomes resulted in readings that were equal to the background levels of Na^+ and K^+ obtained from filters alone.

Synaptosomes in electron micrographs consist of morphologically intact nerve ending particles and vesiculated membranes devoid of cytoplasmic matrix and organelles. We have estimated in electron micrographs of our preparations the mean volume fraction of nerve endings and empty vesicles at about 80 and 20%, respectively. The intrasynaptosomal water spaces measured with $^3\text{H}_2\text{O}$ were $3.4 \pm 0.4 \mu\text{l}/\text{mg}$ protein in Na-syn and $3.0 \pm 0.4 \mu\text{l}/\text{mg}$ protein in Ch-syn (means $\pm$ SD, $n = 6$), which after correction for the volume fraction of empty vesicles gives 2.7 and 2.4 $\mu\text{l}/\text{mg}$ for the volume of morphologically intact synaptosomes in the respective preparations. With the simplifying assumptions that empty vesicles are metabolically inert and passively equilibrate with Na^+ , K^+ , and Cl^- in the medium, and that synaptosomes can be treated as an “averaged” single compartment, we calculated the intrasynaptosomal concentrations of Na^+ and K^+ ions according to

$$[X]_{\text{syn}} = (X_{\text{T}} - [X_0]V_{\text{v}})/V_{\text{syn}} \quad (1)$$

where X is either Na^+ or K^+ , $[X_0]$ and $[X]_{\text{syn}}$ are molar concentrations of ion X in the medium and synaptosomes, respectively, V_{v} is the volume of inert vesicles (0.7 and 0.6 $\mu\text{l}/\text{mg}$ protein in Na-syn and Ch-syn preparations, respectively), and V_{syn} is the volume of nerve ending particles (2.7 and 2.4 $\mu\text{l}/\text{mg}$ protein for

Table 1. Intrasynaptosomal concentrations of cations

Incuba- tion (min)	Content ($\mu\text{mol}/\text{mg}$ protein)		Concentration (mM)		Membrane potential (mV)
	Na ⁺	K ⁺	Na ⁺	K ⁺	
Na-syn					
0	0.309 $\pm$ 0.075	0.209 $\pm$ 0.058	77 $\pm$ 20	77 $\pm$ 21	—
10	0.332 $\pm$ 0.030	0.223 $\pm$ 0.025	85 $\pm$ 8	83 $\pm$ 9	−51
20	0.369 $\pm$ 0.048	0.234 $\pm$ 0.028	99 $\pm$ 13	87 $\pm$ 10	−52
30	0.382 $\pm$ 0.088	0.232 $\pm$ 0.038	104 $\pm$ 24	86 $\pm$ 14	−52
Ch-syn					
0	0.014 $\pm$ 0.008	0.293 $\pm$ 0.054	6 $\pm$ 3	122 $\pm$ 22	—
10	0.010 $\pm$ 0.003	0.287 $\pm$ 0.064	4 $\pm$ 1	120 $\pm$ 27	−83
20	0.014 $\pm$ 0.005	0.263 $\pm$ 0.045	6 $\pm$ 2	109 $\pm$ 19	−80
30	0.013 $\pm$ 0.006	0.236 $\pm$ 0.036	5 $\pm$ 2	98 $\pm$ 15	−78

Incubations were at 30°C; zero time: synaptosomes at 4°C. The results are means $\pm$ SD; $n = 4$. Cation concentrations were calculated on basis of intrasynaptosomal $^3\text{H}_2\text{O}$ spaces, corrected for volume fraction of contaminating structures (see text for details). Membrane potential was estimated according to the constant-field equation with the Cl^- term omitted.

Na-syn and Ch-syn, respectively). The membrane potentials were estimated from the reduced constant-field equation:

$$V_{m,30^\circ} = 60 \text{ mV} \log \frac{P_{\text{Na}}/P_{\text{K}}[\text{Na}]_o + [\text{K}]_o}{P_{\text{Na}}/P_{\text{K}}[\text{Na}]_i + [\text{K}]_i} \quad (2)$$

with $P_{\text{Na}}/P_{\text{K}} = 0.05$ (Blaustein and Goldring, 1975; Keen and White, 1971).

