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# Rate of $Na^+/Ca^{2+}$ exchange across the plasma membrane of synaptosomes measured using the fluorescence of chlorotetracycline. Implications to calcium homeostasis in synaptic terminals

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**Abstract**

It is shown that the fluorescence of chlorotetracycline (CTC) can be used to continuously monitor $Ca^{2+}$ fluxes mediated by the $Na^+/Ca^{2+}$-exchanger of the plasma membrane of synaptosomes. The kinetics of $Ca^{2+}$ uptake can be followed from the kinetics of the increase of CTC fluorescence with external $Ca^{2+}$ concentrations in the micromolar range. Since the fluorescence of CTC is not sensitive to $Ca^{2+}$ concentration below 20 $\mu$M this avoids any significant contribution of $Ca^{2+}$ flux through $Ca^{2+}$ channels to CTC fluorescence. By replacing KCl by choline chloride in the buffer to avoid plasma membrane depolarization it is shown that the amplitude of the CTC fluorescence change is dependent upon the $Na^+$-gradient preimposed across the plasma membrane, and the rate constant of the kinetic process is dependent upon the $Ca^{2+}$ concentration. The rate constant of the $Ca^{2+}$ influx measured with depolarized and non-depolarized synaptic plasma membrane vesicles at 37°C and pH 7.4 were $0.55 \pm 0.10$ and $0.25 \pm 0.02$ $min^{-1}$, respectively. The overall rate of $Na^+/Ca^{2+}$ exchange calculated under conditions close to physiological $Na^+$ and $Ca^{2+}$ gradients and membrane resting potential ranged from 15 to 25% of the activity of the plasma membrane $Ca^{2+}$ pump under these experimental conditions. The results also point out that membrane depolarization increases approx. 2-fold the rate of $Na^+/Ca^{2+}$ exchange in synaptic plasma membrane vesicles.

*Keywords:* Sodium–calcium ion exchange; Chlorotetracycline; Fluorescence; Calcium ion flux; Plasma membrane; Synaptosome

## 1. Introduction

The relevance of calcium fluxes across the plasma membrane during nerve cell activity is now well established, for review see [1–3]. Two transport mechanisms of the plasma membrane are accepted to play a major role in the control of $Ca^{2+}$ homeostasis in nerve terminals: (1) an ATP-dependent mechanism, the $Ca^{2+}$-pump, and (2) the $Na^+/Ca^{2+}$ exchange which is dependent of the $Na^+$ electrochemical gradient generated by the $Na^+$ pump. Both systems can transport $Ca^{2+}$ to the extracellular medium against a concentration gradient. In addition, cytoplasmic $Ca^{2+}$-binding proteins, endoplasmic reticulum, mitochondria and voltage dependent $Ca^{2+}$ channels are involved in the regulation of the cytosolic $Ca^{2+}$ concentration [4–6]. The role of $Na^+/Ca^{2+}$ exchange in the regulation of cytosolic $Ca^{2+}$ in resting nerve endings has been questioned [7,8], because a significant reduction in the $Na^+$ electrochemical gradient does not lead to measurable changes in $Ca^{2+}$ distribution across the plasma membranes of synaptosomes. In contrast, ATP depletion increases the $Ca^{2+}$ content of nerve endings without significant change of the $Na^+$ gradient [7]. Gill et al. [4] have suggested that the $Ca^{2+}$-pump provides the main pathway for $Ca^{2+}$ extrusion from the cytosol in resting nerve cells, becoming more important the role of the $Na^+/Ca^{2+}$ exchange after stimulation.

The relevance of the $Na^+/Ca^{2+}$ exchanger versus the plasma membrane $Ca^{2+}$ pump in the control of cytosolic calcium in nerve terminals is thus a long standing issue. The rate of $Na^+/Ca^{2+}$ exchange decays as the $Na^+$ gradient decreases, and it is modulated by the membrane potential due to its electrogenic activity [9]. Different estimations of the rate of calcium transport by this exchanger using similar experimental conditions have been reported, ranging from 1.5 to 7 nmol/min/mg plasma membrane protein for $Ca^{2+}$ concentrations between 20 and 50 $\mu$M and a physiological $Na^+$-gradient [4,10–13]. The

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changes of the membrane potential of the plasma membrane and the contribution of $Ca^{2+}/Ca^{2+}$ exchange are likely to be the major reasons underlying these discrepancies. Because of the inherent errors in discontinuous data obtained using $^{45}Ca^{2+}$ and Millipore filtration methods in synaptosomes (further complicated by the contribution of the $Ca^{2+}/Ca^{2+}$ exchange, see [8]), alternate methodologies to monitor the $Na^{+}/Ca^{2+}$ exchange in these preparations are needed to obtain continuous data that can be subjected to proper kinetic analysis.

Earlier we have shown that the fluorescence of chlorotetracycline (CTC) can be used to study the $Ca^{2+}$ fluxes across the plasma membrane of synaptosomes while overcoming some of the limitations indicated above [14,15]. In this paper we have worked out the experimental conditions to measure the kinetics of the $Na^{+}/Ca^{2+}$ exchange in synaptic plasma membrane vesicles using the fluorescence of CTC. From the rate constants of operation of the $Na^{+}/Ca^{2+}$ exchanger obtained in this study we have reassessed the relative contributions of the $Na^{+}/Ca^{2+}$ exchange and of the ATP-dependent $Ca^{2+}$ transport to the overall $Ca^{2+}$ efflux across synaptosomal plasma membranes.

