Kinetics of the non-specific calcium leak from non-mitochondrial calcium stores in permeabilized A7r5 cells

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We have investigated the detailed kinetics of the passive Ca\(^{2+}\) leak from non-mitochondrial Ca\(^{2+}\) stores in permeabilized A7r5 cells. The decrease in the content of stored Ca\(^{2+}\) in the presence of 2 \(\mu\)M thapsigargin deviated from a single-exponential curve in the initial phase of the efflux. The deviation persisted after correcting this efflux for passively bound Ca\(^{2+}\). The non-single-exponential nature of the spontaneous release also occurred when the initial store Ca\(^{2+}\) content was reduced to 40% of its original value by pretreatment with 200 nM inositol 1,4,5-trisphosphate (Ins\(_P_2\)). The passive Ca\(^{2+}\) leak could be modelled by two exponential curves with discrete rate constants of 0.06 min\(^{-1}\) and 0.98 min\(^{-1}\), and not by any other type of non-exponential decay. We concluded that individual store units are heterogeneous with respect to their passive Ca\(^{2+}\) permeability.

INTRODUCTION

Many hormones, neurotransmitters and growth factors induce the hydrolysis of PtdIns(4,5)\(_P_2\) and thereby produce inositol 1,4,5-trisphosphate (Ins\(_P_2\)) as an intracellular messenger [1]. Once threshold concentrations of Ins\(_P_2\) are reached and conditions for the regenerative release of Ca\(^{2+}\) are created [2], Ins\(_P_2\) mobilizes Ca\(^{2+}\) from the non-mitochondrial stores through interaction with the Ins\(_P_2\) receptor. The kinetics of the Ins\(_P_2\)-induced Ca\(^{2+}\) release are well known. They deviate from a single exponential curve, although the nature underlying this behaviour is still controversial. It may represent the steady-state control of the release by luminal Ca\(^{2+}\) [3–7], intrinsic inactivation of the Ins\(_P_2\) receptor [8], heterogeneity in affinity of Ins\(_P_2\) receptors present in compartmentalized Ca\(^{2+}\) pools [10–12]. In contrast, much less is known about the kinetics of the non-specific basal Ca\(^{2+}\) leak from the stores in the absence of Ins\(_P_2\). The non-specific leak has been fitted by a single exponential curve [10]. In other instances, the kinetics deviate from a single exponential curve [13,14]. However, in most reports dealing with Ins\(_P_2\)-induced Ca\(^{2+}\) release, the detailed kinetics of the non-specific leak were not addressed. Since the Ins\(_P_2\)-induced Ca\(^{2+}\) release is always measured as the increase in the basal Ca\(^{2+}\) leak induced by the application of Ins\(_P_2\), we have investigated the detailed kinetics of the passive Ca\(^{2+}\) leak from the non-mitochondrial Ca\(^{2+}\) stores in permeabilized A7r5 cells in the absence of Ins\(_P_2\). The decrease in store-Ca\(^{2+}\) content after correction for passive binding did not follow single-exponential kinetics. The non-single-exponential nature of the spontaneous release persisted after reducing the initial store-Ca\(^{2+}\) content to 40% of its original value. The efflux traces could be fitted by two exponential rates, suggesting that individual store units were heterogeneous with respect to their passive Ca\(^{2+}\)-permeability.

MATERIALS AND METHODS

A7r5 cells, an established cell line derived from embryonic rat aorta, were used between the 7th and the 17th passage after receipt from the American Type Culture Collection (Bethesda, MD, U.S.A.), and were subcultured weekly by trypsin treatment. The cells were cultured in an atmosphere containing 9% CO\(_2\) in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, 3.8 mM l-glutamine, 0.9% (v/v) non-essential amino acids, 85 i.u.·ml\(^{-1}\) penicillin and 85 \(\mu\)g·ml\(^{-1}\) streptomycin at 37 °C. The cells were seeded in 12-well dishes (4 cm\(^2\); Costar Europe, Badhoevedorp, The Netherlands) at a density of approximately 10\(^4\) cells·cm\(^{-2}\).

