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Effect of a cyclohexenonic long-chain fatty alcohol on calcium mobilization

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Abstract

Cyclohexenonic long-chain fatty alcohols constitute a family of synthetic compounds with trophic, secretagogue and antioxidant properties. Despite their multiple biological actions in neuronal and non-neuronal tissues, the intracellular mechanisms underlying CFA activity remain unknown. In the present study, we show that 3-(15-hydroxypentadecyl)-2,4,4-trimethyl-2-cyclohexen-1-one (tCFA15) directly mobilizes Ca^{2+} in the pituitary neural lobe synaptosomes and in primary sensory neurons from dorsal root ganglia. This effect is dependent on the presence of extracellular Ca^{2+}, but does not involve transmembrane voltage-operated calcium channels. Using a combination of pharmacological agents that block or deplete intracellular Ca^{2+} stores, our results suggest the implication of a calcium induced–calcium release mechanism evoked by tCFA15-induced Ca^{2+} influx. To our knowledge, these findings constitute the first attempt towards the comprehension of the biological actions of cyclohexenonic long-chain fatty alcohols at a molecular level.

Keywords: Calcium; Fura-2; Long-chain fatty alcohol; Pituitary neural lobe; Primary sensory neuron; Trimethyl-cyclohexenonic alcohol

1. Introduction

Since the discovery of the in vitro neuritogenic activity of n-hexacosanol, a long-chain ω-alkanol isolated from extracts of the tropical plant *Hygrophila erecta*, Hochr. (Acanthaceae) (Borg et al., 1987), we have focused our attention on the synthesis of cyclohexenonic long-chain fatty alcohols, which contain, in addition to an ω-alkanol side chain, a functional nucleus which enhances the physicochemical properties of these highly lipophilic molecules (reviewed by Luu et al., 2000). Cyclohexenonic long-chain fatty alcohols exhibit a wide variety of biological actions. The monomethyl and dimethyl derivatives with an appropriate length of the side chain are potent inducers of neurite outgrowth in cultured neurons from foetal rat cerebral hemispheres (Girlanda-Junges et al., 1998). In the series of trimethyl derivatives, 3-(15-hydroxypentadecyl)-2,4,4-trimethyl-2-cyclohexen-1-one, also referred to as tCFA15, stimulates not only neurite outgrowth but also neuronal survival (Gonzalez de Aguilar et al., 2001). Recent studies extended these findings demonstrating that tCFA15 exerts its growth-promoting effect specifically in axons, and may counteract the inhibitory activity of myelin extracts and members of the semaphorin superfamily which prevent axon regeneration (Hanbali et al., 2004). In vivo administration of tCFA15 ameliorates peripheral nerve conduction velocity in rats suffering from streptozotocin-induced diabetic neuropathy (Watanabe and Miyagawa, 2002). Moreover, tCFA15 also stimulates arginine vasopressin release in mouse pituitary neural lobe terminals (Girlanda-Junges et al., 2000), and prevents the production of free radicals after ischaemia-reperfusion injury in the rat bladder (Saito et al., 2002). Despite these multiple actions, including trophic, secretagogue and antioxidant properties, the intra-

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cellular mechanisms underlying CFA activity await elucidation. In the present study, we provide new insight into the pharmacological effects of tCFA15 by measuring intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) changes. To this aim, we measured $[Ca^{2+}]_i$ in synaptosomes from the neural lobe of the pituitary and in cultured primary sensory neurons isolated from dorsal root ganglia, where we conducted a pharmacological study. We found that extracellular Ca^{2+} is needed by tCFA15 to induce transient $[Ca^{2+}]_i$ increases. However, the pathway followed by Ca^{2+} is independent of voltage-operated calcium channels. We propose that the Ca^{2+} internalised following tCFA15 treatment may be sufficient to generate important $[Ca^{2+}]_i$ transients through a calcium induced–calcium release mechanism.

2. Materials and methods

2.1. Preparation of neural lobe synaptosomes

Animal care followed current EU regulations, and experiments were conducted under the supervision of authorized investigators. Mouse (FBV/N strain) neural lobe synaptosomes were freshly prepared prior to microfluorimetry experiments following the procedure previously described for rat neural lobe synaptosomes (Cazalis et al., 1987). Briefly, sets of neural lobes from four month old mice were homogenized at room temperature in 60 μl of a solution containing (in mM): sucrose 270, EGTA 0.1, Hepes 20, and adjusted to pH 7.2 with Tris–HCl buffer. The homogenates were directly placed onto a bottom glass 35-mm Petri dish, and incubated until use at room temperature in Hanks' balanced salt solution (HBSS) pH 7.4 containing (in mM): $CaCl_2$ 2, NaCl 137.9, $NaHCO_3$ 4, Na_2HPO_4 0.3, KCl 5.3, KH_2PO_4 0.44, Hepes 10, glucose 5.6 and $MgCl_2$ 2.

