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Multiple effects of trichloroethanol on calcium handling in rat submandibular acinar cells

[1]S. Pochet, [1]N. Keskiner, [2]M. Fernandez, [2]A. Marino, [1]N. Chaïb, [1]J.P. Dehaye & *,[1]M. Métioui

[1]Laboratoire de Biochimie et de Biologie Cellulaire, Institut de Pharmacie, Université libre de Bruxelles, B-1050 Brussels, Belgium and [2]Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad del País Vasco, 48080 Bilbao, Spain

1 The effect of trichloroethanol (TCEt), the active metabolite of chloral hydrate, on the intracellular concentration of calcium ($[Ca^{2+}]_i$) was investigated in rat submandibular glands (RSMG) acini loaded with fura-2.

2 TCEt (1–10 mM) increased the $[Ca^{2+}]_i$ independently of the presence of calcium in the extracellular medium. Dichloroethanol (DCEt) and monochloroethanol (MCEt) reproduced the stimulatory effect of TCEt but at much higher concentrations (about 6 fold higher for DCEt and 20 fold higher for MCEt).

3 TCEt mobilized an intracellular pool of calcium, which was depleted by a pretreatment with thapsigargin, an inhibitor of the sarcoplasmic and endoplasmic reticulum calcium-dependent ATPases, but not with FCCP, an uncoupler of mitochondria.

4 TCEt 10 mM inhibited by 50% the thapsigargin-sensitive microsomal Ca^{2+}-ATPase. DCEt 10 mM and MCEt 10 mM inhibited the ATPase by 20 and 10%, respectively.

5 TCEt inhibited the increase of the $[Ca^{2+}]_i$ and the production of inositol phosphates in response to carbachol, epinephrine and substance P.

6 TCEt inhibited the uptake of calcium mediated by the store-operated calcium channel (SOCC).

7 ATP and Bz-ATP increased the $[Ca^{2+}]_i$ in RSMG acini and this effect was blocked by extracellular magnesium, by Coomassie blue and by oxydized ATP (oATP).

8 TCEt potentiated the increase of the $[Ca^{2+}]_i$ and of the uptake of extracellular calcium in response to ATP and Bz-ATP.

9 TCEt had no effect on the uptake of barium and of ethidium bromide in response to purinergic agonists.

10 These results suggest that TCEt, at sedative concentrations, exerts various effects on the calcium regulation: (1) it mobilizes a thapsigargin-sensitive intracellular pool of calcium in RSMG acini; (2) it inhibits the uptake of calcium *via* the SOCC; (3) it inhibits the activation by G protein-coupled receptors of a polyphosphoinositide-specific phospholipase C. It does not interfere with the activation of the ionotropic P2X receptors.

11 The use of chloral hydrate should be avoided in studies exploring the *in vivo* responses to sialagogues.

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Abbreviations: BSA, bovine serum albumin; Bz-ATP, 2′ & 3′-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate; $[Ca^{2+}]_i$, intracellular concentration of calcium; DCEt, dichloroethanol; DMSO, dimethyl sulphoxide; EGTA, ethylene glycol-bis (β-aminoethylether)-N,N,N′,N′-tetraacetic acid; Epi, epinephrine; FCCP, carbonyl cyanide p-(trifluoromethoxy)phenyl hydrazone; Fura-2/AM, fura-2 acetoxymethyl ester; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]; HBS, HEPES-buffered saline; IP_3, inositol 1,4,5-trisphosphate; MCEt, 2-chloroethanol; oATP, adenosine 5′-triphosphate-2′,3′ dialdehyde; PPI-PLC, polyphosphoinositide-specific phospholipase C; RSMG, rat submandibular glands; SOCC, store-operated calcium channel; SP, substance P; TCA, trichloroacetic acid; TCEt, 2,2,2-trichloroethanol; TG, thapsigargin; TRIS, Tris(hydroxymethyl)-aminomethane

Introduction

Most of the anaesthetics are rather lipophilic molecules. Overton and Meyer correlated their anaesthetic property with their solubility in olive oil, suggesting that they interacted with the lipid components of the cell (Lipnick, 1986). Considering their hydrophobic properties, it was thought that membranes would be parts of these components. Indeed, it was demonstrated in more recent works that anaesthetics could affect the properties of lipid bilayers (Trudell, 1991; Cantor, 1997). Nevertheless, various observations did not fit with this model. It was shown that the anaesthetic properties of a molecule were better correlated with its solubility in

*Author for correspondence at: Laboratoire de Biochimie et de Biologie Cellulaire, Institut de Pharmacie C.P. 205/3, Université libre de Bruxelles, Boulevard du Triomphe, B-1050 Brussels, Belgium; E-mail: mmetioui@ulb.ac.be

octanol suggesting that not only hydrophobic interactions contributed to their property but also some polar interaction between the drug and its cellular target (Franks & Lieb, 1978). It was also shown that the anaesthetic potency of molecules with similar structures was abruptly lost as the chain length of n-alkanes or n-alkanols was increased beyond a specific cut-off point (Curatola *et al*., 1991). This observation suggested that the size of the anaesthetic was somehow limiting, and that its interaction with a target involved a hydrophobic pocket of limited size. Since proteins are major components of membranes and since they can form hydrophobic pockets, it could be conceived that the anaesthetics would bind to proteins and affect their properties. This was in line with the early observation by Moore & Roaf (1905) that ether and chloroform were more soluble in solutions containing proteins than in pure water. This was also consistent with the reported effect of anaesthetics on luciferase (Harvey, 1915). Considering (1) that ion channels control most of the ion movements through membranes and (2) that the distribution of ions on both faces of membranes is a major determinant of the membrane potential and (3) that the excitability of the cells is controlled by their membrane potential, it was suggested that anaesthetics could specifically affect the activity of ion channels. Based on their results obtained with luciferase, Ueda *et al*. (1976) suggested that anaesthetics might dehydrate the sodium channel and suppress the conductance of this ion across the membrane. This hypothesis has been extensively confirmed and it is now widely recognized that anaesthetics are good pharmacological tools to modulate the activity of ion channels in order to investigate the role of ion movements in cell function (Yamakura *et al*., 2001).

Anaesthetics are mandatory medications required when testing a drug *in vivo*. In this case, the interaction of the anaesthetic itself with the studied parameter should not be ignored. Chloral hydrate is a sedative and depressant that is widely used to anaesthetize animals during the *in vivo* testing of drugs (Silverman & Muir, 1993). This anaesthetic is so rapidly metabolized to TCEt (Abbas *et al*., 1996) that this halogenated derivative of ethanol is probably the active compound (Sourkes, 1992). It has been reported that TCEt could affect the increase of the intracellular concentration of calcium ($[Ca^{2+}]_i$) in response to N-methyl D-aspartate (NMDA) (Scheibler *et al*., 1999). It has also been shown that TCEt increased the open state probability of HT_3-receptors (Lovinger *et al*., 2000) and that it inhibited the current mediated by the recombinant human $P2X_3$ receptor expressed in HEK cells (Koles *et al*., 2000). The salivary flow in response to various agonists has been studied in animals anaesthetized with chloral hydrate (Nilsson *et al*., 1991). Ion movements are the cornerstone of any exocrine secretion. Acini from salivary glands are responsible for the elaboration of a primary isotonic secretion (Melvin, 1999). The hydromineral secretion of acini is controlled by the $[Ca^{2+}]_i$. Calcium channels located on intracellular membranes and on the plasma membrane contribute to the increase of the intracellular concentration of this ion (Ambudkar, 2000). To our knowledge the effect of TCEt on salivary glands has never been studied. The purpose of this work was to investigate the effect of this drug on the $[Ca^{2+}]_i$ in rat submandibular acini. We also explored its interaction with agonists which increase the $[Ca^{2+}]_i$ either by activating a phospholipase C specific for polyphosphoinositides or by activating the ionotropic P2X receptors expressed by these cells (Marino *et al*., 1999).