## 2. Materials and methods

Synaptosomes and synaptosomal plasma membrane vesicles were prepared from Wistar rat brain as described elsewhere [15]. Protein concentration was measured by the method of Lowry et al. [16], using bovine serum albumin as standard. The total $(Ca^{2+},Mg^{2+})$-ATPase activity of these membrane preparations was measured using the coupled enzyme system pyruvate kinase/lactate dehydrogenase as indicated in [17].

### 2.1. $Ca^{2+}$ transport measurements using $^{45}Ca^{2+}$

$Na^{+}$-gradient-dependent $Ca^{2+}$ uptake measurements were performed with synaptic plasma membrane vesicles preloaded with $Na^{+}$ by incubation during 30 min at 37°C in a medium containing: 0.1 M NaCl and 5 mM Tes (pH 7.4). $Na^{+}$-preloaded vesicles were centrifuged at 13 000 × *g* 15 min, and then resuspended into a small amount of the medium used for preincubation. $Na^{+}$-gradient-dependent $Ca^{2+}$ uptake was started by a large dilution (higher than 30-fold) of synaptic plasma membrane vesicles (90–100 mg protein) preloaded with 100 mM NaCl into 2 ml of a warm (37°C) isotonic solution containing: 5 mM Tes (pH 7.4), 0.1 M KCl and 50 $\mu$M $^{45}CaCl_2$ (21 000 dpm/nmol). Aliquots were pooled at different times after dilution for $Ca^{2+}$ uptake measurements. These aliquots were vacuum filtered through HAWP02500 (Millipore), washed with 9 ml of ice-cold solution containing 50 mM Tes (pH 7.4), 0.1 M KCl and 1 mM $LaCl_3$; and then dissolved with methyl glycol and counted with a Beckman scintillation counter, using 2-(4′-butylphenyl)-5-(4′-biphenylyl)-1,3,4-oxadiazole dissolved in toluene (5 g/l) as the scintillation cocktail.

$Na^{+}$-gradient-dependent $Ca^{2+}$ efflux was measured with synaptosomes preloaded with $^{45}Ca^{2+}$ by incubation at 4°C during 12 h in the following medium: 50 mM Tes (pH 7.4), 0.1 M KCl, 2 mM $\beta$-mercaptoethanol and 1 mM $^{45}CaCl_2$ (7000 dpm/nmol). Aliquots were pooled for $Ca^{2+}$-load measurements at different times after a large dilution (> 30-fold), to a final protein concentration of 0.2 mg/ml, in 50 mM Tes (pH 7.4), 0.1 M NaCl, 2 mM $\beta$-mercaptoethanol and 1 mM EGTA. Synaptosomal $^{45}Ca$-load was measured from the radioactivity retained in the filters as indicated above.

$Ca^{2+}$ transport coupled to ATP hydrolysis by synaptic plasma membrane vesicles was measured by Millipore filtration through HAWPO2500 filters at 25 and 37°C. The reaction medium for $Ca^{2+}$ uptake contained 50 mM Tes (pH 7.4), 0.1 M KCl, 2 mM $MgCl_2$, 1 mM ATP, 50 $\mu$M $^{45}CaCl_2$ (free $Ca^{2+}$ 42 $\mu$M, 21 000 dpm/nmol), 2 mM $\beta$-mercaptoethanol, and 0.13 mg synaptosomal protein per milliliter. Aliquots of the reaction mixture were pooled at 1, 2 and 5 min after addition of calcimycin (19 $\mu$M), treated and counted as indicated above.

### 2.2. $Ca^{2+}$ transport measurements using CTC fluorescence

The fluorescence of CTC can be used to monitor $Ca^{2+}$ fluxes across the plasma membrane of synaptosomes, as shown in the Appendix of Ref. [15]. Briefly, CTC is a moderately lipophilic $Ca^{2+}$-indicator that partitions into the lipid bilayer of biological membranes, and the fluorescence of the $Ca^{2+}$-CTC complex is largely enhanced when bound to a biological membrane [18]. Since the quantum yield of CTC fluorescence in solution, either free or complexed with $Ca^{2+}$ is much lower than that of $Ca^{2+}$-CTC bound to the membrane [18], the fluorescence of CTC in the presence of biological membranes monitors free $Ca^{2+}$ concentration in the medium near the lipid-water interface. Because the dissociation constant of the $Ca^{2+}$-CTC complex is 2.6 mM [18], in the presence of membranes CTC can be used to monitor free $Ca^{2+}$ concentration changes in the range 0.1–30 mM [19]. The rate of $Ca^{2+}$ transport can be estimated from the time dependence of the fluorescence of CTC associated with the membrane [15,19].

As shown in García-Martín et al. [15], for $Ca^{2+}$ transport processes driven by a concentration gradient (e.g., $Ca^{2+}$ or $Na^{+}$-gradients) the time dependence of the fluorescence of CTC fits to the following exponential equation,

$$\Delta F = \Delta F_{max} e^{-kt} \quad (1)$$

where $k$ is the rate constant of the transport process and $\Delta F$ and $\Delta F_{max}$ are the fluorescence change at a time $t$ and after completion of the kinetic process, respectively. Being $F_0$, $F_t$ and $F_\infty$ the fluorescence intensities at times 0, $t$ and

after completion of the kinetic process, respectively, $\Delta F_{max} = F_0 - F_\infty$ and $\Delta F = F_t - F_\infty$. For $Ca^{2+}$ efflux measurements $F_\infty$ is the fluorescence of CTC in the absence of $Ca^{2+}$ (free $Ca^{2+}$ concentration below 0.1 $\mu$M), e.g., CTC free in solution and bound to the membranes in the presence of 1 mM EGTA [15].