2.2. Culture of neurons from dorsal root ganglia

Dorsal root ganglia from as many spinal levels as possible were collected from adult male mice. They were treated with 1 mg/ml collagenase A for 30 min at 37 °C and then with 0.05 % trypsin-0.02 % EDTA (GIBCO BRL) and 5 U/ml DNAse I for10 min at 37 °C. After blocking the enzymatic reactions by adding foetal calf serum (10 %), ganglia were mechanically dissociated into single cells by triturating through a Pasteur pipette. The cells were resuspended in a serum free defined medium: Dulbecco's modified Eagle medium (GIBCO BRL) supplemented with 1 μM insulin, 0.1 mM putrescine, 20 nM progesterone, 100 μg/ml transferrin and 0.15 μM sodium selenite (Bottenstein and Sato, 1979), and then plated on poly-L-lysine-coated bottom-glace Petri dishes and incubated in water-saturated atmosphere (95 % air, 5% CO_2) at 37 °C. The culture medium was renewed 5 days after plating. Experiments were performed using neurons cultured for four to seven days.

2.3. Microfluorimetry

Measurements were carried out with an inverted Olympus IX70 epifluorescent microscope equipped with UPlanFL 20/0.5 and 40/0.75 objectives and a thermostated chamber (Solent Scientific Limited, UK). The system also included a Polychrome IV monochromator and a CCD scan camera (Till Photonics, Germany). Fura-2-loaded neural lobe synaptosomes or cultured neurons were excited at 340 and 380 nm, and fluorescence emission >510 nm was collected and quantified using the Vision computer program (Till Photonics). Autofluorescence and dye bleaching were negligible during the experiments. The recording chamber was maintained at 37 °C and was continuously superfused at a rate of 4 ml/min with standard bath solution or test solutions. Drugs were applied using a perfusion system that allowed rapid manual shifting between solutions.

Synaptosomes were incubated with 150 μl of 4 μM Fura-2 acetoxymethyl ester (Fura-2 AM) in HBSS for 30 min in the dark at 37 °C. Afterwards, they were rinsed at a flow rate of 4 ml/min in order to remove from the recording chamber all free-floating nerve terminals, red blood cells and incompletely dissociated tissue resulting from the homogenisation process. The preparation contained endings and swellings, both being able to release neurohormones upon depolarisation (Nordmann and Dayanithi, 1988; Toescu and Morris, 1990). Using the UplanFL 40× objective, appropriate fields containing well isolated synaptosomes were selected. Measurements in regions of interest containing individual synaptosomes, were expressed as the fluorescence ratio between 340- and 380-nm excitation wavelengths.

Primary sensory neurons were loaded with 5 μM Fura-2 AM for 30 min in the dark at 37 °C, then washed twice in HBSS and transferred to the recording chamber. The $[Ca^{2+}]_i$ values were calculated from the fluorescent ratios (R) acquired at 340- and 380-nm excitation wavelengths under Ca^{2+}-saturating and Ca^{2+}-free conditions using the following equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}]i = K_d(S_{f2}/S_{b2})(R - R_{min})/(R_{max} - R)$$

K_d is the dissociation constant of Fura-2 for Ca^{2+} (K_d=224 nM), S_{f2}/S_{b2} is the fluorescence ratio between the 380-nm signal in the absence of Ca^{2+} and that in the presence of saturating Ca^{2+} (S_{f2}/S_{b2}=1.94±0.03, n=86), R_{min} is the minimal fluorescence value obtained by incubating the neurons in Ca^{2+}-free bath solution supplemented with 10 mM EGTA and 5 μM ionomycin (R_{min}=0.78±0.05), and R_{max} is the maximal fluorescence value obtained by incubating the neurons in standard bath solution supplemented with 5 mM $CaCl_2$ and 5 μM ionomycin (R_{max}=4.46±0.3).

