Thapsigargin inhibits Ca^{2+} entry into human neutrophil granulocytes

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The mechanism of Ca^{2+} entry after ligand binding to receptors on the surface of non-excitabile cells is a current focus of interest. Considerable attention has been given to Ca^{2+} influx induced by emptying of intracellular pools. Thapsigargin, an inhibitor of microsomal Ca^{2+} -ATPase, is an important tool in inducing store-regulated Ca^{2+} influx. In the present paper we show that,

at concentrations above 500 nM, thapsigargin also has an opposite effect: it inhibits store-regulated Ca^{2+} influx into Fura-2-loaded human neutrophil granulocytes. As thapsigargin has been frequently applied at concentrations up to 2 μM , its inhibitory action on plasma-membrane Ca^{2+} fluxes deserves consideration.

INTRODUCTION

The tumour-promoting agent thapsigargin has been shown to inhibit selectively microsomal Ca^{2+} -ATPase with the consequent release of Ca^{2+} from intracellular stores [1,2]. Experiments carried out with this drug have provided significant support for the capacitative model of Ca^{2+} entry into non-excitabile cells [3]. According to this model, depletion of the internal Ca^{2+} stores induces opening of a Ca^{2+} conductance (store-regulated Ca^{2+} pathway) in the plasma membrane of various cell types [4]. The effective concentration of thapsigargin is extremely low, maximal effect being obtained around 50 nM [5–7]. However, in many studies, concentrations of up to a few $\mu\text{mol/litre}$ have been applied [1,8–12]. There have been sporadic observations about additional effects of higher concentrations of thapsigargin. After preliminary observations [13], it has recently been clearly shown that, in the concentration range 0.3–3 μM , thapsigargin inhibits voltage-activated Ca^{2+} channels of adrenal glomerulosa cells [7].

Stimulation of neutrophil granulocytes by chemotactic agents induces a Ca^{2+} signal, the first phase of which is due to Ca^{2+} release from inositol trisphosphate-sensitive stores and the second phase is the consequence of Ca^{2+} influx from the extracellular space [14]. Neutrophil granulocytes do not possess voltage-activated Ca^{2+} channels in their plasma membranes [14] but functioning of the store-regulated pathway has been demonstrated [5,15,16]. Ca^{2+} influx induced by chemotactic stimulation or thapsigargin treatment has the same characteristics, and it has been suggested that Ca^{2+} movement proceeds under both conditions via the same route [6]. Thus, in neutrophil granulocytes, store-regulated Ca^{2+} influx seems to play a major role in the response to physiological stimuli and they therefore provide a good model for investigation of the effects of thapsigargin on non-voltage-regulated channels.

MATERIALS AND METHODS

Materials

Fura-2/AM was obtained from Calbiochem, Percoll from Pharmacia and dimethyl sulphoxide (DMSO), phorbol 12-myristate 13-acetate (PMA), staurosporin and thapsigargin were from Sigma. All other reagents were of research grade.

The routinely used medium (referred to as Ca^{2+} medium) contained (mM) NaCl (145), KCl (5), MgCl_2 (1), CaCl_2 (0.8),

Hepes (10) and glucose (5) (pH 7.4). The Ca^{2+} -free medium consisted of the same constituents except for CaCl_2 .

Cell isolation

Human neutrophils were obtained from blood of healthy volunteers by dextran sedimentation followed by Percoll gradient centrifugation as described in [17]. Contaminating erythrocytes were removed by hypotonic lysis. Cells were finally suspended in Ca^{2+} medium at a density of $5 \times 10^7/\text{ml}$ and kept at room temperature. Typical preparations contained more than 95% neutrophils.

Loading with Fura-2

Neutrophils ($5 \times 10^7/\text{ml}$) were incubated in the presence of 4 μM Fura-2/AM for 30 min at 37 °C. Thereafter they were washed to remove the extracellular dye and resuspended in Ca^{2+} medium at a density of $5 \times 10^7/\text{ml}$. These Fura-2-loaded cells were stored at 4 °C. No increase in the concentration of the external dye was evident for 5 h and the experiments were terminated within this period.

