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FEBS Letters 339 (1994) 307–311

FEBS LETTERS

FEBS 13686

Protein kinase C activates capacitative calcium entry in the insulin secreting cell line RINm5F

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Received 12 January 1994

Abstract

This study examines the calcium store-regulated (capacitative) calcium influx pathway in the endocrine pancreatic cell line RINm5F, utilizing thapsigargin. After preincubation of the cells with the phorbol ester TPA, thapsigargin induced a sustained elevation of cytosolic calcium as well as a sustained stimulation of manganese entry, the latter being used to assess calcium influx. Thapsigargin given alone provoked a smaller and only transient elevation of cytosolic calcium and stimulation of manganese entry. The protein kinase C inhibitor staurosporine antagonized the effect of the phorbol ester. Verapamil, nifedipine, or measures to hyperpolarize the cells exerted no inhibitory action against this effect, which excludes an involvement of voltage-dependent calcium channels. In conclusion, our data shows for the first time that protein kinase C stimulation activates the capacitative calcium influx pathway of endocrine pancreatic insulin-producing cells.

Key words: Calcium entry; Thapsigargin; Protein kinase C; Insulin-secreting cell; RINm5F cell

1. Introduction

In certain types of cells, calcium release from intracellular stores induces calcium influx from outside the cells, across the plasma membrane into the cytosol [1]. This phenomenon is termed capacitative or calcium store-induced calcium entry. Capacitative calcium entry can be triggered by any measure that releases calcium from intracellular stores [2]. Thus, it may account for at least a part of the calcium influx which is mediated by agents that activate phospholipase C, leading to generation of the intracellular calcium store-opening messenger inositol 1,4,5-trisphoshate (IP_3).

The molecular mechanism of capacitative calcium entry is yet unresolved and a matter of intense debate [3,4]. Occurrence of capacitative calcium entry has been demonstrated especially in various types of non-excitable cells, where it can represent the predominant calcium influx pathway [5,6]. In contrast, in excitable cells this phenomenon has been characterized less well.

Capacitative calcium entry can be induced selectively and directly, without prior activation of any signal transduction cascade, by thapsigargin, an inhibitor of intracellular calcium pumps [2]. When calcium uptake into the intracellular calcium stores is inhibited by thapsigargin, the balance between permanent leakage of calcium from the stores and continous reuptake is disrupted, and the stores become depleted rapidly. Using thapsigargin, we have examined capacitative calcium entry in RINm5F rat insulinoma cells, a continous cell line which has been established as a model for β-cells of the endocrine pancreas [7]. We found that in these cells thapsigargin induces a pronounced, sustained entry of calcium when a protein kinase C-stimulating phorbol ester is added prior to thapsigargin, whereas thapsigargin alone causes an only small and short-lived calcium entry. This suggests an activating action of protein kinase C on capacitative calcium entry in RINm5F cells. The implications of our results for capacitative calcium entry in β-cells and other endocrine cells are discussed.

2. Experimental

2.1. Cell culture

RINm5F rat insulinoma cells were cultured as described by Praz et al. [8].

2.2. Loading of cells with the fluorescent calcium indicator fura-2

This was performed as described previously [9]. Cells were detached at subconfluence from the culture flasks by incubation with EDTA/trypsin (0.27 mM/0.025%) for 5 min at 37°C. To recover from the detachment procedure the cells were then maintained in spinner culture for 3 h, at 37°C, suspended in RPMI 1640 supplemented with 10 mM HEPES and 1% (v/v) newborn calf serum (spinner medium). Afterwards, the cells were washed and resuspended in spinner medium at

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Abbreviations: DMSO, dimethyl sulfoxide; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediamine tetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

SSDI 0014-5793(94)00070-C

37°C, followed by addition of fura-2 AM at a final concentration of 5 μM, added from a 5 mM stock solution in DMSO. After 1 h, the cells were washed and resuspended in spinner medium until usage. This spinner incubation was done at room temperature to minimize fura-2 leakage.

2.3. Measurements of the free cytosolic calcium concentration

Fura-2-loaded RINm5F cells were washed and resuspended at a cell density of 1×10^6/ml in a modified Krebs–Ringer buffer containing 136 mM NaCl, 4.8 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 3 mM Glucose, 25 mM HEPES/NaOH, pH 7.4. Fluorescence measurements were performed with a Perkin-Elmer LS-50 fluorescence spectrometer, in continously stirred cuvettes, at 37°C. Excitation and emission wavelengths were set to 340 and 505 nm, respectively. Selected experiments were made with the excitation wavelength alternating between 340 and 380 nm. Lipophilic agents were added as solutions in DMSO. The solvent did not affect the measurements in the respective concentrations. Calibration and calculation of the cytosolic calcium concentration were performed as described [10,11]. Commercial personal computer software was used for calculation and graphical presentation of the data.

