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Short Communication

Measuring Calcium Uptake and Inositol 1,4,5-Trisphosphate-Induced Calcium Release in Cerebellum Microsomes Using Fluo-3

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A simple fluorimetric method is presented for measuring calcium uptake and inositol 1,4,5-trisphosphate ($InsP_3$)-induced calcium release in microsomes using Fluo-3. This method is used to investigate the amplitude of calcium release (quantal release) induced by $InsP_3$ from rat cerebellum microsomes.

KEY WORDS: Fluo-3; fluorescence; calcium; inositol 1,4,5-trisphosphate.

INTRODUCTION

A series of calcium-indicating fluorescent dyes have been developed over the last decade that have become widely used in the measurements of changes in intracellular calcium concentrations. Of these, Quin2 and Fura-2 have been the most commonly used indicators, having dissociation constants for calcium of 115 and 224 n*M*, respectively, when measured under physiological conditions [1,2]. These dyes were synthesized as acetyloxymethyl esters, which are membrane permeable and able to enter the cytosol. Once the ester linkage is cleaved by endogenous esterases, these indicators are able to monitor intracellular calcium changes which are typically in the 50 n*M* to 1 μ*M* range.

Recently, a new series of indicators has been developed based on rhodamine and fluorescein. One of these dyes, Fluo-3, has a much lower affinity for calcium (K_d, 400 n*M*) and a much greater fluorescence change in the presence of calcium than Quin2 or Fura-2 [3].

Calcium flux measurements in subcellular fractions such as microsomes have been fundamental in understanding the processes involved intracellular calcium homeostasis. However, these changes in calcium concentration are very small in comparison to calcium concentration changes observed in striated muscle sarcoplasmic reticulum and therefore require more sensitive methods. Calcium flux measurements in subcellular fractions of non-muscle tissues have employed the use of the radioisotope $^{45}Ca^{2+}$ [4], or monitoring the external calcium concentration using calcium-sensitive electrodes [5], or antipyrylazo III using dual-wavelength spectrophotometry [6]. These methods are either difficult to set up (calcium-sensitive electrodes), require expensive equipment (dual-wavelength spectrophotometry), or do not follow the changes in real time (radioisotopes).

Here it is reported that Fluo-3 in the free acid form is an ideal indicator for monitoring the small changes in calcium influx and efflux in cerebellum microsomes.

METHODS

Fluo-3 was obtained from Molecular Probes; all other reagents were of analytical grade. Rat cerebellum microsomes were prepared as described by Alderson and Volpe [6]. Free calcium concentrations were varied by

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changing the calcium–EGTA concentrations using the dissociation constants given in [7].

The buffer used in this study was 10 m*M* potassium phosphate, 3.5 m*M* potassium pyrophosphate, 100 m*M* KCl, 125 n*M* Fluo-3, pH 7.2, and measurements were carried out at 37°C. Typically 0.3 mg/ml of cerebellum microsomes was added to the cuvette, which was continuously stirred. The calcium concentration of this buffer was estimated to be 2–3 μ*M*. The buffer also contained 10 μg/ml creatine kinase and 10 m*M* creatine phosphate to minimize the buildup of ADP.

Calcium uptake was initiated by the addition of 1.5 m*M* Mg-ATP, and the fluorescence change of Fluo-3 monitored with a Perkin Elmer spectrofluorimeter, exciting at 506 nm and detecting the emission at 526 nm, with slit widths both set at 5 nm. Inositol 1,4,5-trisphosphate ($InsP_3$; Calbiochem) was added directly to the stirred cuvette and calcium changes were monitored. To measure total calcium accumulation, the microsomes were made permeable to calcium by the addition of 12.5 μg/ml A23187.

Fluorescence intensity was related to calcium concentration using the equation:

$$[Ca^{2+}]_{free} = K_d \times (F - F_{min})/(F_{max} - F)$$

where K_d is the dissociation constant for calcium binding to Fluo-3, F is the fluorescence intensity of the sample, and F_{min} and F_{max} are the fluorescence intensities of the sample in 1 m*M* EGTA and 2.5 m*M* $CaCl_2$, respectively.

RESULTS AND DISCUSSION

Varying the free calcium concentration in a buffer containing 10 m*M* Hepes, 100 m*M* KCl, pH 7.2, at 37°C, gave a dissociation constant of 900 ± 80 n*M* for calcium binding to Fluo-3 (Fig. 1). There was also a 35- to 40-fold increase in fluorescence intensity in the presence of saturating concentrations of calcium compared to that in EGTA. From Fig. 1 it can be seen that Fluo-3 is ideal for measuring free calcium concentrations between 0.1 and 4 μ*M*, unlike Quin2 and Fura-2, which have a maximal fluorescence response at submicromolar calcium concentrations. The dissociation constant compares well to the value of 864 n*M* determined by Merritt *et al.* [8] for similar pH and temperature but deviates from that of Minta *et al.* (400 n*M*) measured at 22°C [3].