2.4. Drugs and chemicals

Unless otherwise indicated, chemicals were purchased from Sigma. tCFA15 was synthesized in the Laboratoire de Chimie Organique des Substances Naturelles (Strasbourg, France) as described previously (Girlanda-Junges et al., 2000). The tCFA15 stock solution was 10 mM in ethanol and was diluted to working concentrations containing less than 0.1 % ethanol final concentration. Agents added to either standard bath solution or Ca^{2+}-free bath solution included ATP (magnesium salt), caffeine, 5-hydroxytryptamine (creatinine sulfate complex; 5-HT) and nifedipine; ω-agatoxin TK, ω-conotoxin GVIA and ryanodine (all from Alomone Labs) and thapsigargin (Wako). Stock solutions of 1 mM Fura-2 AM (Molecular Probes), 1 mM ionomycine, and 10 mM thapsigargin were prepared in dimethyl sulfoxide and stored at −20 °C. The Ca^{2+}-free bath solution used for some experiments

was prepared by omitting $CaCl_2$ from standard bath solution and adding 10 mM EGTA.

2.5. Statistics

Data are expressed as the mean ±S.E.M., and statistical significance was determined by one-way analysis of variance followed by Newman–Keuls multiple comparison test (GraphPad InStat version 3.0). Differences were considered significant at $P<0.05$.

3. Results

3.1. tCFA15 induces transient $[Ca^{2+}]_i$ increases in synaptosomes from the pituitary neural lobe

We have previously shown that tCFA15 induced a significant increase in arginine vasopressin secretion from adult mouse pituitary neurointermediate lobes. This effect was abolished by extracellular Ca^{2+} depletion, which led us to postulate the activation of a regulated exocytotic pathway (Girlanda-Junges et al., 2000). To further characterize the pathway activated by tCFA15, we monitored the variations of intracellular Ca^{2+} in neural lobe synaptosomes. To ensure that the synaptosomal preparation was reliable, the response to a brief depolarization by a 25 mM KCl solution was first recorded. This stimulus normally evoked a rise in the intra-synaptosomal Ca^{2+} concentration that was then compared with the effect produced by tCFA15. As shown in Fig. 1A, KCl instantly induced a transient $[Ca^{2+}]_i$ increase that happened to decrease immediately after the KCl solution started to be cleared. Application of tCFA15 also evoked a transient $[Ca^{2+}]_i$ increase that occurred after a short delay. Several responses could be observed from single synaptosomes. In the absence of Ca^{2+} in the bath solution, tCFA15 did not induced any transient $[Ca^{2+}]_i$ changes unless added in a Ca^{2+} containing solution or when Ca^{2+} was present immediately after the tCFA15 pulse (Fig. 2B). Taken together, these observations strongly support the requirement of extracellular Ca^{2+} for the induction of $[Ca^{2+}]_i$ changes by tCFA15. To further characterize the molecular activity of tCFA15 we continue working on primary sensory neurons from dorsal root ganglia which give access to more powerful pharmacological studies. The sensitivity of cells from dorsal root ganglia to tCFA15 was suggested by the observed improvement of peripheral nerve conduction velocity after in vivo administration of the molecule (Watanabe and Miyagawa, 2002).

