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A novel tumour promoter, thapsigargin, transiently increases cytoplasmic free Ca^{2+} without generation of inositol phosphates in NG115-401L neuronal cells

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Thapsigargin, a sesquiterpene lactone with potent irritant and tumour-promoting activities, stimulates a rapid (within 15 s) transient increase in intracellular $[Ca^{2+}]$ in the NG115-401L neural cell line, as measured by the fluorescent indicator dye fura-2. This increase in cytoplasmic free $[Ca^{2+}]$ is concentration-dependent (ED_{50} around 20 nM) and occurs in the absence of extracellular Ca^{2+}. Activation of NG115-401L cells by the inflammatory peptide bradykinin generates inositol phosphates, which parallel increases in intracellular $[Ca^{2+}]$. However, the rise in cytoplasmic $[Ca^{2+}]$ stimulated by thapsigargin occurs in the absence of detectable production of inositol phosphates. Thapsigargin is unlike phorboid tumour promoters in that it has no action on two non-invasive indicators of phorbol stimulation of these cells, i.e. [^{3}H]choline metabolite production and rise in intracellular pH. These data suggest that thapsigargin releases Ca^{2+} from an intracellular store by a novel mechanism, independent of the hydrolysis of phosphoinositides and concomitant activation of protein kinase C. Thus thapsigargin may provide a valuable tool for the analysis of intracellular signalling mechanisms.

INTRODUCTION

The sesquiterpene lactone thapsigargin has been identified as the major skin-irritating principle of the umbelliferous plant *Thapsia garganica* L. (Apiaceae), used in Arabian folk medicine as a counter-irritant (Christensen & Norup, 1985). Previous studies have revealed that thapsigargin is capable of activating a number of different cell types involved in the inflammatory response (Rasmussen *et al.*, 1978; Ali *et al.*, 1985; Jacobsen *et al.*, 1987; Ohuchi *et al.*, 1987) and of platelets (Thastrup *et al.*, 1987*a*,*b*); furthermore, the compound has been classified as a non-TPA tumour promoter (Hakii *et al.*, 1986).

Although thapsigargin has similar effects to the Ca^{2+} ionophore A23187 in its actions on platelets, an ionophoric mechanism for thapsigargin has been effectively ruled out by the following observations: (1) it cannot transfer $^{45}Ca^{2+}$ into an organic phase (Ali *et al.*, 1985); (2) it does not release K^+ from red blood cells (Ali *et al.*, 1985); and (3) it fails to release histamine from isolated guinea-pig mast-cell granules (Patkar *et al.*, 1979).

In the NG115-401L neural cell line, receptor activation by an inflammatory mediator, bradykinin, produces inositol phosphates and a concomitant rise in intracellular $[Ca^{2+}]$ (Jackson *et al.*, 1987). This sensitivity to bradykinin, and other properties (Hanley, 1987), suggest that the NG115-401L cell line is a model for the analysis of the cellular mechanisms of inflammatory mediators. Accordingly, we asked two questions: is thapsigargin active on these cells, and if so, does it work through an established cellular activation mechanism? For purposes of comparison, other inflammatory agents, bradykinin, PDBu and TPA, have been evaluated in parallel.

MATERIALS AND METHODS

Cell culture

NG115-401L cells were routinely cultured as monolayers in DMEM containing 5% (v/v) fetal-calf serum. For lipid-metabolite experiments, cells were plated at a density of 3×10^4 cells/cm^2 on 6- or 24-well plates and grown for 3 days before the addition of radiolabel for the final 24 h: 10 μCi (6-well plates) or 1 μCi (24-well plates) of [^{3}H]choline chloride (NEN; 80.0 Ci/mmol) per well or 1 μCi of *myo*-[^{3}H]inositol (NEN; 12.8 Ci/mmol) [in *myo*-inositol-free DMEM (Gibco) supplemented with 5 μM-*myo*-inositol and 5% dialysed fetal-calf serum] per well. For fluorescence measurements, cells were harvested by scraping with a Teflon-coated spatula, and 5×10^4 cells in 0.2 ml of DMEM were plated on sterile 11 mm × 22 mm glass coverslips and used 24 h later.