The buffers used in these experiments were prepared from deionized water containing pyrophosphate, which chelates calcium; the estimated free calcium concentrations were typically between 2 and 3 μ*M* and within the sensitivity for Fluo-3.

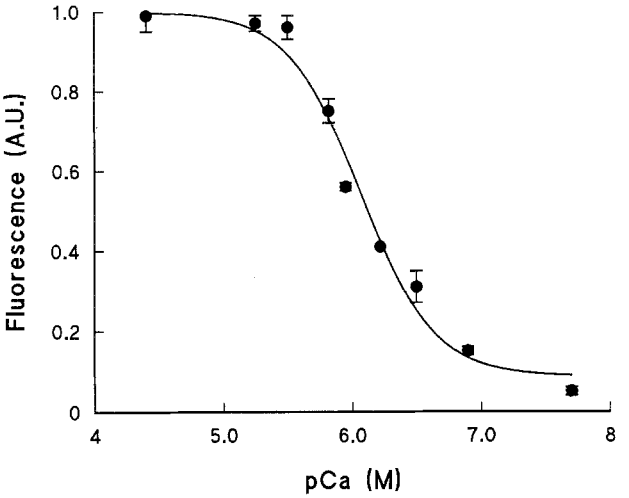

Fig. 1. *Fluorescence change of Fluo-3 with calcium.* Free calcium was varied by the addition of EGTA and calcium according to Ref. 7. Fluorescence was monitored at 526 nm, exciting at 506 nm. Fluo-3, 125 n*M*, was added to the buffer consisting of 10 m*M* Hepes, 100 m*M* KCl, pH 7.2, and 37°C. Points are the mean ± standard error of three separate experiments.

As cerebellum microsomes are relatively enriched in intracellular calcium pumps and channels compared to other nonmuscle tissues [9,10], they were extensively used in this study to evaluate the usefulness of this method.

Figure 2a shows that upon addition of Mg-ATP, the fluorescence intensity of Fluo-3 decreases rapidly as calcium is complexed with ATP (this decrease also occurred in the absence of microsomes). A slower decrease in fluorescence was also observed as extraluminal calcium was pumped into the microsomes. Calcium uptake was aided by the addition of the calcium chelator potassium pyrophosphate, as in its absence little calcium accumulation was detected. Pyrophosphate is also commonly used during calcium flux measurements in sarcoplasmic reticulum to help accumulate calcium within the vesicles [11]. The rate of calcium uptake under the experimental conditions was determined to be 1.6 ± 0.2 nmol/min/mg. The accumulated calcium was completely released by the addition of the calcium ionophore A23187. Sodium vanadate (1 m*M*), an inhibitor of intracellular calcium pumps [9], stopped calcium accumulation, confirming that calcium influx into the microsomes is due to a calcium-dependent ATPase.

The addition of $InsP_3$ caused calcium release from the microsomes, however, this was never more than 40% of that which could be released by A23187. Other stud-