\(^{45}\)Ca\(^{2+}\) fluxes on permeabilized cells were done on a thermostatically controlled plate at 25 °C. The culture medium was aspirated and replaced with 1 ml of permeabilization medium containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 2 mM MgCl\(_2\), 1 mM ATP, 1 mM EGTA and 20 \(\mu\)g·ml\(^{-1}\) saponin. The saponin-containing solution was removed after 10 min and the cells were washed once with a similar saponin-free solution.

RESULTS AND DISCUSSION

Permeabilized A7r5 cells slowly lost their accumulated Ca\(^{2+}\) during incubation in a Ca\(^{2+}\)-free efflux medium containing 2 \(\mu\)M thapsigargin (Figure 1A). The decrease in total Ca\(^{2+}\) content of the stores as a function of time was not linear in this semi-
logarithmic plot, indicating that the curve could not be described by a single exponential function. The total Ca\(^{2+}\) content of the stores represented Ca\(^{2+}\) that was actively accumulated in the stores plus Ca\(^{2+}\) that was passively bound to the store membrane.

To measure the kinetics of the release of passively bound Ca\(^{2+}\), the stores were loaded with \(^{45}\text{Ca}\) in the presence of 10 \(\mu\text{M}\) of the Ca\(^{2+}\) ionophore, A23187, and the efflux was monitored as a function of time (Figure 1A). The amount of passively bound Ca\(^{2+}\) at each time point was then subtracted from the total Ca\(^{2+}\)-content to obtain the time course of the release of the actively accumulated Ca\(^{2+}\) (Figure 1B). The decrease in the content of actively accumulated Ca\(^{2+}\) as a function of time was not linear in this semilogarithmic plot, indicating that the curve could not be described by a single exponential function. A similar result was obtained if 2 \(\mu\text{M}\) thapsigargin, instead of 10 \(\mu\text{M}\) A23187, was used to measure the contribution of the passive Ca\(^{2+}\) binding (results not shown). The use of detergents to measure the passive Ca\(^{2+}\) binding was not possible, since the cells detached under these conditions.

The kinetics of the spontaneous leakage of actively accumulated Ca\(^{2+}\) were better resolved when the data were replotted as fractional loss as a function of time (inset to Figure 1B). The fractional loss is defined here as the amount of actively accumulated Ca\(^{2+}\) leaving the stores in 1 min (i.e. the rate of Ca\(^{2+}\)-release) divided by the content of actively accumulated Ca\(^{2+}\) at that time. The fractional loss was highest at the first time-point and then progressively decreased to become almost constant from 10 min onwards, i.e. at the time when the content of actively accumulated Ca\(^{2+}\) had fallen to about 40\% of its initial value.