Measurement of $[\text{Ca}^{2+}]_i$

Unless otherwise stated, 10^6 Fura-2-loaded cells were suspended in 3 ml of Ca^{2+} medium. Changes in fluorescence were recorded in a Deltascan dual-wavelength spectrofluorimeter (Photon Technology International, South Brunswick, NJ, U.S.A) using 340 nm and 380 nm for excitation and 505 nm for emission wavelength. Measurements were made at 37 °C with continuous stirring. $[\text{Ca}^{2+}]_i$ was calculated from the ratio of fluorescence excited at 340 and 380 nm, by the method detailed in [18].

Measurement of Mn^{2+} influx

This measurement was performed under the same conditions as for $[\text{Ca}^{2+}]_i$ except that the excitation wavelength was 360 nm, at which fluorescence of Fura-2 is independent of $[\text{Ca}^{2+}]_i$. Mn^{2+} influx was initiated by addition of 100 μM MnCl_2 .

RESULTS

Effect of thapsigargin on $[\text{Ca}^{2+}]_i$

A typical rise in $[\text{Ca}^{2+}]_i$ was observed when 50 nM thapsigargin was added to Fura-2-loaded neutrophil granulocytes in the

Figure 1 Effect of thapsigargin on $[Ca^{2+}]_i$ of human neutrophils

Fura-2-loaded cells were exposed to 50 nM (a) or 2 μ M (b) thapsigargin at the time points indicated by the arrow. Cells were suspended in either the standard Ca^{2+} medium (+ Ca^{2+}) or Ca^{2+} medium supplemented with 3 mM EGTA ($-Ca^{2+}$). One representative experiment of ten similar ones is shown.

Figure 2 Effect of thapsigargin on Ca^{2+} entry through the store-regulated pathway

(a) Ca^{2+} entry into Ca^{2+} -depleted cells was followed. Fura-2-loaded cells were kept for 3 h at 4 °C and for another 1 h at room temperature in Ca^{2+} medium supplemented with 3 mM EGTA. Subsequently they were diluted (50 times) in Ca^{2+} -free medium. The first arrow indicates addition of 50 nM or 2 μ M thapsigargin or an equal volume of DMSO; the second arrow marks the time-point of addition of 1 mM $CaCl_2$. (b) Ca^{2+} entry into non-depleted cells was followed in normal Ca^{2+} medium. The first and second arrows indicate addition of 1 μ M cyclopiazonic acid (CPA) and 2 μ M thapsigargin respectively. One representative experiment of five similar ones is shown.

presence of 0.8 mM external Ca^{2+} (Figure 1a). The increase in $[Ca^{2+}]_i$ was complete in about 3 min and reached 375 ± 50 nM (mean $\pm$ S.D. of six different preparations). Basically similar curves were obtained when the concentration of thapsigargin was varied between 10 and 500 nM but the slope of the initial rise became slightly steeper as the concentration of the drug was increased. When external $[Ca^{2+}]$ was buffered by the addition of 3 mM EGTA, the same concentration of thapsigargin induced an initial rise in $[Ca^{2+}]_i$ up to 176 ± 23 nM ($n = 5$) but then the curve declined and $[Ca^{2+}]_i$ gradually returned to the initial value. These results are in good agreement with data reported earlier for both neutrophil granulocytes and other cell types [2,5,7] and they reflect the fact that thapsigargin induces an initial release of Ca^{2+} from intracellular stores (the phase independent of external Ca^{2+}) which is followed by influx from the extracellular space (the phase dependent on external Ca^{2+}).

Basically different results were obtained when thapsigargin was applied at concentrations above 500 nM. Representative curves recorded in the presence of 2 μ M thapsigargin are shown in Figure 1(b). In the presence of 0.8 mM external Ca^{2+} , addition

of thapsigargin induced a biphasic rise in $[Ca^{2+}]_i$. The first phase was similar to that observed with 50 nM thapsigargin, i.e. 250 nM $[Ca^{2+}]_i$ was attained in approx. 40 s. However, a peak of about 400 nM (410 ± 53 nM; $n = 4$) was reached within the next 30 s followed by a sharp decrease in Ca^{2+} concentration. $[Ca^{2+}]_i$ approached the initial value slowly, in 3–5 min. The shapes of the curves obtained in the presence of external Ca^{2+} and in the presence of EGTA were almost identical, although the amplitude of the latter peak was lower (275 ± 60 nM; $n = 4$). The peak-like second phase of the fluorescence increase was observed in both the presence and absence of extracellular Ca^{2+} ; thus it must reflect release of Ca^{2+} from additional intracellular stores or altered kinetics of Ca^{2+} traffic under the influence of high thapsigargin concentration. Apparently, in the presence of 2 μ M thapsigargin the release from internal sources took place but Ca^{2+} influx from the extracellular space was impaired. The curves shown in Figure 1(b) were typically observed when thapsigargin was applied at a concentration of 1–5 μ M. In the range of 500 nM–1 μ M thapsigargin, $[Ca^{2+}]_i$ attained a maximum and then declined moderately (not shown). These findings suggest