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

This was essentially as described previously (Jackson *et al.*, 1987). Briefly, coverslips carrying NG115-401L cells were incubated in DMEM containing 2 μM-fura-2/AM for 45 min at 37 °C, washed, and put into HBS (1 mM-$CaCl_2$, 5.4 mM-KCl, 0.5 mM-$MgCl_2$, 0.2 mM-

Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; DMEM, Dulbecco's minimal essential medium; HBS, Hanks balanced salts solution; Ins*P*, inositol monophosphate; InsP_2, inositol bisphosphate; InsP_3, inositol trisphosphate; InsP_4, inositol tetrakisphosphate; PDBu, phorbol 12,13-dibutyrate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; BCECF/AM, bis(carboxyethyl)carboxyfluorescein acetoxymethyl ester.

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$MgSO_4$, 137 mM-NaCl, 4.2 mM-$NaHCO_3$, 0.4 mM-NaH_2PO_4, 44 mM-glucose, 20 mM-Hepes, pH 7.4) at 37 °C and cooled to room temperature for maintenance before use.

Before addition of agonists, coverslips were placed across the diagonal of a 0.5 cm-path-length quartz cuvette, containing 1.5 ml of HBS with either 1 mM-$CaCl_2$ or 1 mM-NaHEGTA. The cuvette was then placed with the coverslip at 60° to incident light in a Perkin–Elmer 3000 fluorescence spectrophotometer with a cuvette holder thermostatically maintained at 37 °C. Excitation and emission wavelengths of 340 nm and 500 nm respectively were chosen so that increasing $[Ca^{2+}]_i$ gives an increase in fluorescence intensity. Calibration was as previously described; addition of digitonin (25 μg/ml) in the presence of 1 mM-$CaCl_2$ giving maximal fluorescence ($F_{max.}$), followed by addition of 2 mM-$MnCl_2$ to quench the dye ($F_{min.}$; Hallam *et al.*, 1984). Fluorescence data were then calculated by using the equation

$$[Ca^{2+}]_i = K_d\left(\frac{F-F_{min.}}{F_{max.}-F}\right)$$

(Grynkiewicz *et al.*, 1985), where the K_d for fura-2 at 37 °C was taken to be 224 nM.

Measurement of intracellular pH

Coverslips, prepared as described above for $[Ca^{2+}]_i$ measurement, were incubated for 30 min at 37 °C with 5 μM-BCECF/AM (Rink *et al.*, 1982; Paradiso *et al.*, 1984). Cells were then washed with bicarbonate-free HBS (1 mM-$CaCl_2$, 5.4 mM-KCl, 0.5 mM-$MgCl_2$, 0.2 mM-$MgSO_4$, 137 mM-NaCl, 44 mM-glucose, 20 mM-Hepes, pH 7.2), and maintained in this buffer at room temperature until ready for use.

Coverslips were loaded into a cuvette containing 1.5 ml of bicarbonate-free HBS and positioned as previously described. Excitation and emmission wavelengths of 485 nm and 530 nm respectively were chosen so that a rise in intracellular pH gives a corresponding increase in fluorescence intensity.

Assay for [^{3}H]inositol phosphate production

The labelling medium was removed and each well was washed twice with HBS and preincubated in the same medium for 10 min at 37 °C. The preincubation medium was then replaced with fresh prewarmed medium containing appropriate drugs, and with 10 mM-LiCl included for 30 min incubations only. Incubations were terminated by addition of an equal volume of ice-cold 10% (v/v) $HClO_4$; after 5 min on ice, the supernatant was removed and neutralized by addition of tri-n-octylamine/1,1,2-trichlorotrifluoroethane (Freon) (1:1, v/v) (Downes *et al.*, 1986). Soluble [^{3}H]inositol metabolites were then separated by ion-exchange chromatography on 0.5 ml columns of Dowex AG1X8 resin (formate form; Bio-Rad), with batchwise elution as follows; free inositol with 2 × 5 ml of distilled water; glycerophosphoinositol-containing fraction with 2 × 5 ml of 60 mM-sodium formate/5 mM-disodium tetraborate; inositol monophosphates with 0.15 M-ammonium formate/0.1 M-formic acid; inositol bisphosphates with 0.4 M-ammonium formate/0.1 M-formic acid; inositol trisphosphates with 0.8 M-ammonium formate/0.1 M-formic acid; inositol tetrakisphosphates with 1.0 M-ammonium formate/0.1 M-formic acid; remaining inositol polyphosphates were then eluted with 2.0 M-ammonium formate/0.1 M-formic acid. The activity in each fraction was determined by liquid-scintillation spectrophotometry. Elution positions were verified by co-chromatography with known radiochemical standards.