Methods

Cell preparation

The rats were anaesthetized and killed by exsanguination. The submandibular glands were immediately dissected and finely minced. The minced tissue was digested in the presence of 0.4 u ml^{-1} of collagenase P for 20 min at 37°C under constant shaking in 10 ml HEPES-buffered saline (HBS) containing (mM): HEPES (pH 7.4) 24.5, NaCl 96, KCl 6, $MgCl_2$ 1, NaH_2PO_4 2.5, glucose 11.5, sodium pyruvate 5, sodium glutamate 5, sodium fumarate 5, 1% glutamine-free amino acids mixture. Bovine serum albumin (BSA) 0.125% (w v^{-1}) was added to this medium. Ten minutes after the beginning of the digestion, the cells were aspirated several times with 10, 5 and 2 ml glass pipettes. At the end of the digestion the crude suspension was pipetted again, filtered and washed in a saline solution. Acinar and ductal fractions were separated by centrifugation at $4000 \times g$ for 10 min at 4°C through a 40% Percoll solution made isotonic with sodium chloride. The acini were recovered, washed, resuspended in HBS with BSA and kept at 4°C until use.

Preparation of a microsomal fraction

Rats were sacrificed as above and their submandibular glands were immediately dissected and excised in an ice-cold isotonic NaCl solution. The glands were homogenized with a Teflon pestle in 2 ml of a 0.3 M sucrose solution containing 0.02 M histidine, 0.1 mM dithiotreitol, 5 μM phenylmethylsulphonylfluoride, 1 mg ml^{-1} soybean trypsin inhibitor, and 5 mg ml^{-1} bacitracin. Homogenates were centrifuged at $800 \times g$ for 5 min at 4°C. Supernatants were removed, and the pellets were rehomogenized and recentrifuged in the same conditions. The supernatants were then combined and centrifuged at $16{,}000 \times g$ for 20 min at 4°C in a Kontron Centrikon T-2080 centrifuge. Supernatants were recentrifuged at $105{,}000 \times g$ for 20 min at 4°C and the pellets were resuspended in a TRIS 50 mM/histidine 5 mM buffer, pH 8.9. This microsome-containing solution was layered onto a discontinuous sucrose gradient (1.5 M – 0.32 M) and centrifuged at $52{,}000 \times g$ for 1 h at 4°C. Microsomes were collected from the interface of the discontinuous gradient and diluted 1.5 fold. They were concentrated by centrifugation at $105{,}000 \times g$ for 1 h at 4°C and resuspended in a buffer containing 0.25 M sucrose, 1 M KCl, and 50 mM KH_2PO_4. Protein concentration was measured by the Bradford method (Bradford, 1976) and microsomes were aliquoted, frozen in liquid nitrogen and stored at −80°C until use.

Measurement of the production of inositol phosphates

The acini were resuspended in 2 ml HBS medium in the presence of 1 mM $CaCl_2$ and 0.5% (w v^{-1}) BSA. They were incubated for 90 min at 37°C in the presence of 25 μCi ml^{-1} [^{3}H]-inositol. At the end of the labelling period, the acini were washed three times with isotonic NaCl. The final pellet was

resuspended in 12 ml HBS medium without magnesium. The acini were preincubated for 10 min at 37°C in the presence of 10 mM LiCl and thereafter were incubated for 20 min in the presence of the tested agent and 10 mM LiCl. The incubation was stopped by the addition of ice-cold trichloroacetic acid at a 5% final concentration. The samples were sonicated for 10 s at an amplitude of 15 microns (MSE Soniprep 150) and the homogenates were kept at 4°C for 10 min. After centrifugation for 10 min at $1500 \times g$, the pellets were discarded. Fifty μl of 1 N HCl were added to the supernatants, which were extracted four times with 4 ml diethylether saturated with water. The remaining ether was evaporated and the extracts were neutralized with 100 μl Tris 1 M. The samples were placed on top of a 2 ml Dowex AG1X-8 column (100–200 mesh, formate form, Fluka Chemie AG, Buchs, Switzerland). The columns were eluted with 15 ml water and 15 ml of a sodium containing 60 mM ammonium formate and 5 mM sodium tetraborate. The inositol phosphates were then eluted from the column with 7 ml of a solution containing 1 M ammonium formate and 0.1 N formic acid. Ten ml Ecoscint A (National Diagnostics, Atlanta, GA, U.S.A.) were added to the samples and the radioactivity was estimated the next day in order to avoid chemiluminescence.

Determination of the $[Ca^{2+}]_i$

Aliquots of the acinar suspension were incubated in the dark at 25°C in HBS buffer in the presence of a final concentration of 0.5% BSA, 0.25 mM $CaCl_2$ and 2 μM fura-2/AM. After 45 min the acini were washed and resuspended in 2 ml magnesium-free HBS without BSA or amino acids. The cells were constantly stirred in the cuvette and the excitation wavelength was switched every second from 340 nm to 380 nm. The light emitted at 505 nm was recorded. At the end of the assay the traces were calibrated with the successive addition of 120 μg ml^{-1} digitonin and 40 mM EGTA (pH 8.5 with Tris). Autofluorescence was measured after quenching of the fluorescence of fura-2 by the addition of 100 mM $MnCl_2$ and was substracted from all the data before calculation of the ratios. The calcium concentration was estimated by the ratio method of Grynkiewicz *et al.* (1985).

Determination of the uptake of calcium and barium

Barium and calcium modify the excitation spectrum of fura-2 in a similar way, increasing the light emitted after excitation at 340 nm and decreasing the signal after excitation at 380 nm (Kwan & Putney, 1990). As a consequence, the ratio of these two signals increases during the formation of the fura-2-divalent cation complex. To measure the uptake of the two ions, RSMG acinar cells were loaded with fura-2 as described in the previous paragraph. After washing, they were resuspended in HBS medium in the absence of calcium and magnesium. They were constantly stirred in the cuvette of the fluorimeter which was maintained at 25°C. One minute after the start of the measurement, EGTA was added to the cuvette (final concentration, 0.1 mM). After exposure of the cells to the different agonists, calcium (final concentration, 5 mM) or barium (final concentration, 1 mM) was added to the medium. At the end of the assay, the traces where calcium had been added were calibrated as described in the previous paragraph and the ratio of the fluorescence emitted at 505 nm after excitation at 340 nm and 380 nm was converted to calcium concentrations. When the uptake of barium was estimated, no attempt was made to convert fluorescence measurements to barium concentrations. At the end of the incubation, the cells were permeabilised with digitonin and the autofluorescence was estimated by the addition of 100 mM $MnCl_2$ to the medium. After subtraction of this autofluorescence, a new ratio was calculated and used as an index of the intracellular concentration of barium.

Determination of the uptake of ethidium bromide

The permeabilization of the plasma membrane was measured with the fluorescent dye ethidium bromide (Di Virgilio *et al.*, 1989; Chaïb *et al.*, 2000). Acini from one rat were resuspended in 12 ml HBS, and for each assay a 1 ml-aliquot was washed and resuspended in 2 ml HBS medium in the absence of magnesium and amino acids. Ethidium bromide was added to a final concentration of 20 μM and the cells were allowed to equilibrate for 10 min before the start of the measurement performed at 37°C with constant stirring. The samples were excited at 360 nm and the light emitted at 580 nm was measured and recorded every 2 s. At the end of the measurement the maximum uptake was estimated by adding digitonin (120 μg ml^{-1}). The results were plotted as a function of time.

Determination of Ca^{2+}-ATPase activity

Microsomal Ca^{2+}-ATPase activity was determined spectrophotometrically using an enzymatically-coupled assay as previously described for sarcoplasmic Ca^{2+}-ATPase from rabbit white muscle (East, 1993). The assay was carried out at 37°C in a thermostatted cuvette. Each measurement was performed with 5 μg of microsomes in 2.5 ml of the following buffer: HEPES-KOH 30 mM pH 7.2, $CaCl_2$ 1 mM, $MgSO_4$ 5 mM, EGTA 1 mM, phosphoenolpyruvate 0.5 mM, NADH 0.15 mM, pyruvate kinase 3 u ml^{-1} and lactate dehydrogenase 2 u ml^{-1}. The reaction was started by adding a final concentration of 4 mM ATP. The kinetic of disappearance of NADH was monitored at 340 nm during at least 10 min. The decrease in absorbance at this wavelength was linear ($r^2 = 0.97–0.99$) between 1 and 4 min. The Ca^{2+}-ATPase activity was estimated from the rates of NADH consumption, which were calculated from the slopes of the traces between 1 and 4 min after the start of the measurement.