The experimental results have been fit by nonlinear regression to the Eq. (1) with Inplot™ software (GraphPAD Software, Inc., San Diego, California, USA). The kinetic traces of CTC fluorescence shown in the figures are digitalized traces representative of each experimental condition. The noise of the fluorescence signal was always less than 1% of the fluorescence signal.

### 2.3. Other methods

Free $Ca^{2+}$ concentrations were fixed in the submicromolar range using EGTA, with an apparent dissociation constant of the $Ca^{2+}$-EGTA complex of $10^{-7.2}$ at pH 7.4 [20]. When needed the concentrations of free cations, $Ca^{2+}$-ATP and $Mg^{2+}$-ATP in the assay medium were calculated by using a program developed for multiple equilibrium analysis by Perrin and Sayce [21], as indicated in [22]. The following dissociation constants ($K_d$) were used: $K_d$ ($Ca^{2+}$-ATP) = $1.17 \cdot 10^{-4}$ M and $K_d$ ($Mg^{2+}$-ATP) = $2.46 \cdot 10^{-5}$ M [15].

Fluorescence measurements were done with Perkin Elmer mod. 650-40 (operated in ratio mode) and SLM-4800 spectrofluorimeters, equipped with thermostated cell holders. The excitation and emission wavelengths used were 380 and 520 nm (for CTC) and 540 and 580 nm (for merocyanine-540), respectively.

Merocyanine-540 has been used to monitor the membrane potential, as shown in [23]. All the results presented in this paper have been confirmed by triplicate determinations carried out with, at least, three preparations of synaptosomes or synaptic plasma membrane vesicles.

### 2.4. Materials

ATP, Br-calcimycin, calcimycin, chlorotetracycline, EGTA, $LaCl_3$, $\beta$-mercaptoethanol, merocyanine-540, $\omega$-conotoxin GVIA, sucrose, Tes and Tris were obtained from Sigma Chemicals. $^{45}Ca^{2+}$ was supplied by Amersham. Pyruvate kinase and lactate dehydrogenase were from Boehringer Mannheim. Other chemicals were obtained from Merck Darmstadt.

## 3. Results

### 3.1. Calibration of CTC fluorescence in function of $Ca^{2+}$-load of plasma membrane vesicles

Synaptosomes were loaded by incubation with 1 mM $^{45}CaCl_2$ (7000 dpm/nmol) at 4°C during 12 h. $Ca^{2+}$-load was measured by counting the radioactivity retained in HAWP02500 filters at different times after dilution in a buffer containing 1 mM EGTA, as indicated in Section 2. In parallel experiments 10 $\mu$M CTC was added to synaptosomes, incubated as indicated above with cold $CaCl_2$ and the intensity of CTC fluorescence was recorded as a function of time after dilution in an EGTA containing solution (free $Ca^{2+}$ concentration < 10 nM). The tight correlation between the decay of $Ca^{2+}$-load of synaptosomes and the decay of the intensity of CTC fluorescence is shown in Fig. 1A. The kinetics of the decay of the intensity of CTC fluorescence can be fitted to a simple exponential function as indicated in Section 2 (correlation coefficient > 0.99), with a rate constant of $0.178 \pm 0.004$ $min^{-1}$ at 25°C (Fig. 1A) and $0.45 \pm 0.05$ $min^{-1}$ at 37°C (data not shown).

Synaptic plasma membrane vesicles have over synaptosomes a clear methodological advantage, namely the lack of subcellular organelles, which would complicate the

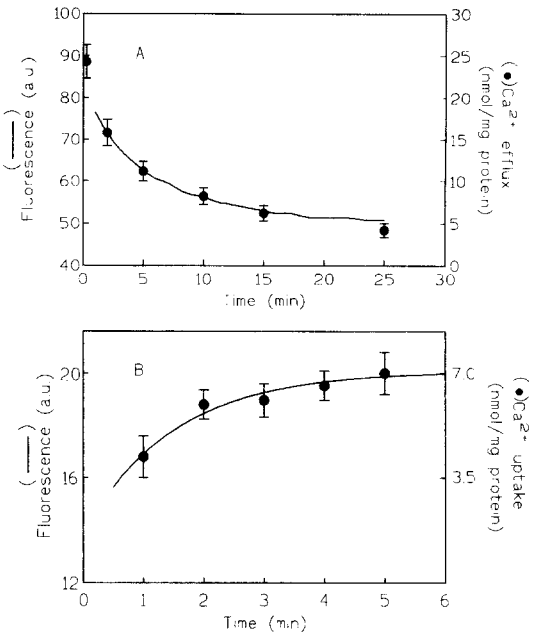


Fig. 1. Overlap between the kinetics of $^{45}Ca^{2+}$ fluxes (efflux and uptake) and the kinetics of CTC fluorescence with synaptosomes and synaptic plasma membrane vesicles. (A) $Ca^{2+}$ efflux from calcium-preloaded synaptosomes. $Na^+$-gradient-dependent $^{45}Ca^{2+}$ efflux measurements were done at 25°C as indicated in Section 2. CTC fluorescence (a.u. means arbitrary units) was recorded as a function of time after 32-fold dilution of calcium-preloaded synaptosomes, to a final protein concentration of 0.1 mg/ml, in 50 mM Tes (pH 7.4), 0.1 M NaCl, 1 mM EGTA and 10 $\mu$M CTC. For CTC fluorescence measurements synaptosomes were preloaded with $Ca^{2+}$ by 3 h incubation at 4°C in 50 mM Tes (pH 7.4), 0.1 M KCl, 2 mM $\beta$-mercaptoethanol, 10 $\mu$M CTC and 20 mM $CaCl_2$. (B) $Ca^{2+}$ uptake by synaptic plasma membrane vesicles. $Na^+$-gradient-dependent $^{45}Ca^{2+}$-uptake measurements were done as indicated in Section 2 (filled circles). $Ca^{2+}$ bound to synaptic plasma membrane vesicles in the absence of $Na^+$-gradient (on average 2 nmol/mg protein) was subtracted. For CTC fluorescence measurements 50 $\mu$M CTC was included in incubation and dilution medium, and cold $CaCl_2$ was used instead of $^{45}CaCl_2$.