The acid-insoluble cell remains were scraped into 250 μl of 1 mM-EDTA, and [^{3}H]inositol-containing lipids were extracted by addition of 900 μl of chloroform/methanol (1:2, v/v) containing 0.1 M-HCl, followed by 300 μl each of chloroform and 0.1 M-HCl. After thorough mixing, the phases were separated by centrifugation (3300 *g* for 5 min), and the lower phase was dried down and the total [^{3}H]inositol lipid content was determined by liquid-scintillation spectrophotometry.

Assay for [^{3}H]choline release

Labelling medium was removed and each well was washed three times with HBS supplemented with 0.1% bovine serum albumin and 1 mM-choline chloride, and preincubated in the same medium for 10 min at 37 °C. The preincubation medium was then replaced with 1 ml of prewarmed medium containing the appropriate drugs. After 30 min, incubations were terminated by transferring a 0.5 ml sample of the supernatant to ice. Samples from 24-well plates were diluted with 1 ml of methanol/water (1:1, v/v), and [^{3}H]choline content was assessed by liquid-scintillation spectrophotometry. Samples from six-well plates were spun at 3300 *g* for 3 min, 100 μl portions were transferred to scintillation vials and the radioactivity was determined as above.

All results are means ± S.E.M.; where significance is indicated, it was assessed by Student's *t* test.

RESULTS

Thapsigargin elevates $[Ca^{2+}]_i$

Addition of thapsigargin to NG115-401L cells loaded with the fluorescent Ca^{2+} indicator fura-2 produces a

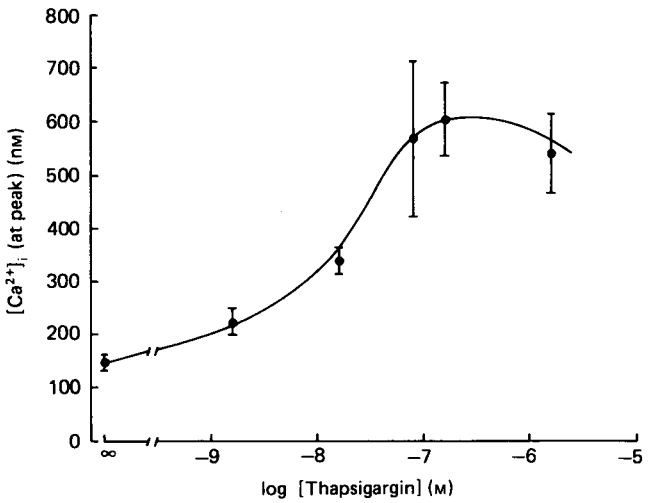

Fig. 1. Dependence of peak intracellular $[Ca^{2+}]$ on added thapsigargin concentration

Peak $[Ca^{2+}]_i$ in NG115-401L cells after addition of appropriate thapsigargin concentrations was determined in fura-2-loaded cells, in the presence of 1 mM extracellular Ca^{2+}, and calibrated as described in the Materials and methods section. Values are means ± S.E.M. for three to 11 determinations.

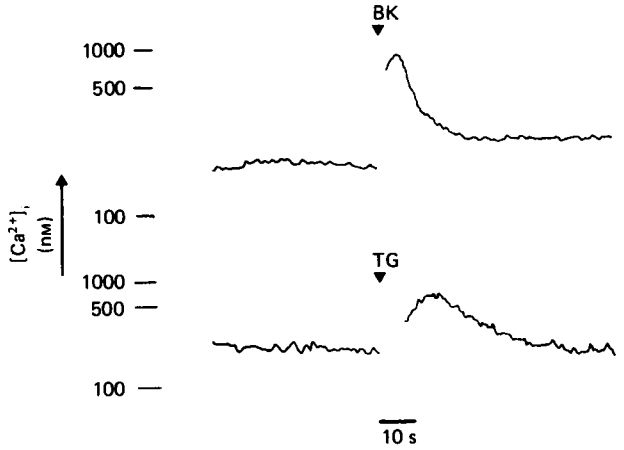

Fig. 2. Intracellular Ca^{2+} transients in response to bradykinin or thapsigargin, in the presence of 1 mM extracellular Ca^{2+}

Transient elevations of $[Ca^{2+}]_i$ (representative of 6 and 11 determinations respectively) in response to 1 μM-bradykinin (BK) or 1.7 μM-thapsigargin (TG) were measured in fura-2-loaded cells (excitation 340 nm, emission 500 nm) as described in the Materials and methods section. Addition of thapsigargin, bradykinin or ionophore to fura-2-loaded cells illuminated at 380 nm with emission at 500 nm gave a decrease in fluorescence, which could be calibrated to $[Ca^{2+}]_i$ (results not shown).