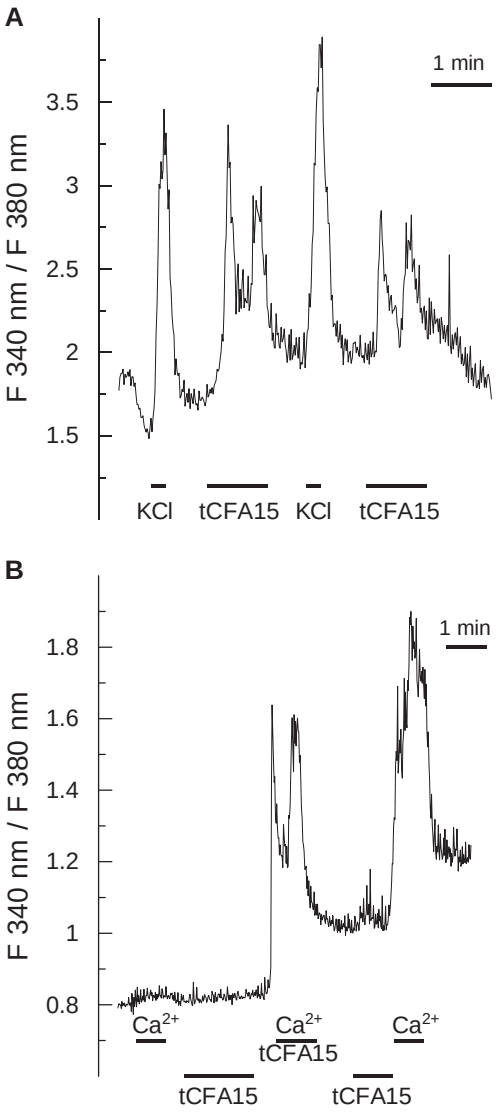

Fig. 1. Effect of tCFA15 on neural lobe synaptosomes. (A) Synaptosomes challenged with a 15-s pulse of 25 mM KCl buffer immediately present a peak of fluorescence. Application of a 0.5 μM tCFA15 solution for 45 s also induces fluorescence variations. Repeated applications of KCl or tCFA15 give similar responses. (B) When incubated in Ca^{2+} free buffer, synaptosomes do not respond to tCFA15 application. However, simultaneous application of tCFA15 and Ca^{2+} induces an important increase of fluorescence. Similarly, if Ca^{2+}-free tCFA15 application is followed by a short pulse of Ca^{2+}-containing buffer, a peak of fluorescence can be recorded.

3.2. tCFA15 induces transient $[Ca^{2+}]_i$ increases in primary sensory neurons

When cultured neurons were incubated in the presence of tCFA15 transient $[Ca^{2+}]_i$ variations were observed in a fraction of the recorded cells. Thus, to exclude from the analysis non viable cells, a 15 s pulse of 1 μM ATP or 1 μM 5-HT was included in the protocol before challenging primary sensory neurons with tCFA15 (Fig. 2A). Only the cells sensitive to these stimuli (about 90 %) were then taken into account. An example of neuron sensitive to tCFA15 is shown in Fig. 2A, this cell responded to ATP and 5-HT application and presented a large $[Ca^{2+}]_i$ variation more than one minute after the tCFA15 was present in the bath. The same cell remained silent during a second application of the molecule. Other cells were silent to a first tCFA15 application but showed $[Ca^{2+}]_i$ changes to a second or responded to more than one application (see also Fig. 3A and B). Therefore, we considered sensitive to tCFA15 those cells that showed, during one of the tCFA15 applications, at least one transient $[Ca^{2+}]_i$ variation with a peak above 40 % of the baseline. In normal conditions, when cells were superfused with a 0.5 μM solution of tCFA15, a transient elevation of $[Ca^{2+}]_i$ was observed in about 30 % of cells (Table 1). Contrary to the ATP or 5-HT responses, which were always instantaneous, tCFA15-

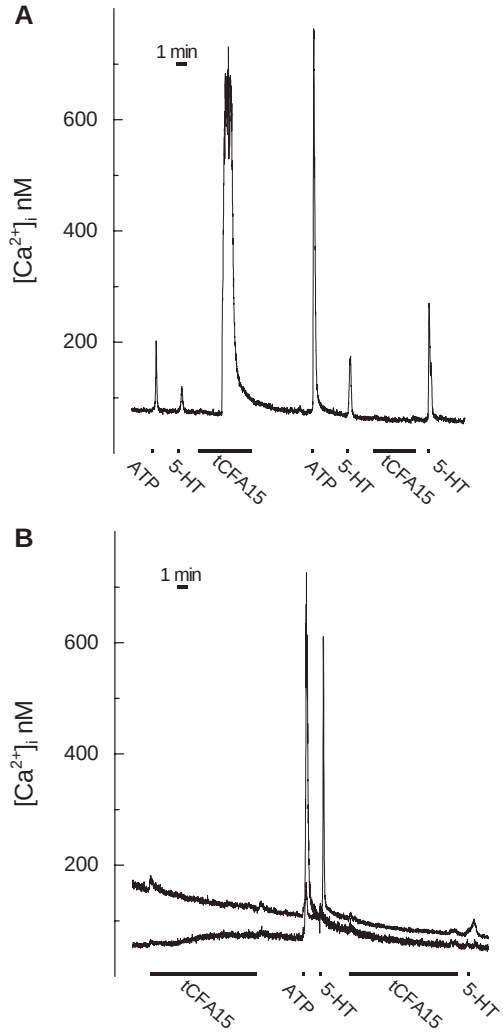

Fig. 2. Transient changes in $[Ca^{2+}]_i$ are induced by 0.5 μM tCFA15 in primary sensory neurons. (A) An example of a neuron from dorsal root ganglia sensitive to ATP, 5-HT and tCFA15. Note the delayed response after 0.5 μM tCFA15 application compared to the immediate effect of other drugs. (B) In the absence of external Ca^{2+}, tCFA15 induced $[Ca^{2+}]_i$ modifications were never observed, although the recorded cells were sensitive to the application of ATP, 5-HT or both. Records of two different cells are shown, the first responded only to ATP application and the second to 5-HT.

induced $[Ca^{2+}]_i$ transients were observed after a variable delay. When Ca^{2+} was suppressed from the bath solution, prolonged applications of tCFA15 failed to elicit $[Ca^{2+}]_i$ mobilization in cells otherwise sensitive to ATP or 5-HT (Fig. 2B).