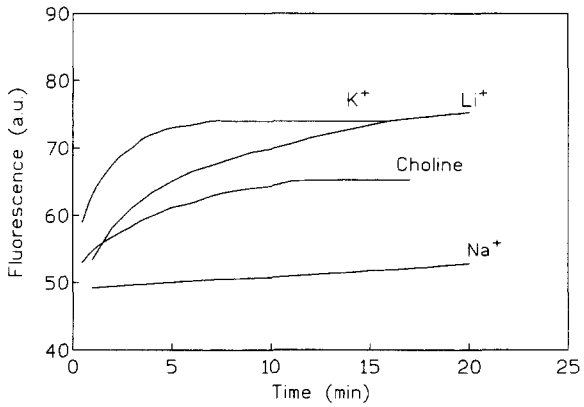


Fig. 2. Kinetics of $Ca^{2+}$ uptake driven by $Na^+$-gradient upon dilution of synaptic plasma membrane vesicles in a KCl, LiCl or choline chloride medium. Dependence of the fluorescence of CTC (a.u. means arbitrary units) upon the time after 32-fold dilution of vesicles preloaded with 0.1 M NaCl and 50 μM CTC in a solution at 37°C containing 50 μM CTC, 2 μM free $Ca^{2+}$, 5 mM Tes (pH 7.4) and 0.1 M of the cation indicated in the figure. Synaptic plasma membrane vesicles were preloaded with 0.1 M NaCl and 50 μM CTC by incubation for 30 min at 37°C with 0.1 M NaCl, 50 μM CTC and 5 mM Tes (pH 7.4). The final concentration of synaptic plasma membrane vesicles after dilution was 0.1 mg protein/ml.

analysis of the kinetic data, particularly for measurements of the rate of $Ca^{2+}$ uptake. The fluorescence of CTC can also be used to monitor $Ca^{2+}$-uptake driven by an outwardly-directed $Na^+$-gradient in synaptic plasma membrane vesicles (Fig. 1B). This point has been further assessed and this is shown below.

### *3.2. Dependence of the CTC fluorescence upon the $Na^+$-gradient across the plasma membrane of synaptosomes*

Synaptic plasma membrane vesicles accumulate $Ca^{2+}$ in response to an outwardly-oriented $Na^+$ gradient, transiently formed by dilution of vesicles preloaded with 100 mM $Na^+$ into a medium made isoosmotic with $K^+$ [10,24]. Fig. 2 shows the effects of several cations in the outer medium on the time dependence of the intensity of CTC fluorescence after addition of plasma membrane vesicles preloaded with 0.1 M NaCl. Dilution of plasma membrane vesicles preloaded with 100 mM NaCl into a medium containing 100 mM KCl results in a large increase of the fluorescence of CTC, which closely follows the uptake of $^{45}Ca^{2+}$ (Fig. 1B) with a first order rate constant of 0.55 ± 0.1 $min^{-1}$ ($n = 9$). In contrast, dilution of synaptic plasma membrane vesicles preloaded with 100 mM NaCl into a medium made containing 100 mM NaCl produced only a slow and much smaller increase of the fluorescence of CTC, likely due to passive $Ca^{2+}$ diffusion through the plasma membrane (Fig. 2). Therefore, these results show that the fluorescence of CTC can be used to monitor $Ca^{2+}$ uptake driven by an outwardly directed $Na^+$-gradient. A $K^+$ gradient is not essential for the $Na^+$-gradient-dependent increase of CTC fluorescence, since it can be replaced by external $Li^+$ or choline chloride (Fig. 2). This is also a property of the $Ca^{2+}$ uptake mediated by the $Na^+/Ca^{2+}$ exchanger [24]. In addition, the results of Fig. 2 show that the kinetics of CTC fluorescence increase is 2 to 3-fold slower with 100 mM $Li^+$ or choline instead of $K^+$ in the dilution medium, yielding first order rate constants of 0.15 ± 0.03 and 0.25 ± 0.02 $min^{-1}$, respectively. This effect of replacing external $K^+$ by $Li^+$ or choline on the kinetics of $Ca^{2+}$ uptake catalyzed by the $Na^+/Ca^{2+}$ exchanger had been noticed earlier using $^{45}Ca^{2+}$ and Millipore filtration [24].

The concentration of $Ca^{2+}$ in the medium was fixed in the micromolar range in these experiments, e.g., 2 μM. Since CTC fluorescence is not significantly affected by $Ca^{2+}$ concentration changes in the micromolar range, because the $K_d$ ($Ca^{2+}$-CTC) is 2.6 mM (see Section 2), this avoids any significant contribution to the increase of CTC fluorescence coming from equilibration of $Ca^{2+}$ across the plasma membrane through voltage operated $Ca^{2+}$ channels. This has been reported to introduce methodological problems when the $Na^+/Ca^{2+}$ exchange is studied using $^{45}Ca^{2+}$ and filtration protocols [8,10]. However, the $Na^+/Ca^{2+}$ exchange accumulates $Ca^{2+}$ against its concentration gradient [24], thus allowing this process to be monitored by the fluorescence of CTC. Accumulated $Ca^{2+}$ can be released by addition of a $Ca^{2+}$ selective ionophore (like Br-calcimycin) or detergents (like 0.1% saponine or 0.15 mM Triton X-100). Consistently, the intensity of CTC fluorescence drops rapidly when the $Ca^{2+}$ selective ionophore, Br-calcimycin, is added to the external medium after completion of the kinetics of increase of CTC fluorescence (Fig. 3). In separate experiments we have assessed that under these conditions Br-calcimycin produced less than 2% quenching of CTC fluorescence (data not shown). Addition of 0.1% saponine or 0.15 mM Triton X-100 instead of Br-calcimycin produced a similar effect on the