The gain of Na^+ suggests a deficient Na,K-ATPase -driven extrusion of this ion and, by extension, an impairment of metabolic function of isolated nerve endings. It is likely that isolated nerve endings may exhibit a range of metabolic states, internal ionic concentrations, and resting membrane potentials. Thus, the assumption of a single "average" compartment used in the foregoing calculations may be questioned. Nevertheless, it may be concluded that *on the average*, synaptosomes equilibrated in standard Na-rich media are Na-loaded and may be depolarized by some 30 mV relative to Na-deficient synaptosomes equilibrated in choline media.

The measurements of free Ca_i with Quin-2 incorporated into synaptosomes indicated low concentrations of ionized Ca_i in both types of preparations. In the absence of Ca^{2+} in the suspension media, the mean ($\pm$ SD) concentrations of Ca_i determined in six preparations were 28.2 ± 8.7 nM in synaptosomes suspended in HKR and 32.4 ± 4.0 nM in synaptosomes in Ch-HKR. Addition of 1 mM Ca^{2+} to the media, resulted in a new steady-state concentration of Ca_i of 77.8 ± 16.7 nM in Na-syn and 71.4 ± 18.4 nM in Ch-syn. These values are similar to 104 ± 8 and 87.5 ± 10 nM Ca_i reported by Richards et al. (1984) and Ashley et al. (1984), respectively.

The maintenance of low Ca_i in Na-loaded synaptosomes may be surprising at first. However, in 140 mM Na_o^+ , the estimated $[\text{Na}_i^+]$ in synaptosomes is ~ 80 – 100 mM (Table 1), so under these conditions extrusion of Ca via Na/Ca carrier may be still operative. On the other hand, lowering Na_o^+ about 90 mM or less by substituting external Na^+ with Li^+ or Ch^+ leads to an increase in steady-state concentrations of Ca_i in Na-syn, and this effect is magnified in the presence of metabolic inhibitors (Ashley et al., 1984; J. B. Suszkiw, unpublished observations). In conclusion, assuming that Quin-2 monitors, rather than sets, free Ca_i concentrations, these measurements suggest that both Ch- and Na-syn are competent in maintaining the steady-state ionized Ca_i at the submicromolar concentrations.

Influx of Ca as a function of time

These experiments compared ^{45}Ca influx in Na-syn and Ch-syn between 0.05 and 5 sec of incubation in low K^+ (5 mM) and

high K^+ (52.5 mM) media with $\text{Ca}_o = 1$ mM (Fig. 2). The basal rate of Ca uptake was 0.21 nmol/sec/mg protein in Na-syn and 0.05 nmol/sec/mg protein in Ch-syn. The higher rate of Ca uptake in Na^+ -loaded synaptosomes in conditions of lowered Na_o^+ (92.5 mM) is consistent with "reversed" Na/Ca exchange, which is inoperative in Na^+ -deficient Ch-syn. Depolarization of synaptosomes in 52.5 mM K^+ resulted in a large, transient augmentation of Ca uptake. In Ch-syn this initial increase in the rate of Ca influx subsided to nearly basal rate after about 1 sec of K^+ depolarization, but in Na-syn the uptake remained somewhat enhanced relative to the basal rate. After subtracting the basal from the K^+ -stimulated Ca uptake, net ΔK^+ -dependent influx of Ca was obtained (Fig. 3). In the case of Ch-syn, ΔK^+ -dependent influx of Ca followed a single-exponential time course and was fitted to the function $j = P(1 - e^{-kt})$, where j is the Ca^{2+} influx measured at times t , P is the plateau value of Ca cumulation, and k is the observed first-order rate constant. Nonlinear least-squares regression of the data gave $k = 1.3 \pm 0.1 \text{ sec}^{-1}$ and $P = 3.02 \pm 0.15 \text{ nmol}/\text{mg}$ protein. In contrast, Ca influx in Na-syn could best be described by a sum of exponential and linear terms: $j = P(1 - e^{-k_1t}) + k_2t$. The second-order rate constant, k_2 , was evaluated by measuring ^{45}Ca influx in synaptosomes after the exponential phase had been abolished (inactivated) by prepolarizing synaptosomes for 5 sec. The rate of ΔK^+ -dependent Ca influx after inactivation of the channels was $0.17 \pm 0.01 \text{ nmol}/\text{sec}/\text{mg}$ protein. The least-squares fit of the data to equation $j = P(1 - e^{-kt}) + 0.17t$ gave $k_1 = 1.0 \pm 0.1 \text{ sec}^{-1}$ and $P = 3.3 \pm 0.1 \text{ nmol}/\text{mg}$ protein for the exponential phase of Ca influx. Interestingly, despite the overall fit of the data to a two-component model of Ca entry in Na-syn, we noted that the earliest time points (i.e., measurements at 49.5, 110.5, and 215 msec) showed a tendency to deviate from the calculated curve and were lower than the values obtained with Ch-syn. At present we have no satisfactory explanation for this observation. However, it is possible that in partially depolarized Na-syn a fraction of channels are inactivated and fewer channels are available for the opening by subsequent high- K^+ challenge. The resultant depression of Ca influx would be most evident at the early time points, when the Na^+ -dependent Ca entry makes relatively negligible contribution.