Statistics

Unless otherwise indicated, values in figures are given as means + standard error (s.e.m.) of n experiments performed with n different cell preparations. When the results were plotted as a function of time, the graph was plotted by using all the data, but only one out of 12 values was represented. Non-parametric tests were used to compare data (the Wilcoxon test for paired data and the Mann–Whitney test for non-paired data).

Drugs and animals

Male Wistar rats (150–200 g) were purchased from the Proefdierencentrum of the Katholieke Universiteit Leuven

(Heverlee, Belgium). The animals were fed *ad libitum* and had free access to water. The Belgian Ministry of Agriculture approved the care and use of rats in this study in agreement with EEC regulations.

Pluronic acid and fura-2/AM were from Molecular Probes (Eugene, OR, U.S.A.). Collagenase P and bovine serum albumin, (BSA, fraction V) were from Boehringer Mannheim (Mannheim, Germany). The glutamine-free amino acid mixture and ethidium bromide were from Gibco BRL (Paisley, U.K.). Adenosine 5′-triphosphate, 2′,3′-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate (Bz-ATP), adenosine 5′-triphosphate -2′,3′ dialdehyde (oATP), 2,2,2-trichloroethanol (TCEt), 2-chloroethanol (MCEt), N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES) and carbonyl cyanide p-(trifluoromethoxy)phenyl hydrazone (FCCP) were obtained from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). 2,2-dichloroethanol (DCEt) was from ChemPure (Karlsruhe, Germany). 2-[^{3}H] myo-inositol (20 Ci mmol^{-1}) was purchased from ICN Biomedicals Inc. (Irvine, CA, U.S.A.). Percoll was obtained from Pharmacia (Uppsala, Sweden). The other materials were purchased from various sources and were reagent grade.

Results

Effect of trichloroethanol on the $[Ca^{2+}]_i$

RSMG acini loaded with fura-2 were resuspended in HBS medium in the absence of magnesium, in the presence of 1 mM calcium. Exposure of these cells to TCEt (3–10 mM range) increased the $[Ca^{2+}]_i$ (Figure 1, left panel). In the presence of 10 mM TCEt, the $[Ca^{2+}]_i$ increased within 1 min from 67.1±4.7 nM to 130.0±10.0 nM ($n=19$). A sustained increase was noted at later times and the $[Ca^{2+}]_i$ averaged 146.2±15.5 nM after 5 min. TCEt (5 and 3 nM) also increased significantly the $[Ca^{2+}]_i$ to 89.7±8.4 nM ($n=14$) and 80.1±7.7 nM ($n=14$), respectively, after 1 min. However, 1 nM TCEt had no significant effect (data not shown). The response to TCEt was reproduced by DCEt but higher concentrations (5 fold higher) were required to observe a similar increase of the $[Ca^{2+}]_i$ (Figure 2). MCEt was even less potent than DCEt: at a 100 mM concentration, MCEt only increased the $[Ca^{2+}]_i$ by 24.3±3.1 nM ($n=5$), an effect similar to the response to 30 mM DCEt or 5 mM TCEt. Ethanol (100 mM) had no significant effect on the $[Ca^{2+}]_i$.

By comparison, 100 μM carbachol increased the $[Ca^{2+}]_i$ within 6 s to a peak value followed by a fast decline (Figure 1, right panel). The $[Ca^{2+}]_i$ remained at a level slightly higher than the initial concentration. The rapid increase was due to the mobilization of the IP_3-sensitive intracellular pool of calcium. The sustained increase was due to the uptake of extracellular calcium *via* the store-operated calcium channel (SOCC) activated by the depletion of the IP_3-sensitive calcium pool (Ambudkar, 2000). The preincubation for 5 min in the presence of 10 mM TCEt significantly inhibited the rapid response to carbachol (from +169.0±5.4 nM, $n=13$, to +47.4±6.5 nM, $n=9$, $P<0.001$). TCEt also blocked the response to the α-adrenergic agonist epinephrine (from +135.7±11.1 nM, $n=5$, to +41.5±4.2 nM, $n=5$, $P<0.001$) and the response to the tachykinin substance P (from +204.0±16.0 nM, $n=3$, to +55.6±9.7 nM, $n=3$, $P<0.001$). These results suggested that TCEt blocked a common step leading from the occupancy by agonists of various receptors to the mobilization of intracellular pools of calcium and the activation of the uptake of this ion.

Effect of TCEt on the production of inositol phosphates

Since both carbachol, epinephrine and substance P activate a polyphosphoinositide-specific phospholipase C (PPI–PLC), the effect of TCEt on this enzyme was tested. These experiments were performed in the presence of 10 mM lithium in order to block the activity of inositol phosphatases. By itself, TCEt 10 mM only slightly increased (+15%) the accumulation of inositol phosphates during a 20-min incubation. In these conditions, 100 μM carbachol, 100 μM epinephrine and 100 nM substance P increased these levels by respectively 14.8, 10 and 3 fold. When the cells were exposed to both TCEt and one of these agonists, TCEt inhibited by 71% the response to carbachol, by 70% the response to epinephrine and by 53% the response to substance P (Figure 3). The cells were also challenged with aluminium fluoride. It has been shown that, in salivary glands, this salt bypasses plasma membrane receptors and directly activates the G protein coupled to phospholipase C (Tojyo *et al*., 1991). Aluminium fluoride increased by 6.5 fold the basal level of inositol phosphates. TCEt inhibited by 47% the response to fluoride (3.45 fold).

Contribution of intracellular pools of calcium to the increase of the $[Ca^{2+}]_i$ in response to TCEt

In order to determine the contribution of the extracellular calcium in the response to TCEt, the effect of this agent was studied in acini resuspended in the absence of extracellular calcium. After 1 min EGTA (100 μM final concentration) was added to the medium. This low concentration of calcium chelator was used in order to inverse the gradient of calcium, favouring its efflux without leading to a spontaneous depletion of intracellular pools of the ion. As shown in the right panel of Figure 4, the addition of 100 μM carbachol to the medium increased the $[Ca^{2+}]_i$ by 17.4±3.3 nM ($n=12$) within 6 s. This sharp increase was followed by a steady decline of the $[Ca^{2+}]_i$ which recovered its basal level after 4 min. Thapsigargin, an inhibitor of intracellular Ca^{2+}-dependent ATPases (Métioui *et al*., 1994), slowly increased the $[Ca^{2+}]_i$ (+15.1±1.7 nM, $n=7$, after 1 min). This peak value was followed by a decrease to the basal value within 5 min. In the same conditions, ATP (1 mM) had no effect on the $[Ca^{2+}]_i$ confirming that, in acini, the activation of purinergic receptors was not coupled to the mobilization of intracellular pools of calcium (Marino *et al*., 1999). TCEt (10 mM) slowly increased the $[Ca^{2+}]_i$: it took nearly 90 s to observe a maximal increase of the $[Ca^{2+}]_i$ (+17.7±2.5 nM, $n=6$) (Figure 4, left panel). However, unlike the other agonists, the $[Ca^{2+}]_i$ was not significantly affected for the next 8 min: the increase of the $[Ca^{2+}]_i$ still averaged +14.0±3.2 nM ($n=6$, $P<0.01$ when compared to control) 10 min after the addition of TCEt to the medium.