Figure 3 Comparison of the inhibition of Ca^{2+} entry by thapsigargin (a) and PMA (b)

The quenching effect of $100 \mu\text{M}$ MnCl_2 (given at 0 s) was measured in Fura-2-loaded cells suspended in Ca^{2+} medium. Additions were as follows: A, 2 μM thapsigargin; B, 300 nM staurosporin and 2 μM thapsigargin; C, 50 nM thapsigargin; D, 10 nM PMA and 50 nM thapsigargin; E, 300 nM staurosporin, 10 nM PMA and 50 nM thapsigargin; F, 50 nM thapsigargin. Staurosporin, PMA and thapsigargin were added 5 min, 3 min and 2 min respectively before MnCl_2 . One representative experiment of six similar ones is shown.

that concentrations of thapsigargin above 500 nM could interfere with Ca^{2+} influx through the store-regulated pathway of the plasma membrane of neutrophil granulocytes.

Effect of thapsigargin on Ca^{2+} influx

The store-regulated Ca^{2+} pathway can be induced to open by depletion of intracellular Ca^{2+} stores by prolonged incubation of the cells in the presence of EGTA [16]. Our results with depleted cells are summarized in Figure 2(a). After Fura-2-loaded granulocytes had been kept in the presence of 3 mM EGTA for 4 h, restoration of the external Ca^{2+} concentration to 1 mM resulted in an increase in $[\text{Ca}^{2+}]_i$ to 500 nM. When 50 nM thapsigargin was added before Ca^{2+} , the rise in $[\text{Ca}^{2+}]_i$ approached 800 nM. The difference may be due to partial refilling of the stores in the absence of thapsigargin or incomplete depletion of the stores by external EGTA. In contrast, when 2 μM thapsigargin was applied before the administration of Ca^{2+} , the signal was significantly reduced, $[\text{Ca}^{2+}]_i$ reaching only 200 nM. This value was in the same range as that obtained by addition of similar Ca^{2+} doses to non-depleted cells in the absence of thapsigargin and it may represent reaction of Ca^{2+} with external dye or Ca^{2+} influx via non-specific pathways.

Higher concentrations of thapsigargin apparently prevent the entry of Ca^{2+} through the plasma-membrane pathway opened by depletion of intracellular Ca^{2+} stores. The experiment in Figure 2(a) indicates that the inhibitory effect of high thapsigargin concentrations does not require the transitory rise in $[\text{Ca}^{2+}]_i$ brought about by the drug, as, in the presence of EGTA, Ca^{2+} depletion occurs gradually, without a significant increase in $[\text{Ca}^{2+}]_i$.

Cyclopiazonic acid (CPA) has also been shown to inhibit microsomal Ca^{2+} -ATPase and to induce similar changes in $[\text{Ca}^{2+}]_i$ to those produced by thapsigargin [5]. When 1 μM CPA was added to Fura-2-loaded neutrophils, effects similar to those shown in Figure 1(a) for thapsigargin were observed. However, up to 30 μM CPA, no sign of any inhibition of Ca^{2+} entry was detected by either of the above techniques. Sustained elevation of $[\text{Ca}^{2+}]_i$ induced by CPA was reversed by thapsigargin added in the micromolar range (Figure 2b).

The operation of a Ca^{2+} -transport pathway in the plasma membrane can also be detected on the basis of the quenching of Fura-2 fluorescence by Mn^{2+} entering via the open Ca^{2+} routes. Typical results obtained by this technique are depicted in Figure

3(a). In the presence of $100 \mu\text{M}$ Mn^{2+} the fluorescence of Fura-2-loaded neutrophils decreases very slowly, with an unchanged rate up to at least 10 min (not shown). Addition of 50 nM thapsigargin before Mn^{2+} induces a sharp decrease in the fluorescence (Figure 3a, trace C). The access of Mn^{2+} to the Fura-2-containing compartment indicates the opening of transport pathways for various bivalent cations in the plasma membrane. In the presence of thapsigargin, this occurs as a consequence of emptying of the intracellular Ca^{2+} stores and opening of the store-regulated route. However, when thapsigargin was applied in concentrations above 500 nM, the quenching effect of Mn^{2+} decreased, almost disappearing in the presence of 2 μM thapsigargin (Figure 3a, trace A). Thus, in the presence of high thapsigargin concentrations, the store-regulated Ca^{2+} pathway did not open, although emptying of the internal Ca^{2+} stores was clearly detectable (see Figure 1b).