Kinetics of voltage-dependent channel inactivation and reactivation

The earlier observations that prepolarization of synaptosomes in high- K^+ media abolished the early phase of Ca entry (Nachshen, 1985; Nachshen and Blaustein, 1980; Suszkiw and O'Leary, 1983) suggested that voltage-dependent inactivation of

Figure 2. Time course of ^{45}Ca uptake. Uptake was measured in Na^+ -loaded (NA-SYN) and Na^+ -deficient (CH-SYN) synaptosomes between 0.05 and 5 sec of incubation. Data are means $\pm$ SD of 4–9 determinations. For details of incubation conditions, see text.

Ca^{2+} channels might account for the time course of Ca entry into synaptosomes. To gain further insight into the relationship between channel inactivation and the decay of Ca influx, we have measured the decay of channel activity as a function of the duration of prepolarization in 52.5 mM K^+ , nominally Ca -free media. Under these conditions, free Ca_i in synaptosomes (monitored with Quin 2) was maintained at ~ 50 nM. The time course of channel inactivation (Fig. 4, left) was fitted to the function $Q_{\text{inact}} = q_1 e^{-k_i t} + q_2$, where Q_{inact} is the fractional ^{45}Ca uptake at time t of prepolarization, q_1 is the fraction of channels undergoing exponential inactivation, k_i is the observed rate constant of this process, and q_2 is the residual fraction of Ca entry showing no inactivation within the time range of these measurements. A nonlinear least-squares regression of the data obtained with Na-syn yielded $q_1 = 0.91 \pm 0.01$, $q_2 = 0.09 \pm 0.01$, and $k_i = 0.29 \pm 0.04 \text{ sec}^{-1}$ ($t_{1/2} \approx 2.4$ sec). These data suggest that 90% of ΔK^+ -dependent Ca influx in Na-syn occurs via the channels that show single-exponential inactivation kinetics. The residual q_2 fraction ($\sim 10\%$) is attributed to the Na -dependent fraction of Ca entry. It may be noted that the rate of channel inactivation in 52.5 mM K^+ solutions is considerably slower than the rate at which channel-mediated Ca entry terminates ($t_{1/2} \sim 0.5$ – 0.7 sec).

In these experiments, inactivation was measured in synaptosomes that were initially equilibrated in standard HKR solution (140 mM NaCl), and Na^+ was lowered to 92.5 mM only in the course of depolarization. We ascertained that this had no effect on the measurements in a separate series of experiments in which synaptosomes were originally suspended in media in which NaCl was reduced to 92.5 mM by isotonic substitution with ChCl ; thus, Na^+ concentration remained constant throughout the experiment, i.e., commencing with exchange of sucrose for the indicated medium. The data obtained with these preparations (triangles in Fig. 4) gave similar parameter estimates: $q_1 = 0.87 \pm 0.02$, $q_2 = 0.12 \pm 0.02$, and $k_{\text{inact}} = 0.29 \pm 0.02$, indicating that transient lowering of $[\text{Na}^+]_o$ had no effect on the