Characterization of the intracellular pool of calcium depleted by TCEt

Several intracellular pools of calcium (IP_3-sensitive or insensitive, thapsigargin- or ryanodine-sensitive, mitochon-

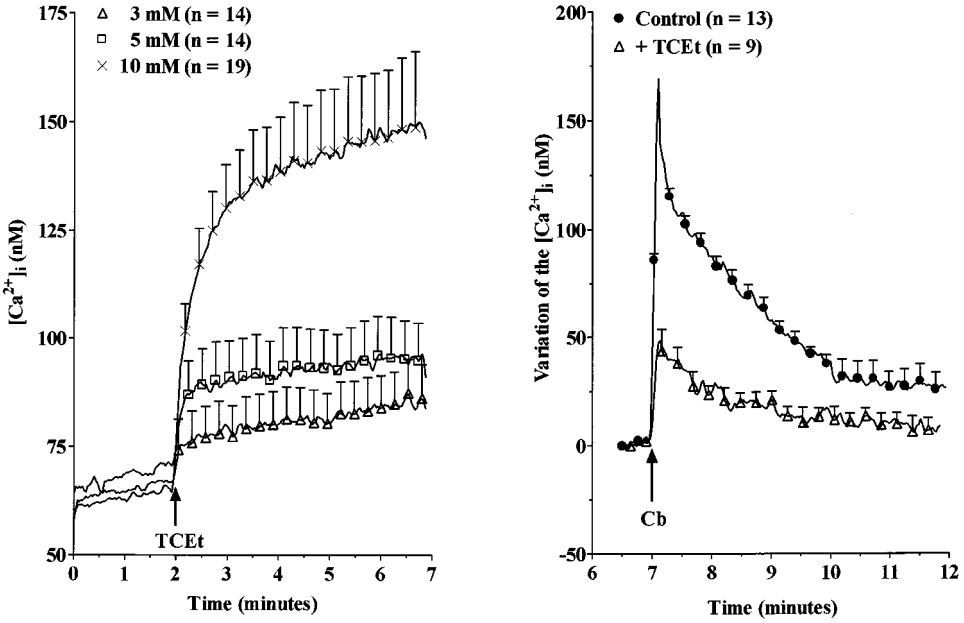

Figure 1 Effect of various concentrations of TCEt on the $[Ca^{2+}]_i$. Rat submandibular acini were loaded with fura-2. After washing, they were resuspended in 2 ml HBS medium, in the presence of 1 mM $CaCl_2$ but in the absence of magnesium. They were maintained at 25°C and constantly stirred in the cuvette of the fluorimeter. Left panel: 2 min after the beginning of the measurement (arrow) they were exposed to various concentrations of TCEt. Right panel: 2 min after the beginning of the measurement, the acini were further incubated either in the presence of DMSO or in the presence of 10 mM TCEt and 5 min later (arrow), 100 μM carbachol was added to the cuvette. The results are the means + s.e.mean of n experiments.

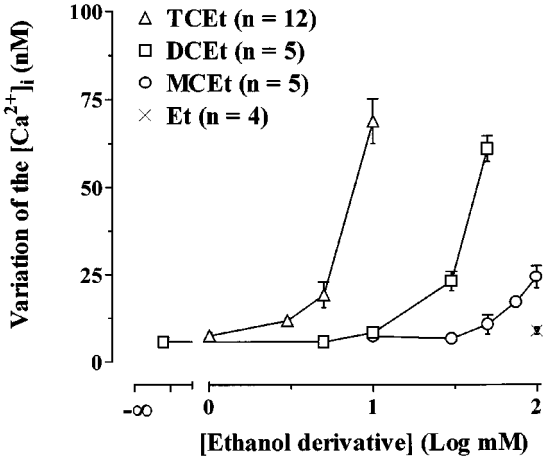

Figure 2 Comparison of the effect of TCEt, DCEt, MCEt and ethanol on the $[Ca^{2+}]_i$. Rat submandibular acini were loaded with fura-2. After washing, they were resuspended in 2 ml HBS medium, in the presence of 1 mM $CaCl_2$ but in the absence of magnesium. They were maintained at 25°C and constantly stirred in the cuvette of the fluorimeter. Two minutes after the beginning of the measurement, they were exposed to various concentrations of TCEt, DCEt, MCEt or ethanol. The results are expressed as the variation of the $[Ca^{2+}]_i$ 1 min after the addition of the ethanol derivative. They are the means ± s.e.mean of n experiments.

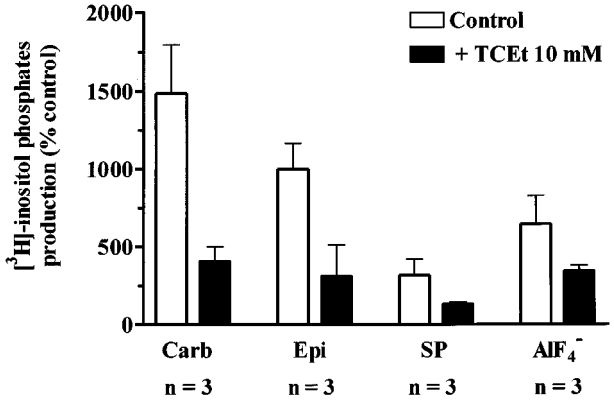

Figure 3 Effect of TCEt on the production of inositol phosphates. Rat submandibular acinar cells were loaded with [^{3}H]-inositol. After washing, they were resuspended in HBS medium in the absence of magnesium and in the presence of 1 mM $CaCl_2$, 1% (w v^{-1}) BSA and 1% (v v^{-1}) amino acid mixture. They were preincubated for 10 min in the presence of 10 mM LiCl. At the end of the preincubation, the cells were incubated for 5 min in control conditions or in the presence of 10 mM TCEt before the addition of 100 μM carbachol, or 100 μM epinephrine, or 100 nM substance P, or 10 mM aluminium fluoride. The incubation was stopped by the addition of 5% (w v^{-1}) TCA. The inositol phosphates were extracted and separated as described in Methods. The results are expressed as per cent of the control and are the means + s.e.mean of three experiments.

drial pools) have been described (Hajnoczky *et al*., 2000). The IP_3-sensitive intracellular calcium pool is mobilized by carbachol, epinephrine or substance P, agonists activating the PPI-PLC. It is also depleted when the cells are incubated in the presence of thapsigargin, an inhibitor of the Ca^{2+}-dependent ATPases responsible for the active uptake of calcium in this pool. In order to examine the contribution of this pool in the response to TCEt, the cells were incubated in a calcium-free medium and preincubated for 5 min either in control conditions or in the presence of 10 mM TCEt. They were then exposed to 1 μM thapsigargin. As shown in the left panel of Figure 5, the preincubation with TCEt completely

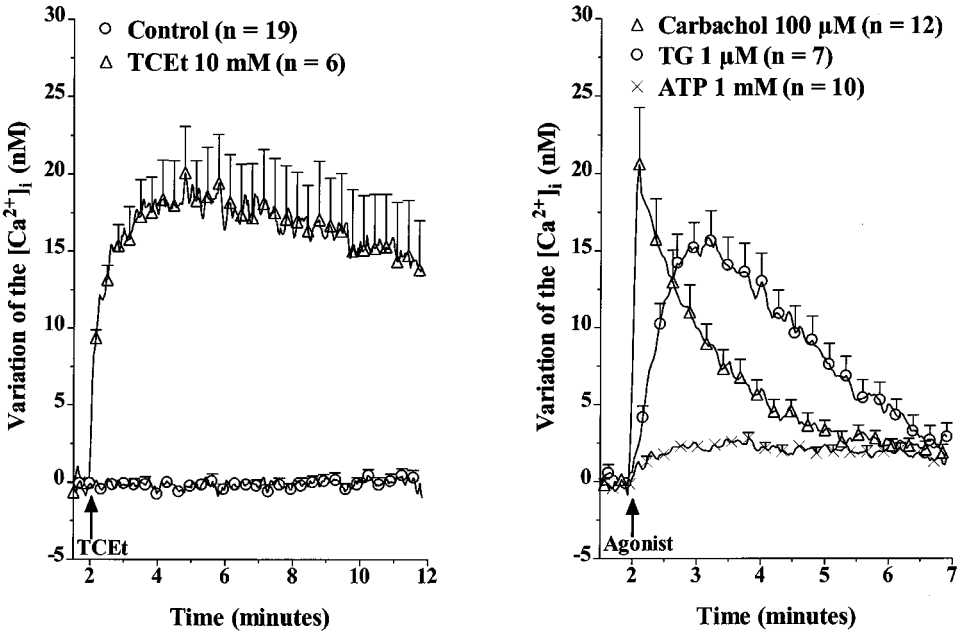

Figure 4 Effect of TCEt and various calcium-mobilizing agonists on the $[Ca^{2+}]_i$ in the absence of extracellular calcium. Rat submandibular acini were loaded with fura-2. After washing, they were resuspended in 2 ml HBS medium, in the absence of calcium and magnesium. They were maintained at 25°C and constantly stirred in the cuvette of the fluorimeter. One minute after the beginning of the measurement, they were exposed to 100 μM EGTA and, 1 min later (arrow), they were either maintained in control conditions or exposed to the mentioned agonists. The results are expressed as the variation of the $[Ca^{2+}]_i$ when compared to the $[Ca^{2+}]_i$ measured 30 s after the addition of EGTA to the medium. The results are the means + s.e.mean of *n* experiments.