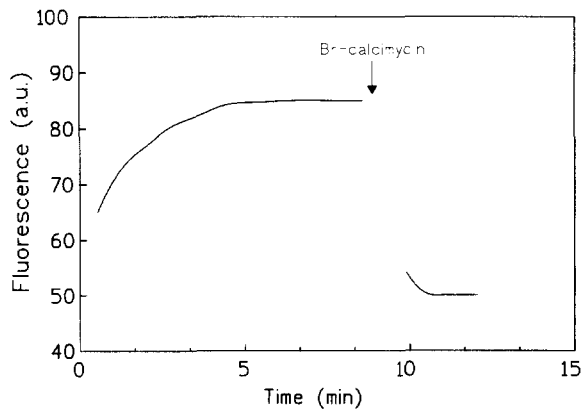


Fig. 3. $Na^+$-gradient-dependent increase of CTC fluorescence is reverted by the calcium ionophore Br-calcimycin. Dependence of the fluorescence of CTC upon the time after 32-fold dilution of synaptic plasma membrane vesicles preloaded with 0.1 M NaCl and 50 μM CTC in a solution at 37°C containing 50 μM CTC, 2 μM free $Ca^{2+}$, 0.1 M KCl and 5 mM Tes (pH 7.4). The arrow indicates the addition of 19 μM Br-calcimycin. Other experimental conditions as indicated in the legend to the Fig. 2.

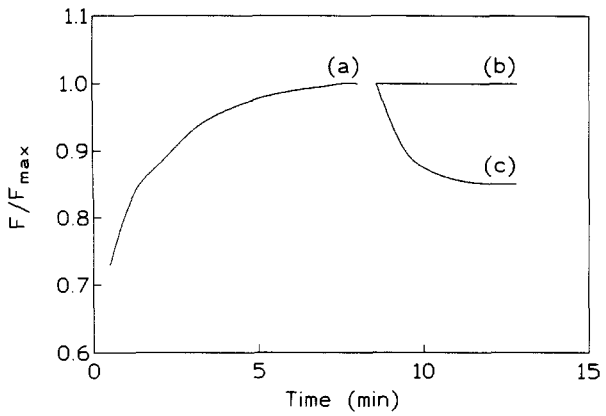


Fig. 4. $Na^+$-gradient-dependent increase of the fluorescence of CTC is reverted by cancellation of the $Na^+$-gradient. (a) Dependence of the fluorescence of CTC upon the time after dilution of 66 μl of synaptic plasma membrane vesicles preloaded with 0.1 M NaCl and 50 μM CTC into 2 ml of a solution at 37°C containing 50 μM CTC, 2 μM free $Ca^{2+}$, 0.1 M KCl and 5 mM Tes (pH 7.4). Other experimental conditions as indicated in the legend to the Fig. 2. At the time indicated by a gap aliquots of the vesicles suspension were 20-fold diluted in the same solution (b) or in a solution with 0.1 M NaCl replacing KCl (c).

fluorescence of CTC in the presence of synaptic plasma membrane vesicles preloaded with $Ca^{2+}$ by operation of the $Na^+/Ca^{2+}$ exchanger.

Moreover, $Ca^{2+}$-preloaded vesicles via $Na^+/Ca^{2+}$ exchange released accumulated $Ca^{2+}$ when diluted in a medium containing high $Na^+$, e.g., when subjected to an inward-directed $Na^+$ gradient [24]. Fig. 4 shows that CTC fluorescence can also be used to monitor this $Ca^{2+}$ release process. These experiments were carried out in two steps. Firstly, vesicles preincubated with 0.1 M $Na^+$ were loaded with $Ca^{2+}$ via $Na^+/Ca^{2+}$ exchanger by incubation at 37°C in a medium containing 0.1 M KCl, 50 μM CTC, 50 μM $Ca^{2+}$, 61.89 μM EGTA (free $Ca^{2+}$ 2 μM), and 5 mM Tes (pH 7.4). After 6 min the vesicles were more than 10-fold diluted in either the same medium (Fig. 4, trace b) or the same medium with 0.1 M NaCl instead of 0.1 M KCl (Fig. 4, trace c). It can be observed that in the presence of an inward-directed $Na^+$ gradient (trace c), $Ca^{2+}$ is rapidly released from synaptic plasma membrane vesicles. These results thus show that the time-course of the intensity of fluorescence of CTC is monitoring the $Ca^{2+}$ uptake driven by $Na^+$-gradient.

### 3.3. Dependence of $Ca^{2+}$ inflow on the extent of outwardly-oriented $Na^+$-gradient and on $Ca^{2+}$ concentration

When NaCl was progressively substituted by KCl to maintain the osmolarity of the outer medium, the modification of both $K^+$ and $Na^+$ gradients result in plasma membrane depolarization, as shown by the membrane potential indicator merocyanine-540 (Fig. 5). Similar results were obtained using Bisoxonol to monitor the membrane potential (results not shown; see also [13]). The rate constant of $Ca^{2+}$ inflow is increased by plasma membrane depolarization (Table 1). The possibility that the increase of CTC fluorescence upon plasma membrane depolarization could also be monitoring $Ca^{2+}$ transport by voltage dependent $Ca^{2+}$ channels can be excluded, since neither the $\Delta F_{max}$ nor the half-time were altered by addition to the incubation or to the assay medium of 1 mM verapamil or 1 μM ω-conotoxin GVIA (a potent and selective inhibitor of neural N-type voltage-sensitive $Ca^{2+}$ channels [25]). In order to maintain the membrane potential and the osmolarity of the extravesicular medium, the magnitude of the $Na^+$-gradient was changed by the appropriate replacement of NaCl with choline chloride in the outer medium as shown by Coutinho et al. [10] and by Taglialatela et al. [13], see also Fig. 5. The change of the $Na^+$-gradient had not a significant effect on the rate constant of $Ca^{2+}$ inflow.