Figure 3. Time course of ΔK^+ -dependent influx of Ca in NA-SYN and CH-SYN. ΔK^+ -dependent influx is ^{45}Ca uptake in 52.5 mM K^+ minus uptake in 5 mM K^+ solutions. The regression curves obtained by the nonlinear least-squares method of data fitting describe equations $j = 3.0(1 - e^{-1.3t})$ for CH-SYN and $j = 3.3(1 - e^{-1.0t}) + 0.17t$ for NA-SYN. The observed half-lives are $t_{1/2} \approx 0.5$ sec and ≈ 0.7 sec in CH- and NA-SYN, respectively.

measurements. Also, since presumably the internal concentration of Na^+ was lower in these synaptosomes, it suggests that inactivation process is not related to Na_i^+ . To exclude a possible effect of Cl^- redistribution on channel activity, we replaced NaCl and KCl with the corresponding methylsulfate salts. The removal of Cl^- from the external medium likewise had no significant effect on the observed decline in channel activity (data not shown).

In the next series of experiments, we measured the rate of channel recovery from inactivation after prepolarized syn-

Figure 4. Time course of voltage-dependent Ca channel inactivation and recovery (reactivation). Inactivation (left side) was measured in NA-SYN prepolarized in Ca -deficient, 52.5 mM K^+ media. ●, Data obtained with synaptosomes preequilibrated in standard, Na^+ -rich HKR are means $\pm$ SD of 3–16 determinations. △, Data obtained with synaptosomes preequilibrated in HKR with reduced Na^+ (92.5 mM Na^+ , 47.5 mM Ch^+) are means $\pm$ SD of 4–8 determinations (for clarity, SD bars have been omitted). Q is fractional Ca influx at times t , relative to control influx at time zero of prepolarization. The regression curve obtained by nonlinear least-squares fitting of the data describes the equation $Q_{\text{inact}} = 0.91e^{-(0.29 \text{ sec}^{-1})t} + 0.09$. The estimated half-life of the channel inactivation is $t_{1/2} \approx 2.4$ sec. Reactivation of channels (right side) was measured after returning prepolarized (10 sec in 52.5 mM K^+) synaptosomes to nondepolarizing 5 mM K^+ conditions. Data are means $\pm$ SD of 4–8 determinations. Q is fractional Ca influx relative to control at time zero of prepolarization–repolarization sequence. The regression curve obtained by the nonlinear least-squares data fitting describes equation $Q = 0.18 \pm 0.63(1 - e^{-0.16t})$. The estimated half-life of recovery is $t_{1/2} = 4.3$ sec.

Figure 5. Initial rates of Ca influx as function of $[Ca^{2+}]_o$. Data are means $\pm$ SD of 4–7 determinations. Nonlinear least-squares fit of the data to Michaelis-Menten equation $J_{Ca} = J_{Ca}^{max} [Ca^{2+}]_o / (K_{Ca} + [Ca^{2+}]_o)$, gave $J_{Ca}^{max} = 2.44 \pm 0.09$ nmol/415.5 msec/mg protein, and $K_{Ca} = 1.48 \pm 0.09$ mM.

aptosomes were returned to resting, 5 mM K^+ conditions. The time course of channel reactivation (Fig. 4, right) was fitted to the equation $Q_{rec} = q_0 + q_1 (1 - e^{-kt})$, where Q_{rec} is the observed fractional channel activity at various times t after return of synaptosomes to the nondepolarizing medium, q_0 is the fraction of noninactivated Ca entry after a 10 sec prepolarization of synaptosomes (this parameter is essentially equivalent to q_2 in the Q_{inact} equation), q_1 is the fraction of channels that recover, and k_r is the observed rate constant of recovery. Nonlinear regression of the data yielded $q_0 = 0.18 \pm 0.05$, $q_1 = 0.63 \pm 0.06$, and $k = 0.16 \pm 0.04$ sec $^{-1}$ ($t_{1/2} = 4.3$ sec). The sum $q_0 + q_1$ indicates that about 81% of the control uptake activity was recovered during the first minute of rest. During an additional 1–2 min, Ca uptake returned to nearly control values (data not shown), but there was considerable variability among preparations. A possible explanation for this variability is that a fraction of synaptosomes became functionally impaired in the course of these experiments and did not completely repolarize or did so only slowly following restoration of 5 mM K^+ conditions.