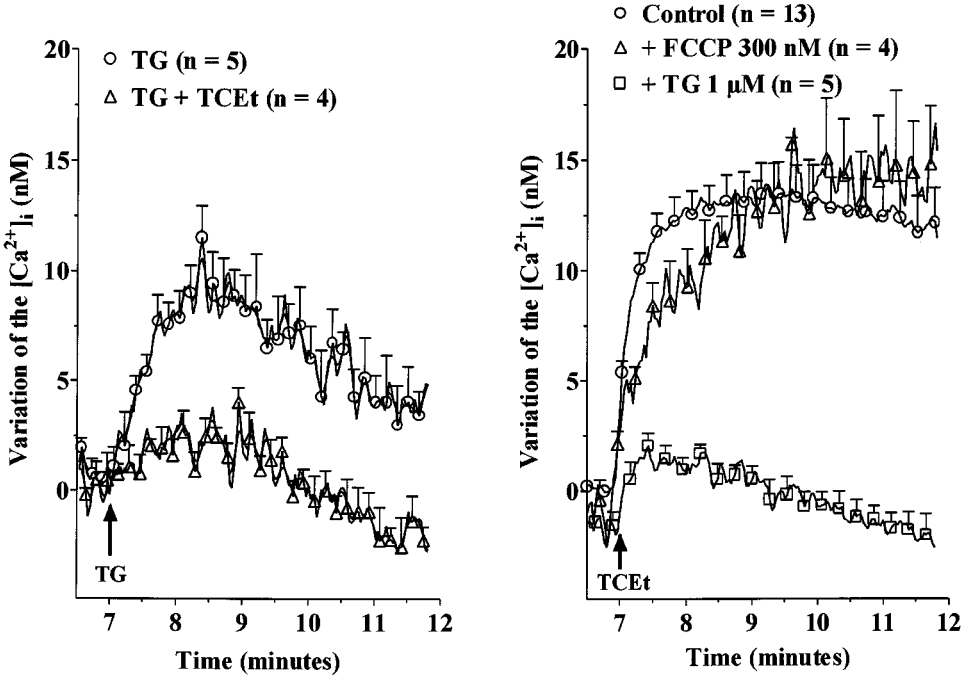

Figure 5 Characterization of the intracellular pool of calcium mobilized by TCEt. Rat submandibular acini were loaded with fura-2. After washing, they were resuspended in 2 ml HBS medium, in the absence of calcium and magnesium. They were maintained at 25°C and constantly stirred in the cuvette of the fluorimeter. One minute after the beginning of the measurement, 100 μM EGTA (final concentration) was added to the cuvette. Left panel: At 2 min the acini were maintained in control conditions or exposed to 10 mM TCEt and 5 min later 1 μM thapsigargin (arrow) was added to the medium. Right panel: At 2 min, the cells were either maintained in control conditions or exposed to 1 μM thapsigargin or to 300 nM FCCP and 5 min later 10 mM TCEt (arrow) was added to the cuvette. The results are expressed as the variation between the $[Ca^{2+}]_i$ measured at 6.5 min and the $[Ca^{2+}]_i$ measured at subsequent times. These results are the means + s.e.mean of *n* experiments.

blocked the increase of the $[Ca^{2+}]_i$ in response to thapsigargin. This result suggested that the IP_3-sensitive intracellular calcium pools could be depleted after exposure of the acini to TCEt. This result was confirmed in a reverse experiment. Acini incubated in a calcium-free medium were exposed to either 1 μM thapsigargin or to 300 nM FCCP, a mitochondrial uncoupler releasing calcium from the mitochondria. As shown in the right panel of Figure 5, the exposure of the acini to FCCP had no effect on the mobilization of intracellular calcium by TCEt: TCEt increased the $[Ca^{2+}]_i$ by 13.1 ± 1.1 nM, $n=13$, after 2 min in acini preincubated in control conditions and by 14.1 ± 1.7 nM, $n=4$, in acini preincubated in the presence of 300 nM FCCP. When the cells had been preincubated with 1 μM thapsigargin, the mobilization of intracellular calcium by TCEt was fully inhibited. These results confirmed that TCEt mobilized thapsigargin-sensitive (probably the IP_3-sensitive) intracellular calcium pools.

Effect of TCEt on the thapsigargin-sensitive microsomal Ca^{2+}-ATPase

The mobilization of thapsigargin-sensitive intracellular calcium pools by TCEt in the absence of any increase of inositol phosphates production prompted us to test whether TCEt, like thapsigargin, inhibited the microsomal Ca^{2+}-ATPase responsible for the active transport of calcium in these pools. For this purpose, microsomes were prepared by differential centrifugation on a sucrose gradient and the Ca^{2+}-ATPase was assayed using an enzymatically-coupled reaction and monitoring the NADH consumption. The reaction was started by the addition of ATP to the incubation medium in the presence or absence of the tested agent. After an initial drop, the decrease in NADH absorbance was linear between 1 and 4 min (Figure 6). The rate of decrease in NADH absorbance, which is a measure of the microsomal Ca^{2+}-ATPase activity, was estimated from the slope of the curve between 1 and 4 min. In basal conditions, this slope averaged -0.087 ± 0.003 units min^{-1}, $n=6$ (Figure 6). Thapsigargin 10 μM inhibited by $\sim$60% the rate of the Ca^{2+}-ATPase-coupled NADH consumption (-0.035 ± 0.004 units min^{-1}, $n=6$). We thus tested the effect of the three halogenated ethanol derivatives on the rate of this reaction. As shown in the insert of Figure 6, TCEt 10 mM inhibited this reaction by $\sim$50% (-0.045 ± 0.003 units min^{-1}, $n=4$), while DCEt 10 mM and MCEt 10 mM only slightly affected NADH disappearance, inhibiting the Ca^{2+}-ATPase by only $\sim$30% (-0.060 ± 0.004 units min^{-1}, $n=3$, for DCEt, and -0.066 ± 0.007 units min^{-1}, $n=3$, for MCEt).

Effect of TCEt on the uptake of extracellular calcium via *the store-operated calcium channels (SOCC)*

According to the capacitative model, the depletion of IP_3-sensitive intracellular calcium pools is responsible for the opening of a calcium channel (SOCC) in the plasma membrane. Considering that TCEt depleted thapsigargin-sensitive calcium pools, it was speculated that, like thapsigargin, it could increase the uptake of extracellular calcium. The uptake was measured by the addition of a high concentration (5 mM) of calcium to acini previously incubated for 11 min in a calcium-free medium. In acini incubated in control conditions, the $[Ca^{2+}]_i$ increased within 1 min by 88.6 ± 4.5 nM, ($n=19$, Figure 7, left panel). In agreement with the capacitative model, 100 μM carbachol increased the uptake of calcium by 145.5 ± 16.4 nM ($n=6$) 1 min after the addition of calcium to cells preincubated for 10 min with the muscarinic agonist ($P<0.001$, when compared to control). When the cells were exposed to 10 mM TCEt 10 min before the addition of calcium to the medium (Figure 7, left panel), the $[Ca^{2+}]_i$ increased by only 30% when compared to basal uptake ($+109.1 \pm 9.1$ nM, $n=6$. $P<0.01$ when compared to control or carbachol). This suggested that either the release of calcium by TCEt from thapsigargin-sensitive pools was not coupled to the activation of SOCC or the activation of these channels was masked by an inhibitory effect of TCEt on them. To test this latter hypothesis, the effect of TCEt on the activation of the SOCC by thapsigargin was studied. The cells were first incubated for 5 min in a calcium-free medium in control conditions or in the presence of 10 mM TCEt. One μM thapsigargin was then added to the medium and five min later the uptake of calcium was measured. As shown in the right panel of Figure 7, TCEt inhibited by 50% the uptake of calcium measured in the presence of 1 μM thapsigargin (from $+255.9 \pm 14.1$ nM, $n=5$, to $+130.9 \pm 18.2$ nM, $n=4$). This inhibition was highly significant ($P<0.01$).