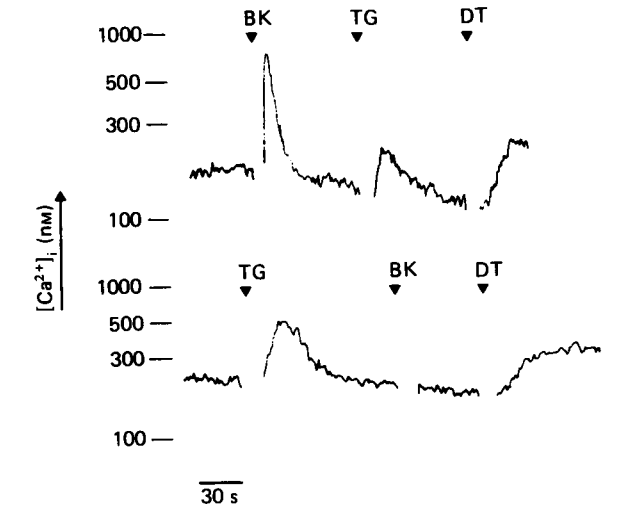

Fig. 3. Intracellular Ca^{2+} transients in NG115-401L cells in response to thapsigargin, bradykinin and digitonin, in the absence of extracellular Ca^{2+}

Transient elevations of $[Ca^{2+}]_i$ (representative of 6 and 8 determinations respectively) in response to 1 μM-bradykinin (BK) and 1.7 μM-thapsigargin (TG), or 1.7 μM-thapsigargin followed by 1 μM-bradykinin, are shown. Digitonin (DT; 25 μg/ml) was added to release any remaining sequestered Ca^{2+}. All measurements were on fura-2-loaded cells as described in the Materials and methods section.

dose-dependent rise in $[Ca^{2+}]_i$ (Fig. 1), with an ED_{50} of approx. 20 nM. In the presence of 1 mM extracellular Ca^{2+}, $[Ca^{2+}]_i$ was maximally elevated by 170 nM-thapsigargin (basal value approx. 150 ± 15 nM; stimulated value approx. 600 ± 80 nM; $n = 6$). The increase in $[Ca^{2+}]_i$ on addition of a maximal thapsigargin concentration (1.7 μM) reached a peak (approx. 550 ± 75 nM; $n = 11$) between 15 and 20 s and declined back to basal values by 90 s (Fig. 2). In contrast, maximal bradykinin (1 μM) stimulated a rise in $[Ca^{2+}]_i$ from a basal value of approx. 110 ± 15 nM to a peak of approx. 700 ± 250 nM within 5 s, which declined to a significantly higher basal value of approx. 170 ± 40 nM ($P < 0.05$; $n = 6$) within 60 s. In the absence of extracellular Ca^{2+} (1 mM-EGTA present for 2 min before stimulation; see Fig. 3), thapsigargin produced a similar rise in $[Ca^{2+}]_i$, from approx. 130 ± 30 nM to a peak of approx. 650 ± 160 nM ($n = 5$) within 20 s, whereas bradykinin produced a rise from a similar basal value to a peak at approx. 540 ± 200 nM within 5 s. Addition of thapsigargin after bradykinin produced a further increase in $[Ca^{2+}]_i$ to approx. 270 ± 40 nM ($n = 6$), which declined back to basal within 90 s, whereas the addition of bradykinin after thapsigargin failed to evoke any further increase. Addition of digitonin after treatment with thapsigargin alone or with bradykinin and thapsigargin gave a further elevation of $[Ca^{2+}]_i$ to approx. 290 ± 50 nM ($n = 8$), indicating that in NG115-401L cells a further pool of sequestered Ca^{2+} exists which is inaccessible to either stimulant. Similarly, in the presence of 1 mM extracellular Ca^{2+}, addition of thapsigargin after a maximal dose of bradykinin allows a further elevation of $[Ca^{2+}]_i$ from approx. 170 ± 40 nM to 350 ± 90 nM ($n = 6$).