3.3. tCFA15-induced $[Ca^{2+}]_i$ transients are independent of voltage operated calcium channels activation

Some aliphatic alcohols and their derived analogues are able to modulate the activity of voltage operated calcium channels (Beedle and Zamponi, 2000; Roullet et al., 1999). Then, we wished to verify whether the observed tCFA15 effects involved activation of these channels. We blocked all the tetrodotoxin-sensitive and most of the insensitive-sodium channels using 2 μM tetrodotoxin. We applied 2 μM nifedipine to block L-type Ca^{2+} channels, 2 μM ω-conotoxin-GVIA to block N-type channels and 1 μM ω-agatoxin-TK to block P/Q-type channels. The effect of tCFA15 was always observed in the presence of these drugs whether applied separately or in different combinations. As observed in control conditions, about 30 % of cells showed $[Ca^{2+}]_i$ transients induced by tCFA15 (Table 1). Two typical examples are given in Fig. 3. Here the cells were first incubated in the presence of 2 μM ω-conotoxin-GVIA and 1 μM ω-agatoxin-TK (15 min), and then constantly superfused with 0.2 μM tetrodotoxin and 2 μM nifedipine. In these conditions, we still observed transient increases in $[Ca^{2+}]_i$, after the beginning of tCFA15 administration. These cells remained sensitive to ATP

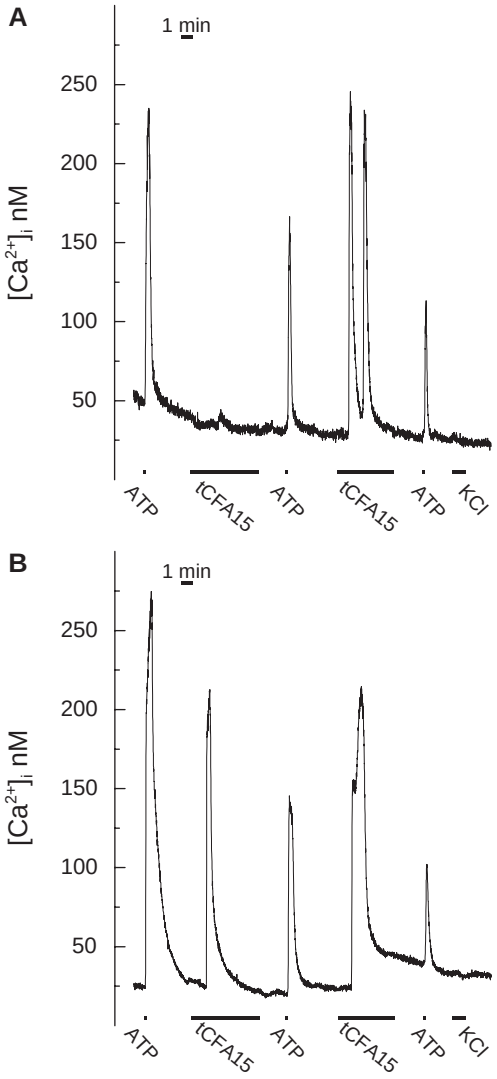

Fig. 3. The block of voltage operated calcium channels do not changes the tCFA15-induced Ca^{2+} mobilization in primary sensory neurons. (A and B) Two different examples of cells recorded in the presence of voltage operated calcium channels blockers: 2 μM nifedipine (to block L-type Ca^{2+} channels), 2 μM ω-conotoxin-GVIA (to block N-type Ca^{2+} channels) and 1 μM ω-agatoxin-TK (to block P/Q-type Ca^{2+} channels). Cell depolarisation due to voltage-gated Na^+ channel activation was also prevented by adding 0.2 μM tetrodotoxin. The efficiency of the voltage operated channels block is confirmed by the application of a 25 mM KCl solution. The same percentage of cells showing tCFA15 induced $[Ca^{2+}]_i$ transients was recorded in the presence of channel blockers than in control conditions.