Since basal Ins\(_P_3\) levels in a permeabilized system can be high enough to trigger Ca\(^{2+}\) release [15], we explored the possibility that Ca\(^{2+}\) release through some open, Ins\(_P_3\)-sensitive Ca\(^{2+}\) channels contributed to the non-single-exponential nature of the passive Ca\(^{2+}\) leak. The endogenous level of Ins\(_P_3\) was measured using a commercial Ins\(_P_3\)-binding-protein kit. The basal level was 1 nM, which was far below the accurately determined threshold for Ins\(_P_3\)-induced Ca\(^{2+}\) mobilization in these cells [16]. This low Ins\(_P_3\) concentration, when compared with the value observed in permeabilized rat hepatocytes [15], was probably because the cell density obtained with the present experimental conditions was much lower (3 \(\times\) 10\(^6\) cells \(\text{ml}^{-1}\) compared with 10\(^7\) rat hepatocytes \(\text{ml}^{-1}\)) and because new medium was added at 2 min intervals, thereby preventing a progressive accumulation of Ins\(_P_3\). Channel openings do not occur in cerebellar endoplasmic-reticulum vesicles fused with planar lipid bilayers in the absence of Ins\(_P_3\) [17]. This finding does not support the hypothesis that the basal Ca\(^{2+}\) leak observed in the present work represented a non-specific Ca\(^{2+}\) flux through the Ins\(_P_3\) receptor. However, it should be noted that the basal Ca\(^{2+}\) leak observed in intact cells is also partially related to opening of the Ins\(_P_3\) receptor by basal Ins\(_P_3\) concentrations [18–21] and can be reduced by heparin [19–21]. We therefore also studied the kinetics of the passive leak of Ca\(^{2+}\) in the presence of 100 \(\mu\text{g} \cdot \text{ml}^{-1}\) of heparin. Figure 2(A) illustrates that, under these conditions, the non-specific Ca\(^{2+}\) leak still presented non-single-exponential kinetics. The Ins\(_P_3\) receptor was inhibited under these conditions, since the stores failed to respond to up to 3.2 \(\mu\text{M}\) Ins\(_P_3\) in the presence of this concentration of heparin (Figure 2B).

The kinetics of the spontaneous release of actively accumulated Ca\(^{2+}\) in control stores and in stores in which the initial Ca\(^{2+}\) content was reduced to 40\% of its control value by application of 200 nM Ins\(_P_3\) during the last 3 min of loading are shown in Figure 3(A). The non-single-exponential nature of the spontaneous release also occurred in the less-filled stores. Figure 3(B) shows that the fractional loss, as a function of time, did not depend on the initial level of store loading. The fractional loss from the stores which were full initially, at 10 min, and the loss at the first time point from the initially less-well-filled stores, differed by a factor of 5, although the Ca\(^{2+}\) content of the stores was similar. This finding excludes the possibility that Ca\(^{2+}\) was released from a homogeneous population of stores with a rate
that was determined solely by the Ca\(^{2+}\) gradient across the store membrane.

Another explanation for the non-single-exponential kinetics of the non-specific Ca\(^{2+}\) leak is that there are at least two populations of stores releasing their Ca\(^{2+}\) in an exponential way but with different rate constants. The model calculations represented in Figure 4(A) illustrate how the overall Ca\(^{2+}\) content of stores that were initially 100\% filled or 40\% filled would decrease if 70\% of the stores released their Ca\(^{2+}\) with a rate constant of 0.05 min\(^{-1}\) and 30\% of the stores with a rate constant of 1 min\(^{-1}\). This model calculation could explain the following experimental observations. (1) The fractional loss of Ca\(^{2+}\) was highest at the first time-point, then progressively decreased and finally became almost constant (Figure 1B, inset). (2) The fractional loss of Ca\(^{2+}\), as a function of time, was independent of the initial level of store loading (Figure 3B). (3) The fractional loss of Ca\(^{2+}\), after 10 min, for the initially filled stores and the fractional loss at the first time point for the initially less filled stores (Figure 3B) differed by a factor of 5, although the overall store Ca\(^{2+}\) content was similar.

Since the experimentally observed passive loss of accumulated Ca\(^{2+}\) can be modelled by two exponential curves, we have
Figure 4 Model calculation of the net Ca\(^{2+}\) leak from two populations of stores releasing their Ca\(^{2+}\) with a different rate constant.

(A) shows the decrease in store Ca\(^{2+}\) content during incubation in efflux medium (logarithmic scale). The store Ca\(^{2+}\) content at time \(t\) is given by the following equation:

\[ C_t = C_0 \cdot 0.7 \cdot e^{-0.05t} + C_0 \cdot 0.3 \cdot e^{-1.00t} \]

where \(C_0\) is the Ca\(^{2+}\) content at time 0 and was set at either 100\% (●) or 40\% (○). (B) The fractional loss as a function of time for the curves in (A) on a linear scale.