Correction for extracellular fura-2 was performed according to a described procedure [12]: Addition of small concentrations of manganese followed rapidly by an excess of the manganese chelator diphenyltetraminepentaacetate (DTPA) causes a transient quenching of extracellular fura-2 without manganese entering the cells. Repeated application of this procedure allowed an estimation of extracellular fura-2 at various points during an experiment. In line with other reports about insulinoma cell lines [12,13], we found the extracellular dye concentration to increase linearly during measurements at 37°C. Therefore, as a routine, we added 50 μM $MnCl_2$, 100 μM DTPA in the beginning and 100 μM $MnCl_2$, 200 μM DTPA at the end of each experiment that was conducted at 37°C to assess the development of the extracellular dye concentration, and corrected the fluorescence data accordingly. No increase of the extracellular dye concentration was observed in the presence of 250 μM sulfinpyrazone or when the temperature was reduced to 30°C. Since lanthanum chloride interfered with manganese- induced quenching of extracellular fura-2, experiments involving lanthanum chloride could not be conducted with the routine correction procedure. Therefore, the respective experiments were performed in the presence of sulfinpyrazone to minimize dye efflux, and the amount of initially present extracellular dye was estimated with a single, initial addition of $MnCl_2$/DTPA in a separate run under the same conditions, but without lanthanum chloride.

2.4 Assessment of calcium influx by manganese-induced quenching of fura-2

This was performed essentially as described by Sage et al. [14]. Excitation and emission wavelengths were 361 and 505 nm, respectively. Since extracellular fura-2 was present, the initial drop of fluorescence after addition of manganese was to a large extent due to quenching of this extracellular dye. Therefore, to obtain a better resolution of the initial phase of manganese influx, in one set of experiments addition of DTPA a short time after manganese served to determine the part of extracellular dye quenching in the initial quench reaction. The figures for these experiments show the fluorescence traces after correction for quenching of extracellular dye

3. Results

Addition of thapsigargin to RINm5F cells in the presence of extracellular calcium produced a rapid increase in the cytosolic concentration of calcium ions (Fig. 1, trace a). After this initial spike, the calcium concentration declined again to the original level. In contrast, when the protein kinase C-stimulating phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 100 nM) was added before thapsigargin, the thapsigargin-induced elevation of cytosolic calcium was not transient, but the cytosolic calcium concentration reached an elevated plateau (Fig. 1, trace b). In line with the concept of capacitative, store-regulated calcium entry, such a thapsigargin-mediated sustained elevation of cytosolic calcium usually, in various types of cells, is associated with calcium entry across the plasma membrane [2]. The following experiments were performed to test whether the sustained calcium elevation evoked in RINm5F cells by thapsigargin with prior addition of TPA was due to calcium entry, too. One prominent way to assay this kind of calcium entry is the readdition of calcium to cells after the intracellular calcium stores have been depleted in the absence of extracellular calcium [15]. If the store-regulated calcium entry mechanism is active, such a readdition of calcium causes a sustained calcium elevation; if not, usually an only transient elevation occurs. This method allows to examine thapsigargin-induced calcium entry well resolved from the release of calcium from intracellular stores mediated by this agent. In RINm5F cells, readdition of calcium after depletion of intracellular calcium stores with thapsigargin induced an only transient elevation of cytosolic calcium (Fig. 1, trace c), whereas calcium readdition after thapsigargin combined with TPA was associated with a sustained calcium elevation (Fig. 1, trace d). This, again, suggested that a combination of TPA and thapsigargin in RINm5F cells can induce calcium entry, whereas thapsigargin alone apparently cannot, or only to a minor extent. Furthermore, the calcium readdition experiments demonstrated that TPA combined with thapsigargin did not effect the sustained calcium elevation via an altered calcium release from intracellular stores: after chelation of extracellular calcium with EGTA, calcium store depletion by thapsigargin with and without prior addition of TPA induced a similarly transient calcium elevation, the calcium spike after prior addition of TPA not being larger or protracted, but even somewhat smaller than without addition of TPA.

TPA alone caused an instant, small and transient decrease of the cytosolic calcium concentration. This effect was dependent on the presence of extracellular calcium. It was not abolished by the presence of the L-type calcium channel antagonists verapamil (20 μM) or nifedipine (5 μM) (data not shown).