Interaction between TCEt and purinergic agonists

It is now widely recognized that submandibular acinar cells express ionotropic $P2X_4$ and $P2X_7$ purinergic receptors (Dehaye *et al*., 1999). As shown in the left panel of Figure 8, 1 mM extracellular ATP increased the $[Ca^{2+}]_i$. The response to ATP was biphasic: a rapid increase ($+74.5 \pm 7.8$ nM, $n=11$, after 1 min) was followed by a more sustained increase for the next 4 min ($+114.7 \pm 11.8$ nM, $n=11$, after 5 min). Only high concentrations of ATP (higher than 30 μM) could significantly increase the $[Ca^{2+}]_i$ (Figure 9). Benzoyl-ATP was a much better agonist than ATP: the $[Ca^{2+}]_i$ increased by 344.4 ± 31.6 nM ($n=6$) in acini exposed for 5 min to 100 μM Bz-ATP (Figure 8, right panel). The response to both ATP and Bz-ATP was blocked by about 70% when 5 mM magnesium was added to the medium. It was also inhibited by 10 μM Coomassie blue or after a 2-h preincubation in the presence of 100 μM oATP (Table 1). A preincubation for 5 min with 10 mM TCEt potentiated the response to 1 mM ATP ($+443.5 \pm 86.7$ nM, $n=10$, after 5 min exposure to ATP) and to 100 μM Bz-ATP ($+858.3 \pm 61.8$ nM, $n=6$). The potentiation by TCEt of the response to purinergic receptors was further investigated by measuring the uptake of extracellular calcium in response to ATP. A preincubation for 10 min with 1 mM ATP steadily increased the uptake of extracellular calcium and the $[Ca^{2+}]_i$ increased by 243.6 ± 42.7 nM ($n=3$) 5 min after the addition of calcium to the medium. The addition of TCEt to the medium 5 min after exposure of the cells to ATP but 5 min before the addition of calcium potentiated the response to ATP ($+487.2 \pm 65.1$ nM, $n=3$) (data not shown). This result further suggested that TCEt potentiated the opening of the non-specific cation channel coupled to P2X receptors. But considering that TCEt inhibited the uptake of calcium by intracellular pools (*vide supra*), these results should be confirmed by measuring the uptake of a cation which is not

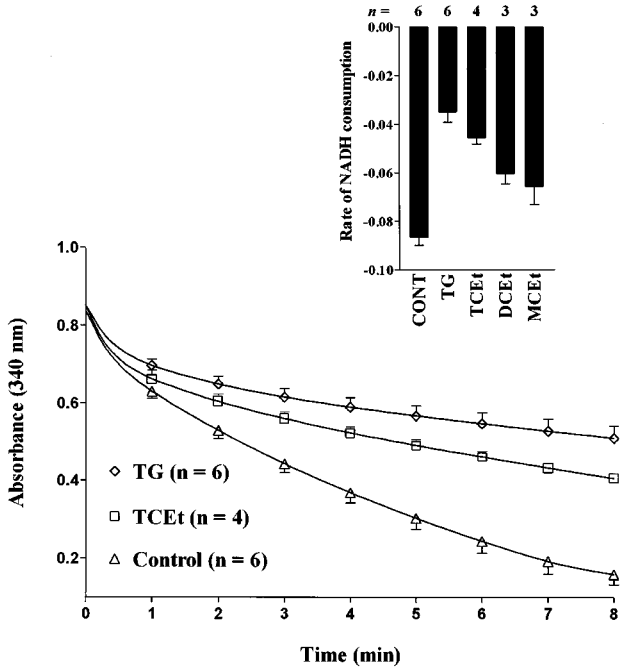

Figure 6 Effect of ethanol derivatives on microsomal Ca^{2+}-ATPase activity. Rat submandibular microsomes were isolated as described in Methods. Five μg of microsomal proteins were used for each assay. They were incubated in the cuvette of a spectrophotometer at 37°C in 2.5 ml of a 30 mM HEPES-KOH buffer, pH 7.2, containing $CaCl_2$ 1 mM, $MgSO_4$ 5 mM, EGTA 1 mM, NADH 150 μM, phosphoenol-pyruvate 0.5 mM, pyruvate kinase 3 u ml^{-1} and LDH 2 u ml^{-1}. They were incubated in the presence of either DMSO 0.5% (CONT) or 10 μM thapsigargin (TG) or 10 mM trichloroethanol (TCEt). The reaction was started by adding 4 mM ATP (final concentration). NADH consumption was measured by monitoring the absorbance at 340 nm during at least 7 min. Insert: Comparison between the effect of 10 μM TG and 10 mM TCEt, DCEt or MCEt. The results are expressed as the slope of the decrease in absorbance calculated between 1 and 4 min. The results are the means ± s.e.mean of n experiments.

a substrate for intracellular Ca^{2+}-ATPase, but which is transported by the channel coupled to purinergic receptors. Barium ions fulfil these conditions (Wiley *et al*., 1993). As shown in the left panel of Figure 10, a preincubation for 10 min with 1 mM ATP increased the uptake of barium (the 340 : 380 ratio increased from 1.38 ± 0.04 to 2.02 ± 0.02, $n = 3$, 5 min after the addition of barium to the medium). By itself, TCEt had no effect on the uptake of barium (Figure 10, right panel). The addition of TCEt to the medium before barium did not significantly affect the variation of the ratio in response to ATP (from 1.49 ± 0.04 to 2.17 ± 0.11, $n = 3$, 5 min after the addition of barium to the medium, $P > 0.05$, when compared to ATP alone). Similar results were obtained when 100 μM Bz-ATP was used as the purinergic agonist.

Among the various P2X receptors, the $P2X_7$ receptors can also form large pores permeable to ethidium bromide (Chaïb *et al*., 2000). To further investigate the possible interaction between TCEt and $P2X_7$ receptors, the effect of TCEt on the uptake of ethidium bromide in response to extracellular ATP was tested. These experiments were performed at 37°C, in the presence of 1 mM extracellular calcium. ATP increased the uptake of ethidium bromide (+14.2 ± 1.0% after 5 min, $n = 4$, $P < 0.001$ when compared to basal uptake). TCEt 10 mM had no significant effect on this uptake (+16.9 ± 2.5%, $P > 0.05$, when compared to ATP alone).

Discussion

TCEt, DCEt and MCEt increased the $[Ca^{2+}]_i$ in rat submandibular cells. These three halogenated derivatives of ethanol differ not only by their molecular volume (TCEt > DCEt > MCEt) but also by their hydrophobicity. The membrane/buffer partition coefficient of TCEt is 6 and 40 fold higher than the coefficients of DCEt and MCEt, respectively (Li *et al*., 1994). The three analogues are difficult to compare with respect to the increase of the $[Ca^{2+}]_i$ because we could not attain the maximal response, making it impossible to determine the half-maximal concentration. The response to 5 mM TCEt could be reproduced by a 6 fold higher concentration of DCEt and a 20 fold higher concentration of MCEt. These results clearly show that the hydrophobicity of TCEt plays a major role in its interaction with rat submandibular acinar cells. In agreement with this conclusion, ethanol had no effect on the $[Ca^{2+}]_i$ in these cells.

The variation of the $[Ca^{2+}]_i$ could still be observed even in the absence of extracellular calcium, confirming that it involved the mobilization of intracellular pools of calcium. The time-course of the effect of TCEt was unique. The increase of the $[Ca^{2+}]_i$ proceeded slowly, as with thapsigargin, but, at the opposite to the other agonists, the $[Ca^{2+}]_i$ remained stable for the next 10 min. This result can be explained either by an equilibrium between the efflux of calcium at the plasma membrane and its slow mobilization by TCEt from intracellular pools or by an inhibition by TCEt of the efflux of calcium outside the acini, an effect that could be due to an inhibition of the plasma membrane Ca^{2+}-ATPase (Lee *et al*., 1997).