Fig. 5. Synaptic plasma membrane depolarization monitored by the fluorescence of merocyanine-540. Synaptic plasma membrane vesicles were preloaded with 0.1 M NaCl as indicated in the legend to the Fig. 2. At the time marked by arrows in the figure, 66 μl of vesicles were diluted into 2 ml of a solution at 37°C containing 5 mM Tes (pH 7.4), 0.5 μM merocyanine-540 and 0.1 M of either NaCl, KCl or choline chloride as indicated in the figure. The final concentration of vesicles was 0.1 mg protein/ml. After dilution in 0.1 M NaCl or choline chloride, KCl was added stepwise to the solution to the final concentrations indicated in the figure.

Table 1
Effect of $Na^+$ gradient and of membrane depolarization on the rate constant of $Ca^{2+}$ uptake monitored with CTC fluorescence and mediated by the $Na^+/Ca^{2+}$ exchanger of synaptic plasma membrane vesicles

| $[Na^+]_o$ (mM) | $[K^+]_o$ (mM) | $[choline^+]_o$ (mM) | $k$ ($min^{-1}$) | $F_\infty - F_0$ (a.u.) [a] | Em [b] |
|---|---|---|---|---|---|
| 0 | 0 | 100 | $0.25 \pm 0.02$ | $12 \pm 1.5$ | P |
| 5 | 0 | 95 | $0.28 \pm 0.03$ | $9.3 \pm 0.5$ * | P |
| 10 | 0 | 90 | $0.29 \pm 0.03$ | $5.4 \pm 0.02$ ** | P |
| 17 | 0 | 83 | $0.29 \pm 0.05$ | $3.2 \pm 0.2$ *** | P |
| 0 | 100 | 0 | $0.55 \pm 0.10$ # | $12 \pm 1$ | DP |

Vesicles preloaded with 0.1 M NaCl, 5 mM Tes (pH 7.4) and 50 $\mu$M CTC were diluted in a warm (37°C) isotonic medium containing: 5 mM Tes (pH 7.4), 50 $\mu$M CTC, 50 $\mu$M $Ca^{2+}$ and with the addition of the cations indicated in the table.
(a) a.u. means arbitrary units.
(b) P and DP means polarized and depolarized synaptic plasma membrane vesicles, see the text for further details. The membrane potential, calculated as indicated in [36] for the different inside/outside ions concentrations listed, are $-80.7 \pm 0.7$ and 0 mV for non-depolarized and depolarized membranes, respectively. Statistical comparison: as compared (Student's $t$-test) with the values obtained with 100 mM choline in the external medium: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; # $P < 0.0001$.

However, the maximum fluorescence change, which monitors the maximum amount of $Ca^{2+}$ taken up by the vesicles (Fig. 1), decreased as the $Na^+$-gradient decreased (Table 1).

We have also studied the ability of the fluorescence of CTC to monitor the $Ca^{2+}$ dependence of $Ca^{2+}$ uptake through the $Na^+/Ca^{2+}$ exchanger with KCl or choline chloride in the external medium (see above). In the absence of $Na^+$-gradient the fluorescence of CTC does not significantly change up to approximately 50 $\mu$M $Ca^{2+}$ in the external medium (Fig. 6A, open circles). As neither $Ca^{2+}/Ca^{2+}$ exchange nor activation of $Ca^{2+}$ channels raise a significant $Ca^{2+}$ concentration gradient this indicated that up to 50 $\mu$M $Ca^{2+}$ in the external medium the change of CTC fluorescence can be safely use to monitor $Na^+/Ca^{2+}$ exchange. Fig. 6A also shows the effect of $Na^+$-gradient across the plasma membrane on the dependence upon free $Ca^{2+}$ concentration of the fluorescence of CTC after completion of the kinetic process. The increase of CTC fluorescence produced by the $Na^+$-gradient (Fig. 6B) shows the same $Ca^{2+}$ dependence than that reported for the $Na^+/Ca^{2+}$-exchanger of the synaptic plasma membrane vesicles using $^{45}Ca^{2+}$ and the filtration technique [4]. In the choline chloride medium the rate constant of $Ca^{2+}$ influx increases from $0.17 \pm 0.04$ to $0.28 \pm 0.03$ $min^{-1}$ when the $Ca^{2+}$ concentration in the external medium is increased from 0.5 to 100 $\mu$M.