Thapsigargin does not generate inositol phosphates

The $[Ca^{2+}]_i$ rise elicited by bradykinin parallels a rise in production of Ins(1,4,5)P_3 and InsP_2 (Jackson *et al.*, 1987), which has been shown in neural cells to be capable of releasing Ca^{2+} from an intracellular store (Ueda *et al.*, 1986). The receptor-stimulated production of inositol phosphates may be detected by accumulation of Ins(1)P in the presence of 10 mM-LiCl (Berridge *et al.*, 1982). A 30 min incubation with 1.7 μM-thapsigargin does not elicit any detectable production of [^{3}H]Ins$P$ (Fig. 4), whereas 1 μM-bradykinin gives a significant increase in Ins$P$ accumulation, to approx. 500% of control (control value 611 ± 215 c.p.m., $n = 4$; $P < 0.05$). Similarly, in a 15 s incubation in the absence of LiCl, thapsigargin fails to evoke any inositol phosphate production, whereas bradykinin elicits an increase in [^{3}H]InsP_3 to more than 300% of control (control value 492 ± 96 c.p.m., $n = 3$; $P < 0.05$). Thapsigargin does not give rise to any detectable change in total [^{3}H]phosphoinositide amounts (results not shown). After 15 s the simultaneous addition of thapsigargin and bradykinin generates a [^{3}H]InsP_3 response similar to that with bradykinin alone (approx. 270% and 360% of control respectively; $n = 3$; $P < 0.05$ relative to control). Addition of Ca^{2+} ionophore (A23187 or ionomycin) to NG115-401L cells in the presence of 1 mM extracellular Ca^{2+} produces a rise in $[Ca^{2+}]_i$ which is sufficient to saturate fura-2 fluorescence, and thus must represent a $[Ca^{2+}]_i$ of at least several micromolar. At 15 s after addition, the Ca^{2+} ionophore A23187 does not increase [^{3}H]inositol phosphates. In contrast with thapsigargin, however, after 30 min in the presence of LiCl, A23187 produces an increase in [^{3}H]Ins$P$ and [^{3}H]InsP_2, to approx. 370% and 340% of control respectively (control values 591 ± 150 and 184 ± 28 c.p.m., $n = 3$; $P < 0.05$ for stimulated relative to controls) and a significant small rise in [^{3}H]InsP_3, to 135 ± 20% of control (control value 424 ± 88 c.p.m., $n = 3$; $P < 0.1$).

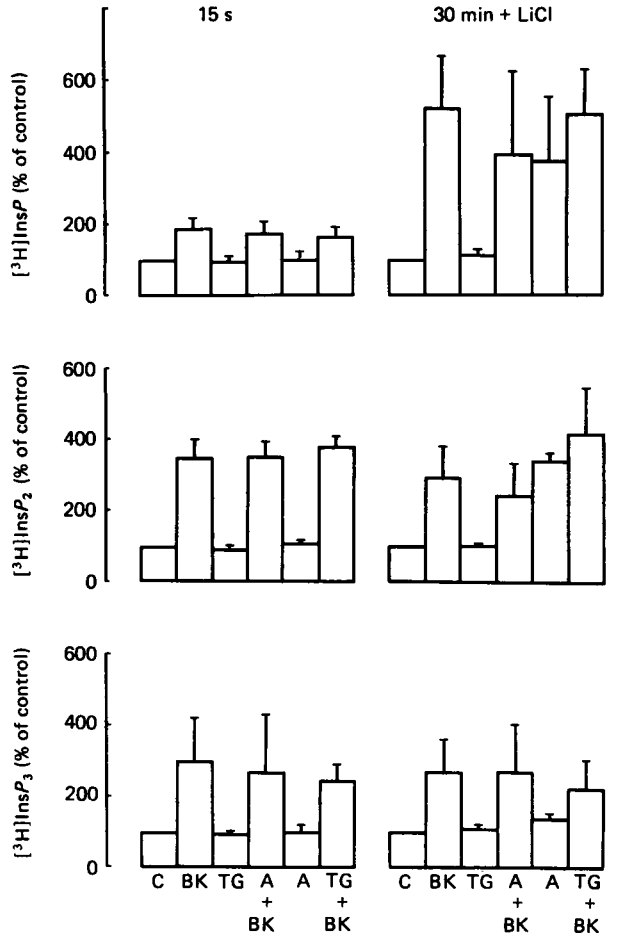

Fig. 4. Production of [^{3}H]inositol phosphates in response to bradykinin, thapsigargin and A23187