For a further characterization of the TPA/thapsigargin-mediated sustained calcium elevation, we examined the effect of various means for the inhibition of voltage-dependent calcium channels (VDCCs). None of these, presence of 20 μM verapamil or 5 μM nifedipine as well as hyperpolarization of the cells by presence of 200 μM diazoxide or by incubation without extracellular potassium, antagonized the sustained calcium elevation induced by thapsigargin with prior addition of TPA (data not shown). In contrast, 100 μM of the unspecific calcium entry blocker lanthanum chloride completely abolished the sustained calcium elevation (Fig. 2, trace a).

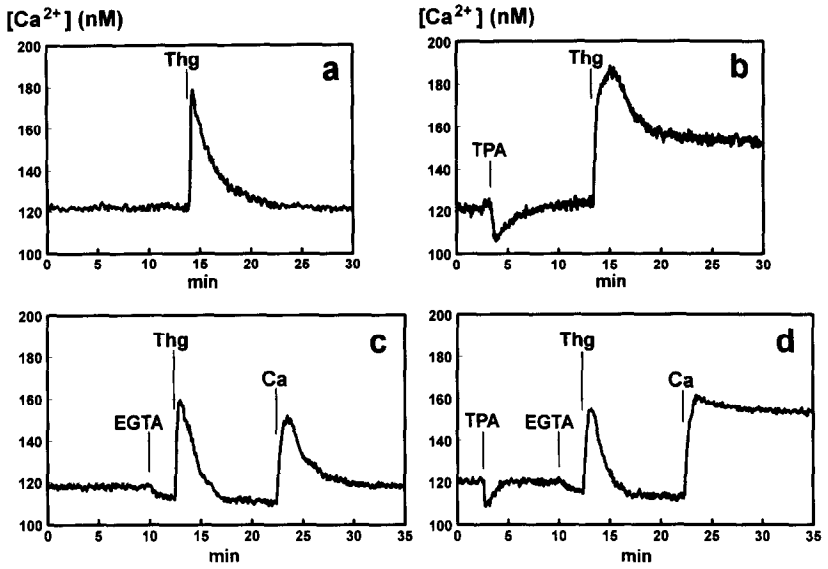

Fig. 1. Effects of thapsigargin, with and without prior addition of the phorbol ester TPA, on cytosolic calcium in RINm5F cells. Addition of 1 μM thapsigargin (Thg) or 100 nM TPA as indicated by the respective dashes. Part a and b, extracellular calcium 2 mM. Part c and d, calcium readdition experiments. Chelation of extracellular calcium (1 mM) with EGTA (1 mM) prior to depletion of intracellular calcium stores with thapsigargin (1 μM), then readdition of calcium (1 mM $CaCl_2$). Results representative of four to six independent experiments.

TPA/thapsigargin then only caused a transient calcium spike, similar to that generated after chelation of extracellular calcium with EGTA.

The modulating effect of TPA on the thapsigargin-mediated calcium elevation could be antagonized completely by addition of 1 μM of the protein kinase C inhibitor staurosporine prior to TPA (Fig. 2, trace b).

The effect of TPA described here was observed similarly with various time intervals between TPA and thapsigargin addition, ranging from 1 to 30 min. The concentration of TPA used, 100 nM, produced a maximal effect. TPA or thapsigargin, alone or in combination, had no effect on the efflux of cytosolic fura-2 into the incubation medium, as tested with manganese/DTPA- mediated reversible quenching of extracellular fura-2 as described in Section 2.

To substantiate our interpretation that TPA/thapsigargin induced calcium entry across the plasma membrane, calcium entry had to be assessed directly. This can be done qualitatively by adding manganese ions to fura-2-loaded cells as a surrogate for extracellular calcium. Quenching of the fura-2 fluorescence by manganese ions entering the cytosol then reflects calcium influx [14]. Fig. 3 shows the respective results without correction for quenching of extracellular fura-2, which is represented by the initial rapid decrease of fluorescence after addition of manganese. In Fig. 4, for a better resolution of the initial manganese entry rate, the fluorescence recordings have been corrected for the contribution of extracellular fura-2. As demonstrated in Figs. 3 and 4, the addition of thapsigargin alone induced an only small and transient acceleration of manganese entry. In contrast, thapsigargin with prior addition of TPA generated a much more pronounced and sustained stimulation of manganese entry. TPA alone had no effect on manganese entry (not shown). The effects of thapsigargin and TPA/