### 3.4. Activity of the $Ca^{2+}$-pump of synaptic plasma membrane vesicles

The high affinity $Ca^{2+}$-stimulated $Mg^{2+}$-ATPase of the synaptic plasma membrane contributes to approximately 40–50% of the total ($Ca^{2+}$,$Mg^{2+}$)-ATPase activity, i.e., $0.09 \pm 0.02$ $\mu$mol ATP hydrolyzed $min^{-1}$ (mg protein)$^{-1}$ and has a $K_{0.5}$ value for $Ca^{2+}$ of $0.13 \pm 0.01$ $\mu$M [26]. The steady state rate of ATP-dependent $Ca^{2+}$ uptake by synaptic plasma membrane vesicles was determined using $^{45}Ca^{2+}$ and Millipore filtration (Fig. 7), as indicated in Section 2, and was found to range between 1.7 and 2 nmol $Ca^{2+}$ $min^{-1}$ (mg membrane protein)$^{-1}$ at 25°C, in agreement with previous reports [4,15,24]. Taking into account that these vesicles are about 40–50% inside out [15,27], and that right-side-out vesicles do not contribute to $Ca^{2+}$ transport coupled to ATP hydrolysis, because the plasma membrane is not permeable to ATP, we obtain a maximum $Ca^{2+}$-pump activity of 4–5 nmol $Ca^{2+}$ $min^{-1}$ (mg protein)$^{-1}$ at 25°C. These experiments were also performed at 37°C from which we have obtained a maximum

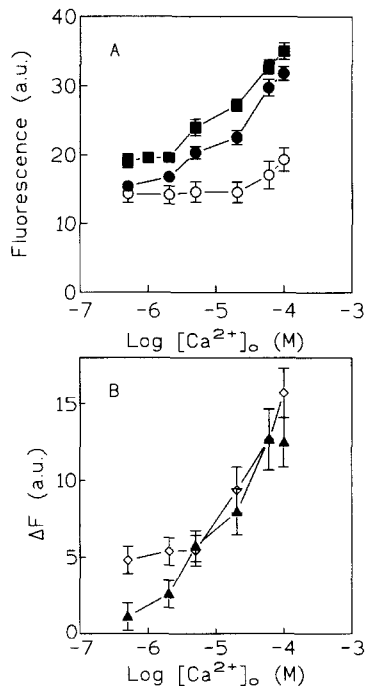


Fig. 6. Dependence of the fluorescence of CTC upon the $Ca^{2+}$ concentration in the presence of synaptic plasma membrane vesicles. (A) Dependence of the fluorescence of CTC upon the $Ca^{2+}$ concentration in the dilution medium, $[Ca^{2+}]_o$: (■,●) values reached after completion of the kinetics of CTC fluorescence increase, $F_\infty$, upon dilution of vesicles preloaded with 0.1 M NaCl as indicated in the legend of the Fig. 2 in a 5 mM Tes solution (pH 7.4) containing 0.1 M $K^+$ (filled squares) or 0.1 M choline (filled circles). Empty circles are the $F_0$ values obtained from plots of $\ln(F_\infty - F_t)$ versus the time after dilution of vesicles preloaded with 0.1 M NaCl in a solution containing 0.1 M KCl or 0.1 M choline chloride, as indicated in Section 2. For each $Ca^{2+}$ concentration, the values of $F_0$ obtained were identical to the CTC fluorescence measured upon dilution in a solution containing 0.1 M NaCl, e.g., without $Na^+$-gradient. (B) Dependence of $F_\infty - F_0$ ($\Delta F$) upon the concentration of $Ca^{2+}$ in the external medium. The values of $F_\infty$ and of $F_0$ have been taken from Fig. 6A: $\Delta F$ values for dilution in the KCl medium (◇) and in the choline chloride medium (▲).

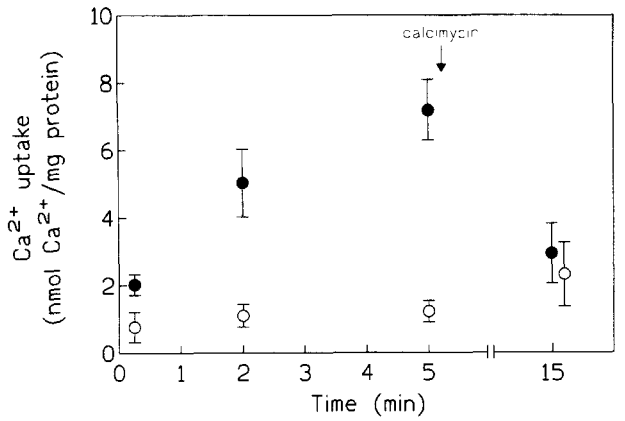


Fig. 7. ATP-dependent $Ca^{2+}$ uptake by synaptosomal plasma membrane vesicles. $^{45}Ca^{2+}$ uptake measurements were carried out at 25°C as indicated in Section 2 in the absence (empty circles) and in the presence of 1 mM ATP (filled circles). Aliquots were pooled at the times indicated in the abscissae, filtered and counted as indicated in Section 2. At the time indicated by an arrow 19 $\mu$M calcimycin was added and approximately 9 min later aliquots were taken for $^{45}Ca^{2+}$ uptake measurements. The data shown are the average ± S.D. of triplicate experiments carried out with three separate vesicle preparations ($n = 9$).

$Ca^{2+}$ pump activity of 8–9 nmol $Ca^{2+}$ $min^{-1}$ (mg protein)$^{-1}$ (data not shown).

## 4. Discussion

The results presented in this paper show that the fluorescence of CTC can be used to continuously monitor the kinetics of $Ca^{2+}$ transport catalyzed by the $Na^+/Ca^{2+}$ exchanger both with synaptosomes and with synaptic plasma membrane vesicles. However, these vesicle preparations are about 40–50% inverted with respect to the orientation of the plasma membrane in synaptosomes [15]. Therefore, an average of the rate constant of the $Na^+/Ca^{2+}$ exchanger operating in the two possible transport modes, e.g., extruding $Ca^{2+}$ from or introducing $Ca^{2+}$ to the cytosol of the nerve terminals is obtained with synaptic plasma membrane vesicle preparations.