Several intracellular pools of calcium have been described in exocrine acinar cells (Krause *et al*., 2002). The IP_3-sensitive pools play a major role in the physiology of acinar cells and were first considered. We could not test the interaction between physiological agonists and TCEt on the depletion of the pools because we observed that TCEt blocked the stimulation of the PPI-PLC by agonists such as carbachol, epinephrine and substance P. It also blocked the activation of this enzyme by aluminium fluoride, an activator of G-proteins in salivary glands (Tojyo *et al*., 1991). These results suggested that TCEt could affect the activity of the PPI-specific phospholipase C or its activation by G-proteins. These results also implied that the interaction between TCEt and an agonist like carbachol could not be used as a tool to study the role of the IP_3-sensitive intracellular pools of calcium in the response to TCEt. This led us to study the interaction between TCEt and thapsigargin. This compound inhibits intracellular Ca^{2+}-dependent ATPases, which are responsible for the active transport of calcium in some intracellular pools (Métioui *et al*., 1994). This inhibition, coupled to the leak of calcium from these pools, will eventually increase the $[Ca^{2+}]_i$. The response to thapsigargin was nearly fully blunted when the cells had been previously exposed to TCEt. Similarly the depletion by thapsigargin of some intracellular pools completely suppressed the response to TCEt. Since chloroethanols can uncouple mitochondria (Bhat *et al*., 1991), we also tested for the contribution of

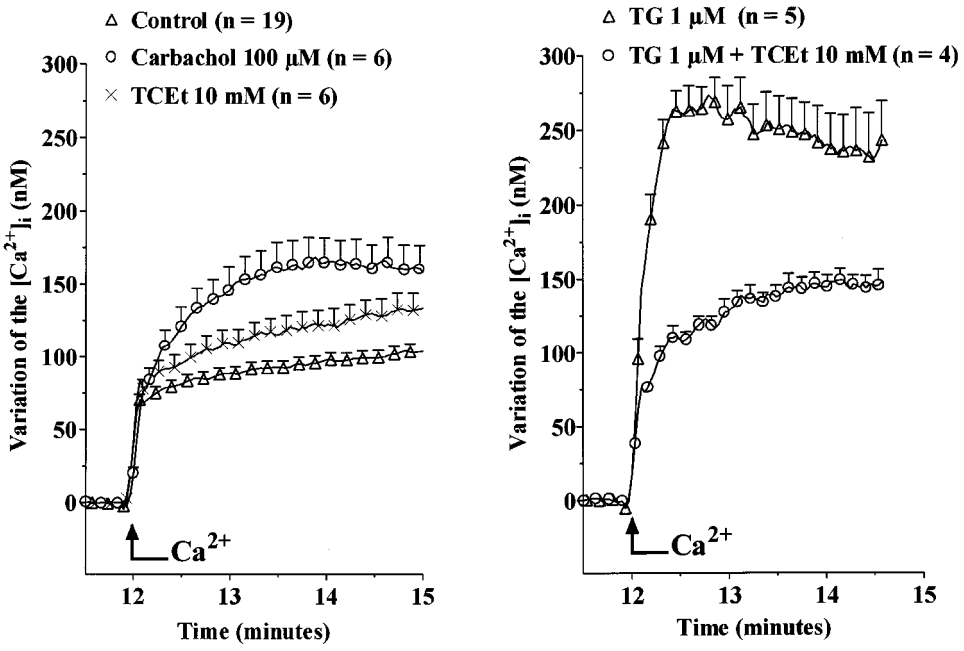

Figure 7 Effect of TCEt on the uptake of extracellular calcium. Rat submandibular acini were loaded with fura-2. After washing, they were resuspended in HBS medium in the absence of calcium and magnesium but in the presence of 100 μM EGTA. They were maintained at 25°C and constantly stirred in the cuvette of the fluorimeter. Left panel: Two minutes after the beginning of the measurement, the cells were either maintained in control conditions (Control) or exposed to 100 μM carbachol or to 10 mM TCEt. Right panel: Two minutes after the beginning of the measurement the cells were either maintained in control conditions or exposed to 10 mM TCEt and five minutes later, 1 μM thapsigargin was added to the medium. At 12 min (arrow), all the cells (the two panels) were challenged with 5 mM $CaCl_2$. The results are expressed as the variation between the $[Ca^{2+}]_i$ measured at 11.5 min and the $[Ca^{2+}]_i$ measured at subsequent times. The results are the means + s.e.mean of n experiments.

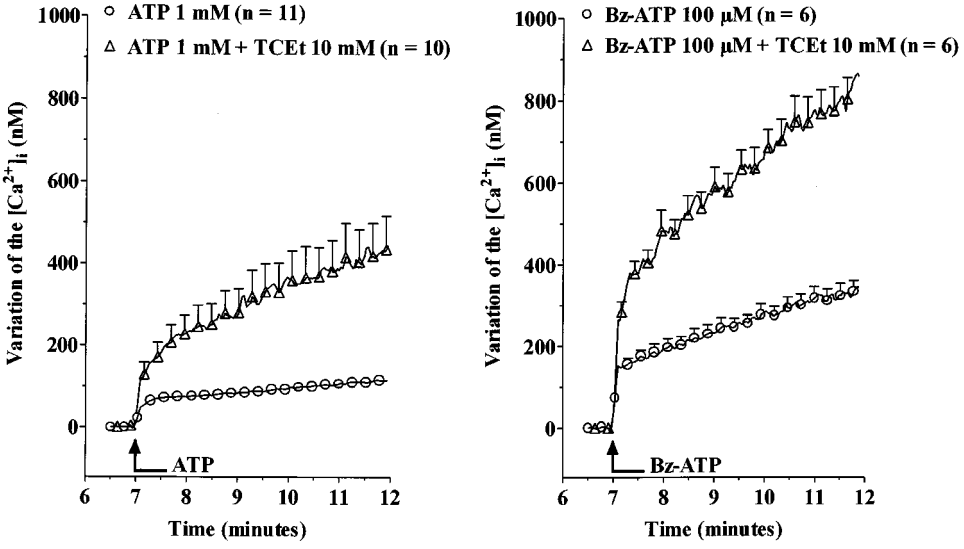

Figure 8 Effect of TCEt on the variation of the $[Ca^{2+}]_i$ in response to purinergic agonists. Rat submandibular acini were loaded with fura-2. After washing they were resuspended in 2 ml HBS medium, in the presence of 1 mM $CaCl_2$ but in the absence of magnesium. They were maintained at 25°C and constantly stirred in the cuvette of the fluorimeter. Two minutes after the beginning of the measurement, they were either exposed to 10 mM TCEt or maintained in control conditions and 5 min later (arrow), they were stimulated with either 1 mM ATP (left panel) or 100 μM Bz-ATP (right panel). The results are expressed as the variation between the $[Ca^{2+}]_i$ measured at 6.5 min and the $[Ca^{2+}]_i$ measured at subsequent times. These results are the means + s.e.mean of n experiments.

mitochondrial calcium to the response to TCEt. The cells were first exposed to FCCP. This organic acid is a protonophore and, as such, it dissipates intracellular proton gradients, especially at the mitochondrial level. The increase of the $[Ca^{2+}]_i$ in the presence of FCCP has been correlated with the release of calcium from mitochondria (Duchen,

1990). In our hands, a preincubation for 5 min with FCCP did not prevent the increase of the $[Ca^{2+}]_i$ in response to TCEt. It can thus be concluded that TCEt mobilized thapsigargin-sensitive but not mitochondrial pools of calcium.

Like thapsigargin, TCEt was able to deplete IP_3-sensitive intracellular calcium pools but had no effect on the production of inositol phosphates. The effect of TCEt and its analogues on the Ca^{2+}-ATPase responsible for the accumulation of calcium in these pools was thus investigated. The three halogenated derivatives of ethanol, TCEt, DCEt and MCEt, inhibited at various degrees the thapsigargin-sensitive microsomal Ca^{2+}-ATPase, the former displaying an inhibitory effect that was similar to thapsigargin. The depletion of intracellular pools by TCEt thus probably reflected an inhibition of this Ca^{2+}-ATPase. TCEt was shown to interact with a tryptophan residue of the ATP-induced myosin subfragment-1 and this interaction increased the Mg^{2+}-ATPase activity of the subunit (Papp & Highsmith, 1993). This activation required, however, much higher concentrations (half-maximal concentration around 25 mM) than the concentrations used in our study. It has also been reported that halogenated alkanes can bind to Ca^{2+}-ATPases located both on the plasma membrane and on the endoplasmic reticulum (Lopez & Kosk-Kosicka, 1997).