Influx of Ca as a function of $[Ca]_o$

The relationship between the initial rates of Ca influx and external concentration of Ca_o (Fig. 5) was satisfactorily fitted to the Michaelis-Menten type equation:

$$J_{Ca} = (J_{Ca}^{max} [Ca]_o) / (K_{Ca} + [Ca]_o) \quad (3)$$

where J_{Ca} is the measured Ca influx at various concentrations of Ca in the medium, J_{Ca}^{max} is the maximal rate of influx at saturating concentration of Ca^{2+} , and K_{Ca} is the external concentration of Ca at which $J_{Ca} = J_{Ca}^{max}/2$. The initial rates of Ca influx in Na-syn were measured with Ca_o concentrations ranging from 0.1 to 10 mM. Uptake of ^{45}Ca was measured during 415.5 msec of depolarization by 52.5 mM K^+ and was corrected for the basal Ca uptake in 5 mM K^+ . Nonlinear least-squares regression analysis of the data gave $J_{Ca}^{max} = 5.9 \pm 0.2$ nmol/sec/mg protein, and $K_{Ca} = 1.5 \pm 0.1$ mM.

The average surface-to-volume ratio estimated from electron micrographs of synaptosomes is 83 cm 2 /μl (Marchbanks and Campbell, 1976). The synaptosomal surface area in our preparations calculated from corrected 3H_2O space of 2.7 μl/mg was about 2×10^{10} μm 2 /mg protein. Assuming that accumulation of ^{45}Ca in synaptosome during the 415 msec incubations represents a net unidirectional flux (e.g., see Nachshen, 1985; Nachshen and Blaustein, 1980), J_{Ca}^{max} corresponds to the flux of 1.7×10^5 Ca ions/sec/μm 2 synaptosomal surface area.

Figure 6. Scatchard plot of equilibrium binding of 3H -nitrendipine to synaptosomes. Experiments were done with CH-SYN in Na-deficient media, with nitrendipine concentrations in the range of 0.04–1.5 nM. The data points have been corrected for nonspecific binding in the presence of 1 μM nitrendipine and are from six preparations. The least-squares analysis of the data gave $B_{max} = 203.5 \pm 42$ (SD) fmol/mg protein and $K_D = 0.12 \pm 0.08$ nM.

Binding of NTD to synaptosomes and effect of NTD on Ca influx

The purpose of these experiments was to reexamine the relationship of NTD binding sites to Ca channels in synaptosomes. In order to avoid possible complications associated with the presence of Na^+ (Turner and Goldin, 1985), we measured equilibrium binding of 3H -NTD and the effect of NTD on ΔK^+ -dependent ^{45}Ca influx in Ch-syn in choline media. The equilibrium binding of 3H -NTD was examined between 0.03 and 1.5 nM concentration of the drug and was corrected for nonspecific binding in the presence of 1 μM unlabeled NTD. The Scatchard plot of the data in Figure 6 indicates the presence of a single class of high-affinity binding sites with $B_{max} = 203.5 \pm 42$ fmol 3H -NTD sites/mg protein and $K_D = 0.12 \pm 0.08$ nM. Figure 7 shows that binding of NTD to Ch-syn in Ch-HKR was not significantly altered in the presence of 52.5 mM K^+ in the medium but was slightly depressed in 1 mM Ca_o . A combination of high K^+ and Ca_o had no additional effect on binding of NTD to synaptosomes. Having established that conditions employed in ^{45}Ca flux measurements did not substantially decrease in NTD binding, we examined the effects of NTD on the K^+ -stimulated influx of Ca in synaptosomes pre-equilibrated with the drug (10 μM total added, ~ 2.5 μM free NTD) for 60 min under conditions used in binding experiments. As the results in Figure 8 indicate, preincubations of synaptosomes with NTD had no significant ($p \geq 0.2$; t test) effect on the K^+ -stimulated influx of Ca measured during 215.5 msec depolarization with either 1 or 0.1 mM Ca_o .