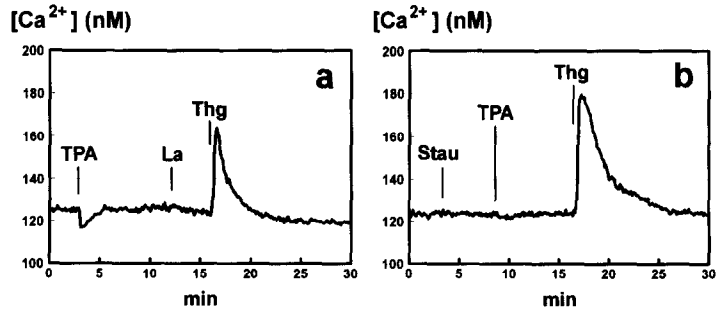

Fig. 2. Part a, effect of thapsigargin (1 μM) with prior addition of TPA (100 nM) on cytosolic calcium, after addition of 100 μM lanthanum chloride (La). Part b, effect of prior addition of staurosporine (Stau, 1 μM) on the action of thapsigargin (1 μM) combined with TPA (100 nM). Part a and b, extracellular calcium 2 mM. Results in each case representative for four independent experiments.

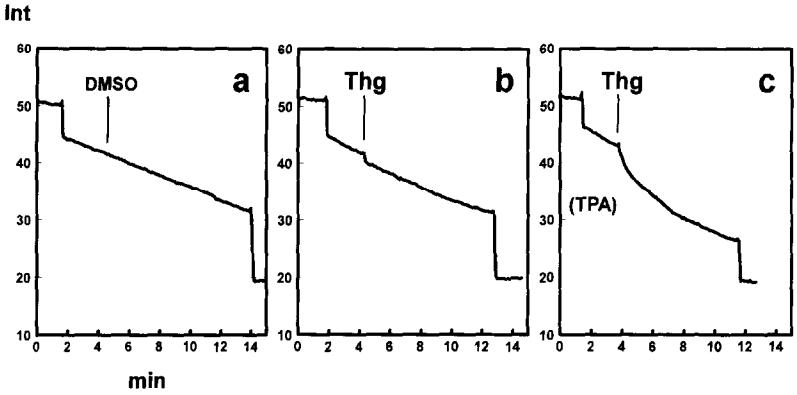

Fig. 3. Effect of thapsigargin, with and without TPA preincubation, on manganese entry in RINm5F cells. Manganese entry assayed as quenching of fura-2 fluorescence (Int, arbitrary units), excitation at 361 nm. The initial sharp drop of fluorescence marks the addition of 200 μM $MnCl_2$ (quenching of extracellular fura-2). The sharp drop of fluorescence at the end of the measurements marks addition of 0.1% (w/v) Triton X-100 for permeabilization of the cells. 2 mM extracellular calcium. Part a, control, addition of 0.1% (v/v) DMSO. Part b, addition of 1 μM thapsigargin (Thg, in 0.1% DMSO). Part c, addition of 100 nM TPA 5 min prior to thapsigargin. Each trace representative for at least three independent experiments.

thapsigargin on manganese entry were not antagonized by various means to inhibit VDCCs, namely addition of 20 μM verapamil or 5 μM nifedipine, or hyperpolarization of cells by omission of extracellular potassium or addition of 200 μM diazoxide (data not shown).

4. Discussion

In RINm5F rat insulinoma cells thapsigargin in the presence of the protein kinase C-stimulating phorbol ester TPA induces a sustained elevation of the cytosolic calcium concentration while thapsigargin alone generates a transient calcium elevation only. Since the sustained calcium elevation mediated by thapsigargin combined with TPA depends on the presence of extracellular calcium, can be inhibited by the unspecific calcium entry blocker lanthanum chloride and is associated with increased influx of manganese ions, it appears to be based on increased entry of calcium across the plasma membrane into the cytosol. Theoretically, two other mechanisms would be conceivable, namely a more complete and prolonged emptying of intracellular calcium stores by thapsigargin when added after TPA, or an inhibition of calcium extrusion by TPA. Our results, however, argue against these interpretations. After chelation of extracellular calcium, when exclusively intracellular calcium release was monitored, thapsigargin combined with TPA did not generate a larger or more prolonged calcium elevation than thapsigargin alone. To imply an action of TPA via an alteration of the intracellular calcium release process this would have had to be the case. On the contrary, intracellular calcium release by thapsigargin in the absence of extracellular calcium generated a smaller calcium peak in the presence than in the absence of TPA. This and the transient decrease of the