Figure 5 Kinetics of Ins\(_P\)-induced Ca\(^{2+}\) release

(A) shows the decrease in Ca\(^{2+}\) content of the stores during incubation in efflux medium from 10 min onwards and its modification by the addition of 0.5 \(\mu\)M Ins\(_P\) as indicated by the bar above the trace (logarithmic scale). (B) shows the fractional Ca\(^{2+}\) loss as a function of time (linear scale). The results shown in the graphs are typical of four experiments.

because it was tightly bound to the inside of the store [10]. Alternatively, more than two exponentials would be needed for a perfect fit of all the data.

The physiological implication of the double-exponential nature of the spontaneous release could be that there are at least two store compartments that release their Ca\(^{2+}\) with a 17-fold difference in rate constant (0.06 min\(^{-1}\) and around 0.98 min\(^{-1}\)). It is unlikely that mitochondria are one of these compartments, since NaN\(_3\) (10 mM) was used as a mitochondrial inhibitor and since the free Ca\(^{2+}\) concentration of the uptake medium (100 nM) was set below the threshold for mitochondrial Ca\(^{2+}\) uptake. Ca\(^{2+}\) uptake under these conditions was not inhibited by adding 10 \(\mu\)M oligomycin or carbonyl cyanide \(p\)-trifluoromethoxyphenylhydrazone in addition to NaN\(_3\) (results not shown). Finally, more than 95\% of the accumulated Ca\(^{2+}\) was released by the addition of Ins\(_P\) [14]. All of these findings indicate that mitochondrial Ca\(^{2+}\) uptake did not occur under our experimental conditions.
conditions. The two compartments were, therefore, of non-mitochondrial origin. They both seemed to be InsP_2-sensitive, since the stimulation with 200 nM InsP_2 decreased the initial Ca^{2+} content of both compartments by a factor of about 2.5. Indeed, the two equations given above indicate that the calculated size of the slowly leaking compartment, which represented 29.1% of the total store capacity at zero time in the absence of InsP_2, decreased to 10.5% of the total store content at zero time following preincubation in 200 nM InsP_2. In addition, the calculated size of the more leaky compartment, which represented 67.0% of the total store capacity at zero time in the absence of InsP_2, decreased to 28.4% of the total Ca^{2+} store content at zero time following preincubation in 200 nM InsP_2. Preincubation with 200 nM InsP_2 therefore must have released Ca^{2+} from both compartments.

The release of Ca^{2+} induced by InsP_2 does not follow a single-exponential function [6,9,16,22], but these kinetics are unrelated to the non-single-exponential kinetics of the spontaneous Ca^{2+} release. Figure 5 shows the effect of adding 0.5 µM InsP_2 after 13.5 min of efflux, i.e. at a time when the spontaneous release became single-exponential. The InsP_2-induced Ca^{2+} release under these conditions was still characterized by an initial fast release, followed by a progressive decrease in fractional loss as the time of incubation in InsP_2 was prolonged (Figure 5B). The finding that the InsP_2-induced Ca^{2+} release could not be fitted by a single-exponential function at a time when the spontaneous release was mono-exponential, as well as the finding (Figure 3) that preincubation in the presence of 200 nM InsP_2 did not change the double-exponential kinetics, indicate that both phenomena were unrelated.

In conclusion, we have shown that individual non-mitochondrial Ca^{2+} store units are heterogeneous with respect to their passive Ca^{2+} permeability and that at least two compartments with different modes of passive Ca^{2+} leak exist. The nature of these compartments, which are InsP_2-sensitive, is unknown, although it is possible that peripheral endoplasmic reticulum, central endoplasmic reticulum and the nuclear envelope may have different passive Ca^{2+}-release kinetics. Recent data indicate that one or both of these components of the non-specific leak pathway may play a role in the Ca^{2+}-release induced by osmotic shock [23].


**REFERENCES**


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