Discussion

The main conclusion of these experiments is that although synaptosomes comprise heterogeneous populations of nerve endings, the kinetics of voltage-dependent Ca influx are consistent with a single compartment containing a homogeneous population of voltage-dependent Ca channels. In several respects, our results of fast-kinetic measurements of Ca entry in synaptosomes are in substantial agreement with a similar study recently reported by Nachshen (1985).

Time course of phasic Ca influx

A single-exponential, rapidly decaying influx of Ca is most clearly demonstrated in Na-deficient preparations, i.e., Ch-syn. In 1 mM Ca_o^{2+} , the half-time for the termination of ΔK^+ -dependent Ca entry is ~ 0.5 sec, and after 1–2 sec of depolarization there is little additional ^{45}Ca accumulation in K^+ -depolarized syn-

Figure 7. Effect of 52.5 mM K^+ and 1 mM Ca^{2+} on binding of 3H -nitrendipine. Binding was assayed with 0.17 nM 3H -nitrendipine in Ch-HKR. Control conditions were the same as in Figure 6, i.e., 5 mM K^+ and no Ca^{2+} . Incubations were carried out for 60 min at 4°C. Results are means $\pm$ SD of three experiments.

aptosomes. In agreement with the earlier observations of Nachshen and Blaustein (1980, 1982), the entry of Ca into synaptosomes in Na media (Na-syn) can be resolved into fast-exponential ($t_{1/2} \sim 0.7$ sec) and slow linear phases. The fast-exponential Ca entry is consistent with passive influx of Ca into nerve endings through voltage-sensitive channels that inactivate in the course of depolarization (Nachshen, 1985). The presence of slow ΔK^+ -stimulated, linear Ca entry component in Na-syn but not in Ch-syn, suggests a carrier-mediated, membrane-potential-sensitive (electrogenic) Na/Ca exchange (Coutinho et al., 1984; Turner and Goldin, 1985).

Kinetics of channel inactivation and recovery

The first indication that Ca channels inactivate during prolonged electrical or K^+ depolarization was provided by Baker et al. (1973) in the squid giant axons. That channels in synaptosomes inactivate in the course of K^+ depolarization was first shown by Nachshen and Blaustein (1980, 1982) and later confirmed by us (Suszkiw and O'Leary, 1983). More recent data of Nachshen (1985) provide a further indication that the decay of Ca entry in synaptosomes may be primarily a voltage-dependent process, but the involvement of Ca has also been implicated by the observation that Ca influx decays faster at higher Ca_o concentrations. Assuming a homogenous population of Ca channels, inactivation should follow a simple exponential time course. In agreement with this supposition, our results show that during prepolarization of synaptosomes by 52.5 mM K_o^+ in Ca-deficient media, 90% of channel activity decays with first-order rate constant, $k_i \approx 0.3 \text{ sec}^{-1}$ ($t_{1/2} \sim 2.4$ sec). The rate of voltage-dependent channel inactivation in our preparations is similar to the estimated half-time, 1–2 sec, for inactivation of channels in synaptosomes depolarized by 77 mM K_o^+ in Nachshen's (1985) experiments. The somewhat faster rate of inactivation in 77 mM K_o^+ is consistent with the dependence of the rate of channel inactivation on the level of membrane depolarization (Nachshen, 1985; M. E. O'Leary and J. B. Suszkiw, unpublished observations).

The recovery of channels from inactivation is likewise a single-exponential process, with a half-life, $t_{1/2} = 4.3$ sec, although it should be pointed out that the actual rate of channel recovery could, in fact, be somewhat faster than indicated, since we cannot be sure that our measurements are not attenuated by the

Figure 8. Effect of nitrendipine on initial rate of ΔK^+ -dependent ^{45}Ca influx in CH-SYN. Synaptosomes were pre-equilibrated with 10 μM (2.5 μM free) nitrendipine for 60 min at 4°C. After prewarming synaptosomes to 30°C, influx of ^{45}Ca was assayed at 215.5 msec with 1.0 and 0.1 mM Ca_o . The results are means $\pm$ SD of three experiments, each performed in duplicate.

rate at which synaptosomes repolarize on their return to 5 mM K^+ media. Similar rates of channel inactivation and reactivation might be expected in the case of a purely voltage-dependent process.