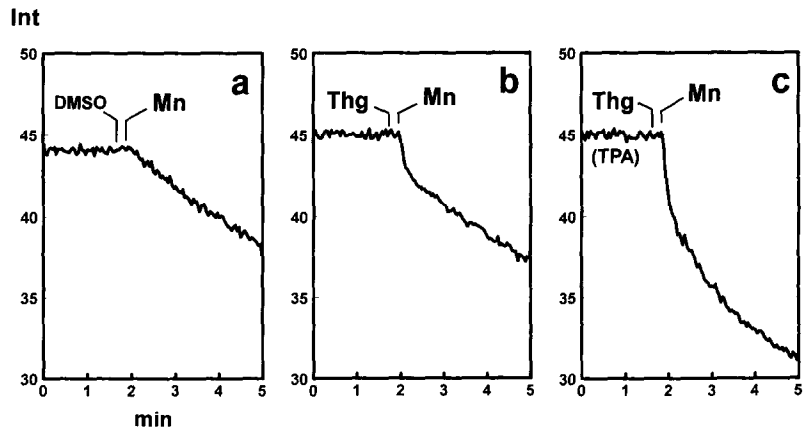

Fig. 4. Effect of thapsigargin, with and without TPA preincubation, on the initial manganese entry rate, manganese (200 μM) added shortly after thapsigargin. Extracellular calcium 2 mM. Results displayed after correction for quenching of extracellular fura-2. Part a, control, only solvent added (0.1% DMSO). Part b, addition of 1 μM thapsigargin (Thg). Part c, addition of 100 nM TPA 5 min before thapsigargin. Each trace representative for at least three independent experiments.

cytosolic calcium concentration immediately after addition of TPA suggest, furthermore, rather a stimulatory than an inhibitory action of TPA on calcium extrusion.

L-type and other types of depolarization-activated calcium channels are not involved in calcium influx induced in RINm5F cells by thapsigargin combined with TPA, since L-type channel blockers and measures to hyperpolarize the cells did not inhibit this influx. Furthermore, it has to be considered that TPA did not increase cytosolic calcium independently of thapsigargin but only modulated the action of thapsigargin. In summary, it can be concluded that in RINm5F cells the action of thapsigargin combined with TPA is similar to the described effect of thapsigargin alone in several other types of cells [2], representing capacitative, calcium store depletion-induced calcium entry.

Since TPA was effective at low concentrations and its effect was antagonized by the protein kinase C inhibitor staurosporine, TPA acted via activation of protein kinase C, according to its properties as protein kinase C-stimulating phorbol ester. In RINm5F cells, thapsigargin alone, without TPA-mediated stimulation of protein kinase C, induced calcium entry only transiently and to a smaller degree, regarding the respective manganese entry data. Apparently protein kinase C can activate the capacitative calcium entry mechanism in RINm5F cells. Any analysis of the mechanism responsible for this will be mostly hypothetical, since knowledge about the mechanisms of capacitative calcium entry is still scarce in general [4]. It is, however, an interesting point in our study that the very initial acceleration of manganese entry after addition of thapsigargin alone often was of a similar magnitude as after addition of thapsigargin in combination with TPA. This, as well as the transient calcium increase in the calcium readdition experiment without TPA, might suggest that capacitative calcium entry in RINm5F cells principally can fully be triggered by thapsigargin-mediated store depletion but is subject to rapid inactivation, and that protein kinase C acts by inhibiting this inactivation.

Irrespective of considerations about the mechanism of protein kinase C-mediated activation of capacitative calcium entry, our study has implications for the evaluation of capacitative calcium entry in RINm5F and other cells. Agents that activate phospholipase C in RINm5F cells cause calcium release from intracellular stores and calcium entry across the plasma membrane [16]. The mechanism of this calcium entry has been unclear. For example, a detailed investigation on calcium entry in RINm5F cells mediated by the phospholipase C agonist vasopressin addressed the involvement of voltage-dependent or second messenger-operated calcium channels, but could not provide evidence supporting such explanations [17]. In contrast, our study shows that in RINm5F cells the capacitative, store-regulated calcium entry pathway can be active during stimulation of protein kinase C. Therefore, since agonists of phospholipase C via generation of diacylglycerol cause a stimulation of protein kinase C, capacitative calcium entry now has to be considered as a main mechanism for the respective agonist-induced calcium entry in RINm5F cells. Since, moreover, these cells are related to endocrine pancreatic β-cells, a role for capacitative calcium entry in the latter has to be considered, too.

Finally, it will be of interest whether in those other types of cells where addition of thapsigargin alone produces no or only little calcium entry, like adrenal chromaffin cells [18] and NG115–401L neuronal cells [19], a similar modulating action of protein kinase C can be observed as described in our study. For example, the effect of thapsigargin in bovine adrenal chromaffin cells [18,20] appears similar to the transient elevation of cytosolic calcium by thapsigargin without TPA in RINm5F cells, and might as well be modulatable by protein kinase C. Investigations addressing this question could lead to a reevaluation of capacitative calcium entry in these cells.

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