Taking an internal volume of 3–7 $\mu$l per mg of protein of plasma membrane vesicles [11,28], for a $Ca^{2+}$ concentration of 1 mM (e.g., the $Ca^{2+}$ concentration of extracellular fluids) the results obtained for non-depolarized membranes yield a rate of calcium uptake of 0.7–1.5 nmol $Ca^{2+}$ (mg protein)$^{-1}$ after 1 min of operation of the transporter in the influx mode at 37°C. The values produced from CTC fluorescence measurements for the rate of $Na^+/Ca^{2+}$ exchange operating in the influx mode are in good agreement with those reported by Snelling and Nicholls [8] by using the $^{45}Ca^{2+}$/Millipore filtration technique and non-depolarized synaptic plasma membrane preparations similar to ours. The rate of $Ca^{2+}$ transport mediated by the $Na^+/Ca^{2+}$ exchanger is to be compared with the maximum rate of ATP-dependent $Ca^{2+}$ uptake measured with synaptic plasma membrane vesicles, approx. 4–5 and 8–9 nmol $Ca^{2+}$ $min^{-1}$ (mg plasma membrane protein)$^{-1}$ at 25 and 37°C, respectively. Since the plasma membrane $Ca^{2+}$-pump has a $K_m$ for $Ca^{2+}$ well below 1 $\mu$M [6], our results confirm that in the 1–10 micromolar $Ca^{2+}$ concentration range the plasma membrane $Ca^{2+}$-pump is more efficient than the $Na^+/Ca^{2+}$ exchanger to extrude $Ca^{2+}$ from the cytosol. Thus our results confirm that in non-depolarized synaptic plasma membranes the $Ca^{2+}$ pump is the main pathway for $Ca^{2+}$ extrusion from synaptic terminals, because the cytosolic free $Ca^{2+}$ concentrations measured within synaptic nerve terminals under conditions simulating repetitive nerve stimulation (trains of < 60 impulses) are always less than 1 $\mu$M [29,30]. Only transiently during depolarization $[Ca^{2+}]_i$ can reach up to 100 $\mu$M locally near the plasma membrane [31]. However, it is to be recalled that the $Na^+$-gradient is largely decreased and even reverted during plasma membrane depolarization.

Plasma membrane depolarization increased 2 to 3-fold the rate of the calcium flux through the plasma membrane of synaptosomes. Plasma membrane depolarization activates the voltage sensitive calcium channels present in synaptic terminals [2] and it has been reported that the $Ca^{2+}$ flux through voltage-dependent $Ca^{2+}$ channels may produce overestimation of the $^{45}Ca^{2+}$ flux through the $Na^+/Ca^{2+}$ exchanger in depolarized plasma membrane of synaptic terminals [8,10,13]. However, in this regard the use of CTC provide a clear advantage over the use of $^{45}Ca^{2+}$, because by fixing $Ca^{2+}$ concentrations in the micromolar range in the extravesicular medium the activation of voltage-operated $Ca^{2+}$ channels will result in micromolar $Ca^{2+}$ concentrations inside the vesicles or synaptosomes, a concentration range in which CTC fluorescence is not significantly increased ([19] and this paper). Therefore, we conclude that plasma membrane depolarization produced a 2 to 3-fold stimulation of the $Na^+/Ca^{2+}$ exchanger operating in the $Ca^{2+}$ uptake mode, as it has been shown for the $Na^+/Ca^{2+}$ exchange of cardiac muscle preparations [32]. As the $Na^+/Ca^{2+}$ exchanger operates with an exchange stoichiometry of 3 $Na^+$/1 $Ca^{2+}$ which generates an inside-negative membrane potential, the stimulation of the exchanger by membrane depolarization could be due to the rapid counterbalance of transient inside-negative membrane potential. Alternatively, the change of the membrane potential may produce a conformational shift of the $Na^+/Ca^{2+}$ exchanger, as it has been suggested earlier [32]. During nerve cell activation, complex changes in membrane potential and transient reversal of the $Na^+$-gradient occur. Therefore, this exchanger could play a versatile role in regulating cytosolic $Ca^{2+}$ during the activity of synaptic terminals [9,33], as it does in cardiac myocytes [34,35].

With synaptosomes we have obtained a rate constant of $Ca^{2+}$ efflux through the plasma membrane of 0.45 ± 0.05 $min^{-1}$ ($n = 12$) at 37°C under depolarizing conditions

(100 mM KCl in the external medium), a value which is close to the rate constant measured for the $Ca^{2+}$ influx catalyzed by the $Na^+/Ca^{2+}$ exchanger with depolarized synaptic plasma membrane vesicles, $0.55 \pm 0.10$ $min^{-1}$ at 37°C (Table 1). As the preparation of synaptic plasma membrane vesicles contains approximately 50% inverted vesicles, these results suggest that the $Na^+/Ca^{2+}$ exchanger operates at a similar rate in the $Ca^{2+}$ influx and in the $Ca^{2+}$ efflux mode. In addition, these results further validate the use of synaptic plasma membrane vesicles to study the $Na^+/Ca^{2+}$ exchanger of the plasma membrane of synaptosomes.

In conclusion, the fluorescence of CTC can be used to analyze the kinetics of the $Na^+/Ca^{2+}$ exchanger of synaptic plasma membrane vesicles. The experimental conditions to avoid a significant contribution to CTC fluorescence changes of $Ca^{2+}$ fluxes through voltage-sensitive $Ca^{2+}$ channels and of $Ca^{2+}/Ca^{2+}$ exchange are reported in this paper. The results show that the $Na^+/Ca^{2+}$ exchanger is stimulated by membrane depolarization, thus suggesting the implication of this exchanger in $Ca^{2+}$ inflow to the synaptic terminal upon stimulation.

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