Table 1
Percentages of cells responsive to tCFA15 under different pharmacological treatments

Treatment	Number of cells[a]	% of responsive cells[b]	Statistical significance[c]
Control	547 (11)	30.9±2.25	
Block of VOCC[d]	491 (7)	33.1±4.31	
Presence of ryanodine	274 (3)	52.0±7.51	$P<0.01$ vs. control and VOCC block $P<0.001$ vs. thapsigargine
Thapsigargine or ryanodine plus caffeine	326 (4)	14.5±1.32	$P<0.01$ vs. control and VOCC block

[a] The number of independent experiments is indicated in brackets.
[b] Cells were considered responsive when they showed at least one transient $[Ca^{2+}]_i$ variation with a peak of 40 % of the baseline.
[c] See Section 2.5.
[d] VOCC: voltage operated calcium channels. Block of VOCC was obtained with 2 μM nifedipine, 2 μM ω-conotoxin-GVIA and 1 μM ω-agatoxin-TK.

but were not responsive to the application of a depolarising 25 mM KCl solution (Fig. 3A and B), indicating that voltage operated channels were blocked.

3.4. tCFA15 may operate through the activation of intracellular Ca^{2+} stores

As the tCFA15 effect did not depend on voltage operated channels and moreover involved a delayed Ca^{2+} response we hypothesized the recruitment of intracellular Ca^{2+} stores. Since submicromolar concentrations of ryanodine have been reported to induce an increase in the permeability of endoplasmic reticulum Ca^{2+} stores (Sutko et al., 1997) we imagined that the tCFA15 effect could be emphasized under these conditions. Cultured cells were incubated for 15 min in the presence of 0.5 μM ryanodine before tCFA15 application (Fig. 4A) or were bathed in a solution containing 0.5 μM ryanodine and 0.5 μM tCFA15 (Fig. 4B). In both cases, the most significant effect was an increase in the number of cells (over 50 %) showing $[Ca^{2+}]_i$ transients in the presence of tCFA15 (Table 1). Since the extracellular Ca^{2+} is necessary to observe tCFA15 induced $[Ca^{2+}]_i$ transients, a calcium induced–calcium release mechanism could be postulated. In this case, the depletion of intracellular Ca^{2+} stores should reduce the number of cells sensitive to tCFA15.

3.5. Blocking or depleting internal Ca^{2+} stores prevent tCFA15-induced $[Ca^{2+}]_i$ transients

Cells were incubated in the presence of 1 μM ryanodine and 10 mM caffeine. This treatment has been shown to irreversibly modify ryanodine receptors so that they are insensitive to Ca^{2+}(Rousseau et al., 1987). Under these conditions, only 15 % of cells showed $[Ca^{2+}]_i$ modifications in the presence of tCFA15 (Fig. 5A and Table 1). Finally, by interacting with sarco/endoplasmic reticulum Ca^{2+}-ATPases, thapsigargine can deplete the internal Ca^{2+} stores. In the presence of 1 μM thapsigargine, following depletion of internal Ca^{2+} stores, the number of cells responding to tCFA15 was reduced to 15 % (Fig. 5B and Table 1).

4. Discussion

An effect of tCFA15 on arginine vasopressin release from nerve terminals has been demonstrated and the involvement of Ca^{2+} in this effect has been suggested (Girlanda-Junges et al., 2000). In the present study, we measured Fura-2 fluorescent changes in adult mouse pituitary neural lobe synaptosomes and in primary sensory neurons and we showed that tCFA15 directly promotes intracellular $[Ca^{2+}]$ modifications. The effect depended on external Ca^{2+}, confirming that tCFA15 may act directly