NG115-401L cells were prelabelled with [^{3}H]inositol, as described in the Materials and methods section, and stimulated for either 15 s in the absence of, or 30 min in the presence of, 10 mM-LiCl. [^{3}H]Inositol phosphates were extracted and separated on Dowex AG1X8 (formate form) columns as described in the Materials and methods section. Bars correspond to: C, control; BK, 1 μM-bradykinin; A, 2 μM-A23187; A+BK, 2 μM-A23187+ 1 μM-bradykinin; TG, 1.7 μM-thapsigargin; TG+BK, 1 μM-bradykinin+ 1.7 μM-thapsigargin. Values represent means $\pm$ S.E.M. for four to six separate determinations each performed in triplicate.

Table 1. Effects of PDBu and/or thapsigargin on [^{3}H]choline release from prelabelled NG115-401L cells

Cells, grown on six-well tissue-culture treated plates, were metabolically prelabelled with [^{3}H]choline, incubated with 1 μM-PDBu and/or 1 μM-thapsigargin, and released radioactivity was assayed as described in the Materials and methods sections. Values represent means $\pm$ S.E.M. for three experiments each performed in triplicate: *different from basal ($P < 0.05$); †different from PDBu alone ($P < 0.05$).

	[^{3}H]Choline released (c.p.m.)
Basal	11754 $\pm$ 854
PDBu	18294 $\pm$ 709*
Thapsigargin	8183 $\pm$ 898*
PDBu + thapsigargin	14691 $\pm$ 841*†

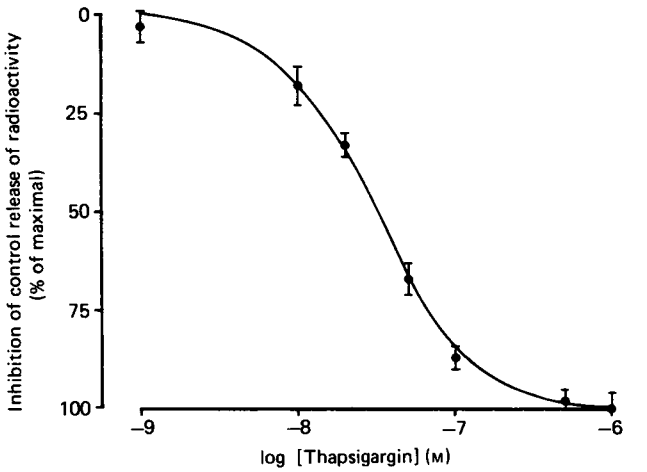

Fig. 5. Dependence of inhibition of basal [^{3}H]choline release on thapsigargin concentration in NG115-401L cells

Cells, grown on 24-well tissue-culture treated plates, were metabolically prelabelled with [^{3}H]choline, incubated with thapsigargin as appropriate, and extracellular radioactivity was determined as described in the Materials and methods section. Values represent means $\pm$ S.E.M. for three to six experiments each performed in triplicate.

Thapsigargin does not have phorbol-diester-like activities

Analysis of [^{3}H]choline release provides as assay for the activity of inflammatory and tumour-promoting phorbol esters in intact cells (Liscovitch *et al.*, 1987; Takuwa *et al.*, 1987). Addition of PDBu (1 μM) to cells preloaded with [^{3}H]choline produces an increase in [^{3}H]choline release into the extracellular medium (stimulated value approx. 18000 $\pm$ 700 c.p.m.; basal value approx. 12000 $\pm$ 800 c.p.m.; $n = 3$; $P < 0.05$, see Table 1 for exact numbers). Addition of thapsigargin, on the other hand, produces a decrease in the basal [^{3}H]choline release compared with control ($P < 0.05$). However, thapsigargin does not impair the PDBu response when correction is made for the decrease in basal values by thapsigargin (no difference in PDBu stimulation with or without thapsigargin; $P > 0.5$). The inhibition of basal [^{3}H]choline release is dose-dependent (ED_{50} approx. 20–30 nM; Fig. 5), with maximal inhibition at 1 μM-thapsigargin. Accordingly, the dose/response relationships of thapsigargin on $[Ca^{2+}]_i$ and inhibition of [^{3}H]choline release are very similar.