Possible role of phosphorylation

Previous reports have demonstrated that Ca^{2+} entry after emptying of the internal stores by thapsigargin could be inhibited by both the chemotactic agent formylmethionyl-leucylphenylalanine (fMLP) and phorbol esters [19–21]. The effect of fMLP was transient, enhanced by phosphatase inhibitors and only partially inhibited by staurosporin. The inhibition obtained in the presence of the phorbol ester was permanent, independent of phosphatase inhibitors and completely blocked by staurosporin [20,22]. These data raise the possibility of the involvement of a phosphorylation reaction in the regulation of the store-operated Ca^{2+} influx and suggest that the inhibitory effect of fMLP and phorbol esters may be mediated via different kinases.

As thapsigargin has been shown to induce protein phosphorylation in platelets [1], we compared the effects of the phorbol ester PMA (Figure 3b) and high concentrations of thapsigargin (Figure 3a) on Ca^{2+} entry induced by emptying of the internal stores. PMA effectively inhibited Mn^{2+} entry into neutrophils pretreated with a low concentration (50 nM) of thapsigargin (Figure 3b, traces F and D). This effect of PMA was completely prevented by staurosporin (trace E). In contrast, the inhibitory action of high thapsigargin concentration (2 μM) was not affected by staurosporin (Figure 3a, trace B). Preincubation of the Fura-2-loaded neutrophils with 2 μM okadaic acid for 5–15 min did not result in any detectable change in Ca^{2+} movement induced by various concentrations of thapsigargin (not shown).

DISCUSSION

In the experiments summarized in Figures 1–3, three different lines of observation suggest that high (above 500 nM) concentrations of thapsigargin inhibit store-regulated Ca^{2+} influx in neutrophil granulocytes: (1) in the presence of 0.8 mM Ca^{2+} , the sustained increase in $[\text{Ca}^{2+}]_i$ after addition of thapsigargin is missing and the recorded curve showed a similar shape to that obtained in the absence of external Ca^{2+} ; (2) the rise in $[\text{Ca}^{2+}]_i$ in previously depleted cells on addition of 1 mM Ca^{2+} is strongly inhibited; (3) Mn^{2+} quenching that was clearly detectable in the presence of low thapsigargin concentration was almost completely blocked by a high concentration of the drug. Inhibition of store-regulated Ca^{2+} influx seems to be an inherent property of the tumour-promoting drug thapsigargin and not the consequence of a transient rise in $[\text{Ca}^{2+}]_i$ initiated by emptying of intracellular stores.

A partial inhibition of the voltage-activated Ca^{2+} channels (both T and L type) by thapsigargin has recently been reported in an excitable tissue, the adrenal glomerulosa cells [7]. The present paper extends these data to another Ca^{2+} -transporter, the store-regulated pathway of neutrophil granulocytes, a typically non-excitabile cell type. In our experiments the inhibition is almost complete. The effect of thapsigargin may be more general and not limited to specific channel and specific cell types. The inhibitory effect of thapsigargin on Ca^{2+} entry is only evident when the drug is applied at concentrations above 500 nM whereas Ca^{2+} release from intracellular stores is achieved by concentrations below 50 nM. The inhibition demonstrated in our experiments deserves serious consideration, as thapsigargin was (and probably is) widely used in concentrations of up to 1 or 2 μM [1,8–12]. Application of thapsigargin in these concentrations may thus lead to erroneous conclusions.

Our investigations suggest that inhibition of the store-regulated Ca^{2+} influx induced by high thapsigargin concentration is probably mediated via a pathway not involving protein kinase C. Participation of other kinases cannot be excluded although the present experiments did not provide any clear indication for it. Elucidation of the mechanism of the inhibitory action of thapsigargin could help to reveal the physiological regulation of Ca^{2+} entry into granulocytes.

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