Comparison of the half-times for the decay of Ca influx ($t_{1/2} \approx 0.5$ – 0.7 sec) and voltage-dependent channel inactivation ($t_{1/2} \approx 2.4$ sec) indicates that the latter process is too slow to account for the more rapid decline of Ca entry into synaptosomes. At present it cannot be ruled out that the rapid decay of Ca entry may reflect a combination of factors associated with an increase in Ca_i , namely, a decrease in electrochemical driving force, increased Ca efflux, and, possibly, direct block of Ca channels by Ca_i . An alternative explanation may be that Ca entry either facilitates the voltage-dependent channel inactivation or promotes a distinct Ca_i -dependent inactivation process. Evidence for dual voltage and Ca_i -dependent channel inactivation has been obtained in snail neurons (Brown et al., 1981) and has also been implicated in cardiac tissue (Tsien, 1983). A Ca_i -facilitated channel inactivation ($\tau = 1.5$ sec) has been demonstrated recently in the voltage-clamped (0 mV) giant axon terminals in the squid (Augustine and Eckert, 1984). Interestingly, the recovery from Ca_i -dependent inactivation in the squid ($\tau = 70$ sec) is much slower than in synaptosomes. Preliminary results suggest that recovery of synaptosomal Ca channels from inactivation induced by depolarization of synaptosomes in medium containing 1 mM Ca_o is also much slower ($t_{1/2} \sim 1$ min) than the recovery from purely voltage-dependent inactivation. The functional state of cardiac Ca channels is regulated by cAMP-dependent phosphorylation (Reuter, 1983; Sperelakis, 1984). Evidence indicating that cAMP and ATP slows, and Ca_i promotes, channel inactivation has been obtained in perfused snail neurons (Dorochenko et al., 1982) and in the rat dorsal root neurons (Fedulova et al., 1981). It is thus an interesting possibility that the presynaptic Ca channels may be similarly modulated by phospho-dephosphorylation. An increase in Ca_i might stimulate channel dephosphorylation, possibly by stimulating Ca-regulated phosphatase activity. The rate of channel recovery would then reflect a slower rephosphorylation of channels by a cAMP-dependent mechanism. This hypothesis is presently being tested in synaptosomes.

Steady-state kinetic parameters of Ca influx: estimation of functional channel density in synaptosomes

The relation between unidirectional Ca influx (initial rates of Ca influx measured during 415 msec K^+ depolarization) and Ca_o fits the Michaelis-Menten type of equation and is consistent with the notion of a homogenous population of saturable Ca channels. In 52.5 mM K^+ ($\Delta K = 47.5$ mM), the apparent half-saturation (K_{Ca}) occurs at 1.5 mM Ca_o , i.e., the physiological range of Ca_o concentration. J_{Ca}^{max} , the limiting value of Ca influx at saturation is 5.9 nmol/sec/mg protein and corresponds to the flux of $\sim 1.7 \times 10^5$ Ca^{2+} ions/sec/ μm^2 or a current of ~ 0.06 pA/ μm^2 of synaptolemmal surface area.

The macroscopic Ca flux or current can be expressed as a product of the number of functional channels (N_f), single-channel current (j), and the probability of channel opening (p) (Tsien, 1983):

$$J_{max} = N_f j p. \quad (4)$$

Based on our estimates of intrasynaptosomal Na^+ and K^+ concentrations, the membrane potential in synaptosomes depolarized by 52.5 mM K_o^+ may be in the range -20 to ± 10 mV. At this level of membrane depolarization, and at saturating concentrations of Ca_o , single-channel currents estimated in snail neurons (Krishtal et al., 1981) and chromaffin cells (Fenwick et al., 1982) are about 0.1 pA. Assuming this value for Ca channels in synaptosomes, the minimum number (assumption of $p = 1$) of functional Ca channels in synaptosomes would be $0.6 \mu m^{-2}$. The probability of channel opening in K^+ -stimulated synaptosomes could be as low as 0.1. Thus, the number of functional Ca channels in synaptosomes may be 0.6 – $6 \mu m^{-2}$. This may be compared with channel density of $1 \mu m^{-2}$ in rat clonal pituitary cells (Hagiwara and Ohmori, 1982), 5 – $15 \mu m^{-2}$ in chromaffin cells (Fenwick et al., 1982), and 30 – $60 \mu m^{-2}$ in snail neurons (Krishtal et al., 1981). However, it should be noted that the estimated density of "functional" channels ignores the possibility that a substantial fraction of channels in synaptosomes may be in inactivated form and unavailable for opening.