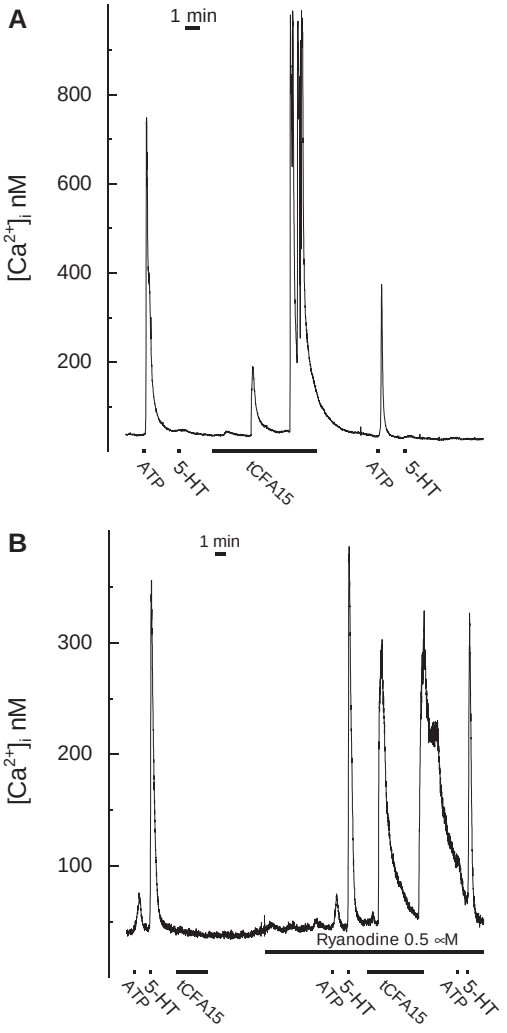

Fig. 4. The presence of ryanodine influences the tCFA15 effect in cultured primary sensory neurons. (A) Example of a cell that was incubated in the presence of 0.5 μM ryanodine for 15 min before recording the $[Ca^{2+}]_i$ changes induced by the application of 0.5 μM tCFA15. Under these conditions, 52 % of recorded cells showed tCFA15 induced $[Ca^{2+}]_i$ transients. (B) Example of a cell that was first controlled for its sensitivity to tCFA15 and then superfused with 0.5 μM ryanodine and 0.5 μM tCFA15. This cell did not responded to a first application of 0.5 μM tCFA15 but, in the presence of ryanodine, showed an activity particularly elevated after tCFA15 application, which suggests a use-dependent ryanodine-induced receptor modification. When this protocol was used, 50 % of cells showed $[Ca^{2+}]_i$ changes during the second application of tCFA15.

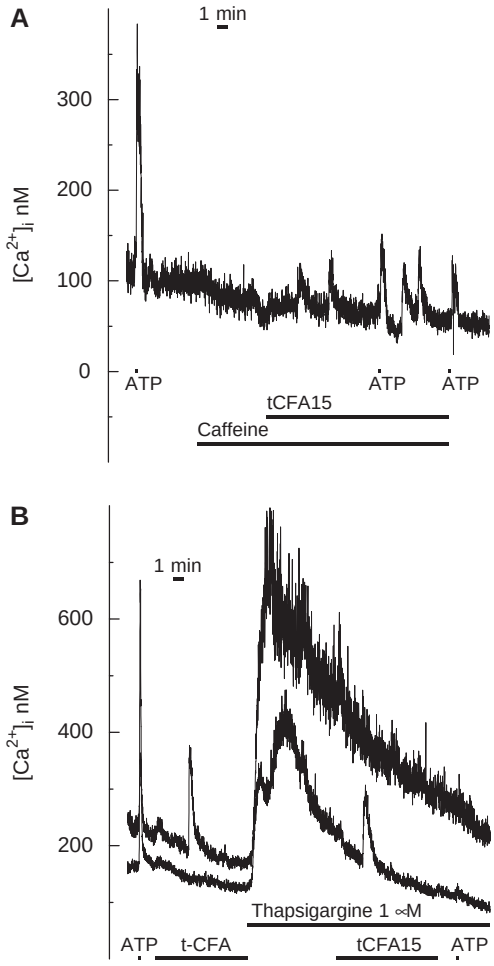

Fig. 5. Block or depletion of intracellular Ca^{2+} stores reduces the percentage of neurons showing tCFA15 induced $[Ca^{2+}]_i$ modifications. (A) Before tCFA15 application, cells were incubated in the presence of 1 μM ryanodine and 10 mM caffeine. The combined action of these drugs makes the ryanodine receptor insensitive to Ca^{2+}. Under these conditions, a reduced number of cells showed tCFA15 induced $[Ca^{2+}]_i$ transients that never went over 50 % of the base line, as it is shown in this particular example. (B) Internal Ca^{2+} stores were depleted by incubating the cells with 1 μM thapsigargine before the application of tCFA15. Examples of two cells are shown. In the first cell, a tCFA15 induced $[Ca^{2+}]_i$ transient was observed before thapsigargine application but not during the internal Ca^{2+} stores depletion. The second cell did not responded during the first incubation with tCFA15, but one transient was recorded during the internal Ca^{2+} stores depletion.

upon nerve terminals inducing Ca^{2+} entry and hence promoting neurohormone release.