Addition of bradykinin to NG115-401L cells loaded with the fluorescent pH indicator BCECF produces a rise in fluorescence, indicating that cytoplasmic alkalinization has occurred (Fig. 6). This change in intracellular pH may be inhibited by prior incubation with 200 μM-amiloride (results not shown). The protein kinase C activator TPA (100 nM) also produces a rise in fluorescence, although this effect is considerably slower in time to reach the peak than is that evoked by bradykinin (1 μM). Addition of thapsigargin (1.7 μM) did not alter BCECF fluorescence, and did not alter the responses evoked by bradykinin or TPA, providing further confirma-

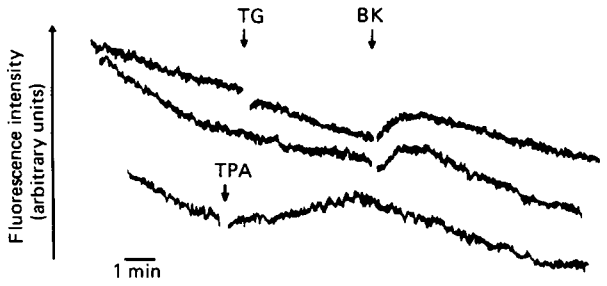

Fig. 6. Effect of bradykinin, TPA and thapsigargin on BCECF fluorescence

TPA (100 nM, bottom trace) and bradykinin (1 μM, middle trace, BK) produce a rise in fluorescence, whereas thapsigargin (1.7 μM, TG) does not, and moreover does not alter the cell response to either bradykinin (upper trace) or TPA (not shown). Responses were measured in BCECF-loaded cells as described in the Materials and methods section. Each trace is representative of at least five separate determinations.

tion that thapsigargin does not share the biological activities of phorbol diesters.

DISCUSSION

The naturally occurring sesquiterpene lactone thapsigargin has been found to activate a broad spectrum of different cell types, including cells involved in the inflammatory response (Rasmussen *et al.*, 1978; Ali *et al.*, 1985; Jacobsen *et al.*, 1987; Ohuchi *et al.*, 1987), and blood platelets, by an apparently novel mechanism of action (Thastrup *et al.*, 1987*a*). Thus it has been found that thapsigargin in human platelets elevates $[Ca^{2+}]_i$ by a non-ionophoric mechanism that seems to be independent of hydrolysis of phosphoinositides (Thastrup *et al.*, 1987*a*, *b*).

Present studies on NG115-401L cells, a cell line that constitutes a useful model system of sensory neurons which participate in acute inflammation (Hanley, 1987), have revealed that thapsigargin is able to produce a transient increase in $[Ca^{2+}]_i$. The peak value of the $[Ca^{2+}]_i$ rise resembled that produced by the endogenous agonist bradykinin. However, the responses to the two agonists can be distinguished by the rate of elevation of $[Ca^{2+}]_i$; thus peak values were reached at approx. 20 s and 5 s after stimulation with thapsigargin and bradykinin respectively. In platelets a similar difference is observed, as the thrombin-stimulated Ca^{2+} response is more rapid than that evoked by thapsigargin (Thastrup *et al.*, 1987*b*). In the absence of extracellular Ca^{2+}, both thapsigargin and bradykinin affected $[Ca^{2+}]_i$ in a reversible manner; thus $[Ca^{2+}]_i$ returned to the basal value after the initial rise. However, when extracellular Ca^{2+} is included, bradykinin was found to provoke a sustained elevation of basal $[Ca^{2+}]_i$, which is presumed to be due to influx of Ca^{2+} across the plasma membrane. No sustained elevation of basal $[Ca^{2+}]_i$ can be detected after stimulation with thapsigargin, the response being identical in the presence or the absence of extracellular Ca^{2+}. The influx component of the bradykinin $[Ca^{2+}]$ response may be due to the opening of receptor-operated Ca^{2+} channels, described in other cell types, but where the mechanism is unknown (Reynolds & Dubyak, 1986, Pozzan *et al.*, 1986; Hallam *et al.*, 1984). Thus it may be significant that, in contrast with bradykinin, thapsigargin produces discharge of intracellular Ca^{2+} without the hydrolysis of polyphosphoinositides, metabolites of which (inositol polyphosphates or diacylglycerol) may participate in the generation of a Ca^{2+} influx (Irvine & Moor, 1986).