Relation of NTD receptors to Ca channels

There are indications of several classes of neuronal Ca channels that differ in their sensitivity to Ca antagonists (Miller, 1985). In the chick dorsal ganglion neurons, three distinct types of Ca channels have been identified, but only one (the L-channel) is responsive to Ca channel agonist Bay K 8644 (Nowycky et al., 1985).

The presence of the high-affinity ($K_D \sim 0.12$ nM) 3H -NTD binding sites, but with a negligible effect of $>10^4 \times K_D$ concentrations of the drug on the K^+ -stimulated, channel-mediated Ca influx in our preparations of synaptosomes, suggests a dihydropyridine-insensitive type of Ca channels in nerve endings. This observation agrees with several previous reports (Daniell et al., 1983; Miller and Friedman, 1984; Nachshen and Blaustein, 1979; Rampe et al., 1984) but differs from the results of Turner and Goldin (1985), who demonstrated that $1 \mu M$ NTD inhibited Ca entry in their synaptosome preparations by about 30–40%.

One criticism (Turner and Goldin, 1985) of the earlier studies is that Ca uptake was measured during relatively long (e.g., 10 sec) incubation times. Since rapid inactivation of the channels appears to be related to changes in Ca_o , it is conceivable that a depression of Ca influx by a channel antagonist might slow the rate of channel inactivation. In the course of long (>1 sec) incubations, this might lead to a spurious result of a similar Ca accumulation in control and drug-treated preparations. Since in the present work we measured the initial rates of Ca influx during very short (215 msec) depolarization and tested the effect of the drug in normal, 1 mM, and reduced, 0.1 mM, Ca_o , this possibility

can be excluded. The failure to detect functional, dihydropyridine-sensitive Ca channels in the Gray and Whittaker (1962) preparation employed by us but apparently not in the Hajos (1975) preparation used by Turner and Goldin could be due to differences in the metabolic states of nerve endings in the two preparations. It has been suggested that the high-affinity binding sites for dihydropyridine Ca antagonists may reflect an inactive state of the Ca channels (Bean, 1984). Also, the results of Nowycky et al. (1985) indicate that the dihydropyridine-sensitive channels are more labile than the dihydropyridine-insensitive channels. With the foregoing considerations in mind, the discrepancy between our results and those of Turner and Goldin could be accounted for by assuming that the high-affinity 3H -NTD sites in our preparations correspond to a distinct class of inactivated, dihydropyridine-sensitive channels. Interestingly, the density of these putative channels, estimated from the $B_{max} \approx 200$ fmol NTD/mg protein and the surface area $2 \times 10^{10} \mu m^2$ /mg protein, would be about $6 \mu m^{-2}$, i.e., similar to the estimated density, 0.6 – $6 \mu m^{-2}$, of functional NTD-insensitive channels.

An alternative explanation for the apparent insensitivity of synaptosomal Ca channels to NTD may be that in our preparations the functional channels and/or the dihydropyridine receptors are in a conformation with a very low affinity for Ca antagonists (Bean, 1984; Nachshen, 1985). Finally, it cannot be completely ruled out that the NTD sites are, in fact, unrelated to Ca channels in nerve endings but rather are associated with the contaminating membranes. This would seem inconsistent with the inhibition by dihydropyridines of 3H -norepinephrine release reported by Turner and Goldin (1985). However, it may be noted that release of transmitters may also occur from dendrites, and there are indications that dendrites may contain the dihydropyridine-responsive type of Ca channels (Miller, 1985). Thus, it is conceivable that the presence of metabolically competent "dendrosomes" in Turner and Goldin's preparations but not in ours might be the source of the observed discrepancies between our results and theirs. Further studies comparing the channels and their responsiveness to dihydropyridines in the two types of synaptosome preparations may help to clarify some of these issues.

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