Intracellular Ca^{2+} was not taken into account in other well characterized effects of tCFA15, such as promoting neurite outgrowth and neuronal survival (Gonzalez de Aguilar et al., 2001; Hanbali et al., 2004) or protecting peripheral nerve conduction velocity against steptozotocin-induced neuropathy (Watanabe and Miyagawa, 2002). The present study shows tCFA15 induced $[Ca^{2+}]_i$ transients not only in neurohypophyseal terminals but also in primary sensory neurons. The $[Ca^{2+}]_i$ peaks were of variable shape and size and did not followed a homogenous kinetic of initiation. Moreover, only a fraction of the simultaneously recorded synaptosomes or cultured neurons showed $[Ca^{2+}]_i$ variations during tCFA15 application. These observations conducted us to analyse records of groups of cells and to define as sensitive only the cells showing a peak of $[Ca^{2+}]_i$ above 40 % of the base lane, during at least one of the tCFA15 applications. In control conditions 30 % of cells showed a tCFA15 induced activity. In the presence of specific blockers of voltage operated calcium channels the percentage of sensitive cells remained unchanged. However, when internal calcium stores where depleted using either caffeine or thapsigargine only 15 % of the cells responded to tCFA15 application, always showing peaks of small amplitude. Moreover, submicromolar concentrations of ryanodine, which are expected to favour the release of Ca^{2+} from intracellular stores (Sutko et al., 1997), induced robust responses to tCFA15 in about 50 % of cells. These results exclude the activation of voltage operated channels by tCFA15 and suggest the implication of a calcium induced–calcium release mechanism evoked by the incoming Ca^{2+}. This is of particular interest to understand the pharmacological activity of tCFA15 even though more work is needed to clarify the first step of its molecular action.

In enteroendocrine cells, the recruitment of a specific signalling pathway has been proposed to explain $[Ca^{2+}]_i$ increases evoked by medium- to long-chain fatty acids (Sidhu et al., 2000). Moreover, many molecules with amphoteric properties can induce transient $[Ca^{2+}]_i$ changes through the activation of specific membrane receptors (Briscoe et al., 2003; Itoh et al., 2003; Kotarsky et al., 2003; Zhu et al., 2001). Thus, tCFA15 could be considered as an agonist pharmacophore of one of this pathways. However, the delay we observed between tCFA15 application and the surge of $[Ca^{2+}]_i$ transients is not consistent with this hypothesis. Earlier reports postulated that incorporation of lipidic substances into biological membranes can modify physicochemical properties that would affect a variety of cellular processes, including enzymatic activation and protein–lipid interactions, which might ultimately favour a Ca^{2+} influx (Alam and Alam, 1986; Rando, 1988; Stubbs and Smith, 1984). The prolonged time between tCFA15 application and intracellular Ca^{2+} mobilization may reflect a slow diffusion or a membrane partition step prior to receptor binding. In this respect, the emerging transient receptor potential (TRP channels) and vanilloid receptor family of Ca^{2+} permeable channels need to be considered. Since these ion channels are predominantly gated by intracellular lipidic ligands (Benham et al., 2002 and references therein), it is tempting to speculate that the bipolar nature of tCFA15 allows it to slowly reach the channel after interacting with the lipid phase of the plasma membrane.

Although the precise route by which tCFA15 drives Ca^{2+} into the cell has not been resolved, the demonstration that tCFA15 effectively mobilizes intracellular Ca^{2+} and, more importantly, that a limited amount of Ca^{2+} can be amplified by a calcium induced–calcium release mechanism may

provide an explanation for other biological effects of tCFA15. Our previous findings identifying a tCFA15 effect on neurite outgrowth in mouse foetal neurons (Gonzalez de Aguilar et al., 2001) are in agreement with transient increases in $[Ca^{2+}]_i$ able to modify intracellular signalling pathways. Moreover, tCFA15 has recently been shown to favour axonal growth while circumventing various selective inhibitors of growth-associated intracellular pathways (Hanbali et al., 2004). This can be understood through a local action of tCFA15 since neuritic processes of developing neurons enclose synaptotagmin carrying organelles that may promote neuritic growth by fusing with the plasma membrane in a Ca^{2+} dependent manner (Schwab et al., 2001). The unique capacity of tCFA15 to induce $[Ca^{2+}]_i$ transients at low concentrations in a variety of tissues further underlines the pharmacological potential of this molecule.

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