The rapid return to basal $[Ca^{2+}]_i$ values after thapsigargin stimulation indicates activation of a mechanism to extrude and/or re-sequester intracellular Ca^{2+}. These observations differ from thapsigargin-stimulated platelets, where, after an initial peak, $[Ca^{2+}]_i$ is sustained at an elevated value (Thastrup *et al.*, 1987*b*). In a number of tissues and cells, Ca^{2+}-ATPases activated by Ca^{2+}–calmodulin have been described (Carafoli, 1984; Gill *et al.*, 1984; Papazian *et al.*, 1984). A Ca^{2+} pump of this type may be activated to cause Ca^{2+} extrusion after thapsigargin stimulation in NG115-401L cells. In contrast, the platelet Ca^{2+}-ATPase may be insensitive to calmodulin (Enyedi *et al.*, 1986; Pollock *et al.*, 1987), so that a thapsigargin-stimulated rise in $[Ca^{2+}]_i$ alone would be insufficient to activate extrusion.

Thapsigargin abolishes the $[Ca^{2+}]_i$ response to bradykinin, suggesting that the two agents release Ca^{2+} from a common intracellular store; however, the ability of thapsigargin to produce a small rise in Ca^{2+} after a maximal dose of bradykinin suggests one of the following: (*a*) an ability to release Ca^{2+} from a second store, (*b*) the ability to discharge partially re-sequestered Ca^{2+}, as described for platelet-activating factor and thrombin in platelets (Pollock *et al.*, 1987), or (*c*) the existence of a sub-population of bradykinin-insensitive cells which retain their sensitivity to thapsigargin. However, neither agent is capable of complete discharge of sequestered intracellular Ca^{2+}, as addition of digitonin gives a further increase in $[Ca^{2+}]_i$.

In the presence of a LiCl block, a 30 min incubation with Ca^{2+} ionophore produces inositol phosphate accumulation, whereas thapsigargin does not. This may indicate that the activation of phosphoinositide hydrolysis does not occur with relatively small, transient, rises in $[Ca^{2+}]_i$, but may be induced by large sustained Ca^{2+} influxes produced by ionophores. Indeed, Renard *et al.* (1987) found that quin2 clamping of $[Ca^{2+}]_i$ to fixed cytoplasmic values (200–1130 nM) has little effect on basal or stimulated polyphosphoinositide hydrolysis in hepatocytes.

Although it has tumour-promoting activity, thapsigargin differs from phorbol diesters in that it does not compete with [^{3}H]PDBu binding (results not shown), does not produce intracellular alkalinization, and does not stimulate enhanced release of [^{3}H]choline from prelabelled cells, these all being responses evoked by the class of inflammatory and tumour-promoting phorbol esters and related substances. Thapsigargin does not alter the release of [^{3}H]choline stimulated by PDBu, but, like bradykinin, it decreases the basal [^{3}H]choline release. A similar decrease in [^{3}H]choline release has been reported to occur in C3H10T1/2 cells on treatment with Ca^{2+} ionophore (Mufson *et al.*, 1981), suggesting that the basal release rate may be a sensitive indicator of increased $[Ca^{2+}]_i$. In keeping with this, the dose-dependencies of $[Ca^{2+}]_i$ increases and [^{3}H]choline release by thapsigargin show close correspondence. Thapsigargin thus appears to exert its action in NG115-401L cells independent of protein kinase C and phorbol diester-sensitive mechanisms, supporting the proposition by Hakii *et al.* (1986) that thapsigargin is a non-TPA-type tumour promoter.

In conclusion, we find that the inflammatory sesquiterpene lactone thapsigargin activates a neuronal cell line by transiently raising cytoplasmic [Ca^{2+}] via discharge from an intracellular store. This store may be the same as that utilized by another inflammatory stimulant, the neural peptide bradykinin. Thapsigargin should therefore provide a valuable tool allowing the generation of intracellular [Ca^{2+}] transients and associated events in the absence of complicating processes resulting from the release of diacylglycerol or inositol polyphosphates.

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