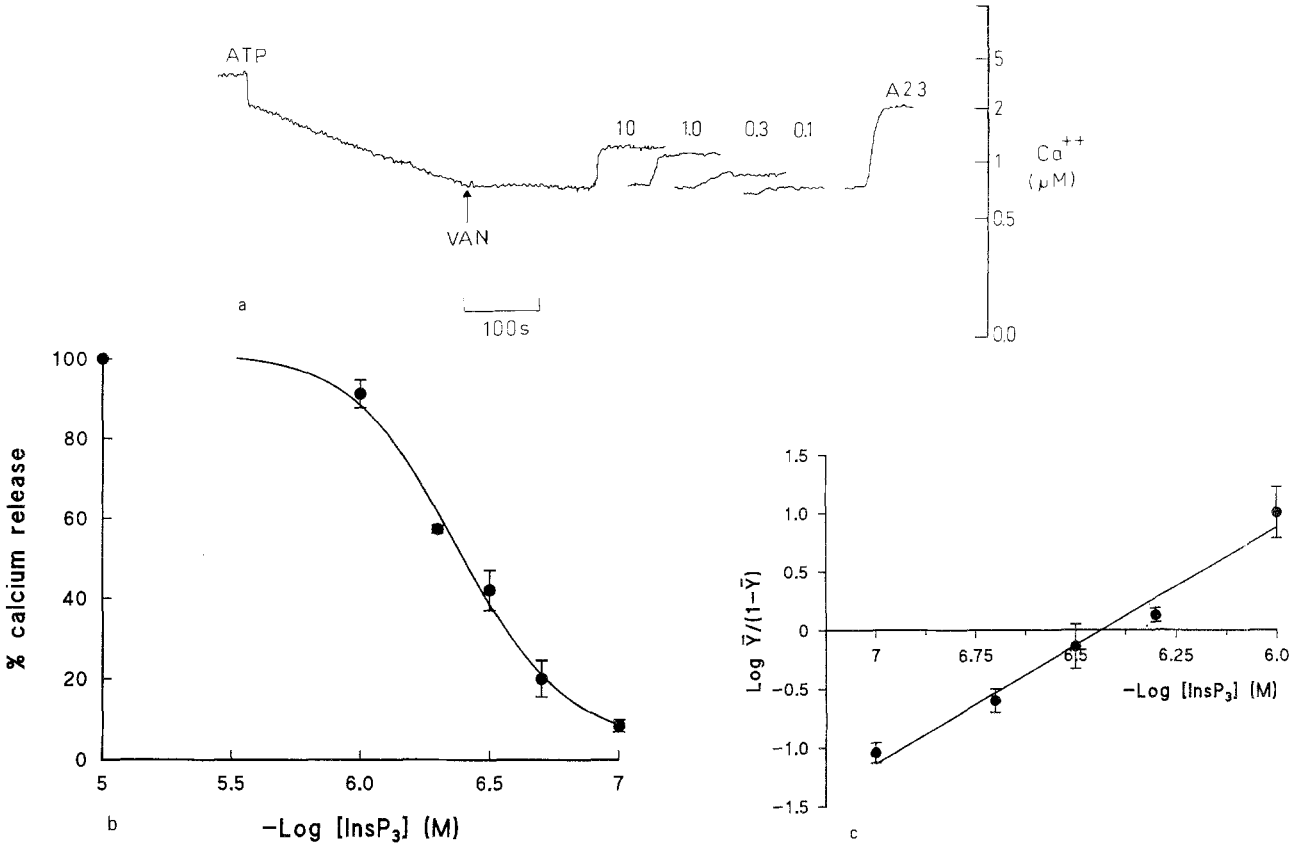

Fig. 2. *Calcium uptake and release in rat cerebellum microsomes monitored using Fluo-3.* (a) Calcium uptake into the microsomes (0.3 mg/ml was initiated by the addition of 1.5 m*M* ATP and inhibited with 1 m*M* vanadate (VAN). Calcium release was induced by various concentrations of $InsP_3$ (numbers given are μ*M*). Accumulated calcium was released by 12.5 μg/ml A23187 (A23). (b) Amount of calcium release (quantal release) as a function of $InsP_3$ concentration, expressed as a percentage of calcium released by 10 μ*M* $InsP_3$. Points are the mean ± standard error of three separate experiments. (c) Hill plot of data presented in b, where $\bar{Y}$ is the fractional response.

ies using cerebellum microsomes have also shown that not more than 40–50% of the calcium accumulated is released by $InsP_3$ [6]. This $InsP_3$-induced calcium release was deemed to be via the $InsP_3$ receptor since it was blocked by heparin (an inhibitor of the $InsP_3$-sensitive calcium channel [12]). It was also noted that, although calcium accumulation increased at higher pyrophosphate concentrations, these higher concentrations (greater than 10 m*M*) inhibited $InsP_3$-induced calcium release, an observation also made by Palade *et al.* [13]. However, at the concentrations used in this study (3.5 m*M*), good levels of calcium accumulation could be obtained with negligible effects on $InsP_3$-induced calcium release as assessed by other studies using cerebellum microsomes.

The maximal amount of release was observed at $InsP_3$ concentrations of between 1 and 10 μ*M*; submaximal concentrations of $InsP_3$ caused a decrease in both the rate and the amount of calcium released. These observations have been made previously in permeabilized hepatocytes and pancreatic acini cells [4,14]. The nonmetabolizable analogue of $InsP_3$, inositol 1,4,5-trisphosphothionate ($InsP_3\gamma S$), also showed similar patterns of calcium release, indicating that this observation is not due to $InsP_3$ breakdown (data not shown). The dependency of the amount of calcium released on $InsP_3$ concentrations was first observed by Muallem *et al.* using $^{45}Ca^{2+}$ [4] and was termed quantal calcium release. In Fig. 2b the amount of calcium released (quantal release) is plotted as a function of added $InsP_3$. It can be seen that a nonlinear dependency exists. Plotted as a Hill plot (Fig. 2c) the data give a linear relationship with a Hill coefficient of 2.0 ± 0.2. This compares well with the value of 1.7 determined for canine cerebellum microsomes using dual-wavelength spectrophotometry [15].

When $InsP_3$ binding was measured in rat cerebellum

microsomes, a simple binding curve was obtained with a Hill coefficient of 1.0 [10]. Therefore, the dependency of quantal calcium release is not simply related to ligand concentration and these data suggest that calcium release occurs in a positive cooperative fashion. The simplest interpretation of such a phenomenon is that the $InsP_3$-sensitive channel exists as an oligomer (an observation already made elsewhere [16]) where the opening of one channel in the oligomeric aggregate enhances the subsequent opening of adjacent channels.

In summary, Fluo-3 free acid is an ideal indicator for monitoring the small calcium concentration changes in microsomes. This method has been used to demonstrate that the amount of calcium released (quantal release) by $InsP_3$ in cerebellum microsomes occurs in a cooperative fashion and is therefore in accordance with the idea that the channel exists as an oligomer, the subunits of which interact allosterically to control the amount of calcium released.

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