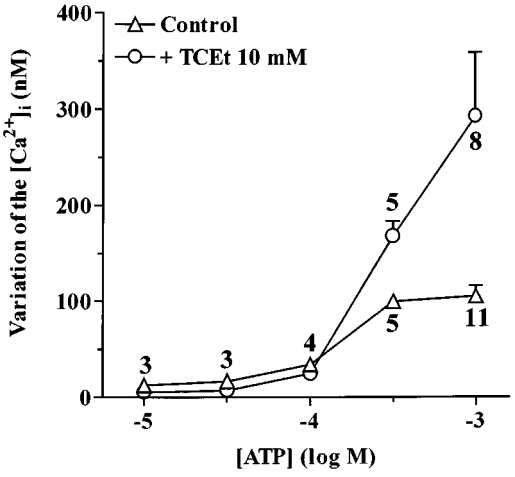

Figure 9 Effect of TCEt on various concentrations of extracellular ATP. Rat submandibular acini were loaded with fura-2. After washing they were resuspended in 2 ml HBS medium, in the presence of 1 mM $CaCl_2$ but in the absence of magnesium. They were maintained at 25°C and constantly stirred in the cuvette of the fluorimeter. Two minutes after the beginning of the measurement, they were either exposed to 10 mM TCEt or maintained in control conditions and 5 min later, they were stimulated with various concentrations of ATP. The results are expressed as the variation between the $[Ca^{2+}]_i$ measured 30 s before and 5 min after the addition of ATP to the medium. These results are the means + s.e.mean of *n* experiments.

Both carbachol and thapsigargin increase the uptake of extracellular calcium. According to the capacitative model, this uptake is secondary to the depletion of intracellular calcium pools and the subsequent activation of the SOCC (Putney & Ribeiro, 2000). TCEt, which depletes the thapsigargin-sensitive calcium pools, should thus likely increase the uptake of extracellular calcium. Yet, the uptake of calcium by cells preexposed for 10 min to TCEt in a calcium-free medium was only very slightly increased when compared to control and was much smaller than the response to carbachol or thapsigargin. This result could be explained by a dual interaction of TCEt with the SOCC: an activating effect secondary to the depletion of the thapsigargin-sensitive calcium pools and an inhibitory effect on the SOCC. To test this hypothesis the cells were preincubated with TCEt before exposure to thapsigargin. The uptake of calcium by these acini was smaller than by cells exposed to thapsigargin in the absence of TCEt. This result confirmed that TCEt blocked the uptake of calcium mediated by the SOCC.

Extracellular ATP acting on P2X receptors increased the $[Ca^{2+}]_i$ in acini without modifying the production of inositol phosphates. This response was blocked by extracellular magnesium suggesting that ATP^{4-} was the agonist of the receptors involved in this response. Coomassie blue, a rather non-specific antagonist of P2X receptors, and oATP, a rather specific inhibitor of $P2X_7$ receptors (Murgia *et al*., 1993) both inhibited this response. Bz-ATP was a better agonist than ATP, a unique property of $P2X_7$ receptors (North & Surprenant, 2000). Both agonists increased the uptake not only of calcium but also of barium, in agreement with the activation of a non-specific cation channel coupled to $P2X_7$ agonists (Wiley *et al*., 1993). ATP also increased the uptake of ethidium bromide by acini. This is probably secondary to the formation of pores by a $P2X_7$ receptor (North &

Table 1 Characterization of the response of rat submanibular acini to purinergic agonists

	Variation of the $[Ca^{2+}]_i$		
	ATP 1 mM	*Bz-ATP* 30 μM	*Bz-ATP* 100 μM
Without preincubation			
Control	158.2 ± 15.8 (*n* = 12)	280.2 ± 20.2 (*n* = 5)	314.2 ± 5.4 (*n* = 3)
+ $MgCl_2$ 5 mM	37.3 ± 4.8 (*n* = 7)	n.d.	105.2 ± 0.5 (*n* = 7)
+ Coomassie blue 10 μM	49.8 ± 4.2 (*n* = 13)	54.5 ± 3.5 (*n* = 5)	134.1 ± 4.9 (*n* = 7)
With preincubation			
Control	158.3 ± 18.1 (*n* = 3)	464 ± 25.5 (*n* = 4)	n.d.
+ oATP 100 μM	80.4 ± 3.3 (*n* = 5)	115.1 ± 14.4 (*n* = 8)	n.d.

Rat submandibular acini were loaded with fura-2. After washing they were resuspended in 2 ml HBS medium in the presence of 1 mM $CaCl_2$, in control conditions or in the presence of 5 mM $MgCl_2$ or in the presence of 10 μM Coomassie blue. Some cells were preincubated for 2 h at 25°C in the absence or in the presence of 100 μM oATP. After the preincubation they were washed and resuspended in 2 ml HBS medium in the same conditions with respect to oATP. Five minutes after the beginning of the measurement, the cells were exposed to 1 mM ATP or 30 or 100 μM Bz-ATP. The results are expressed as the variation of the $[Ca^{2+}]_i$ 30 s before and 5 min after the addition of the agonist in the cuvette. They are the means ± s.e.mean of *n* experiments. n.d.: not determined.

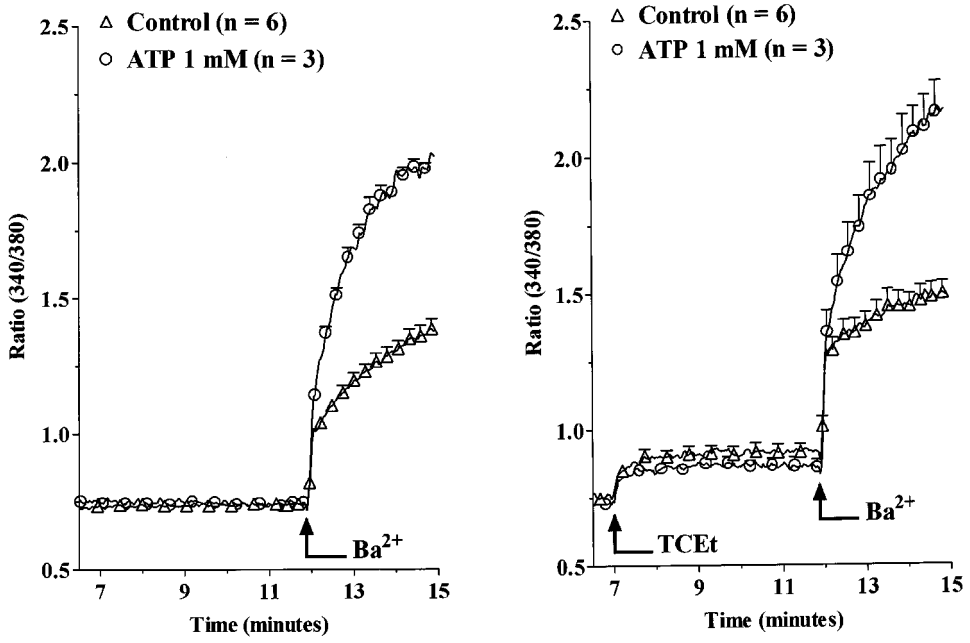

Figure 10 Effect of TCEt on the uptake of barium in response to extracellular ATP. Rat submandibular acini were loaded with fura-2. After washing, they were resuspended in HBS medium in the absence of calcium and of magnesium but in the presence of 100 μM EGTA. They were maintained at 25°C and constantly stirred in the cuvette of the fluorimeter. Two minutes after the beginning of the measurement two groups of acini were maintained in control conditions and two groups of cells were exposed to 1 mM ATP. Five minutes later, the cells were either maintained in the same conditions (left panel) or exposed to 10 mM TCEt (right panel). At 12 min, barium was added to the medium (1 mM final concentration). The results are expressed as the ratio of the light emitted after excitation at 340 nm versus 380 nm. They are the means+s.e.mean of *n* experiments.

Surprenant, 2000). All these results were thus in favour of the presence of $P2X_7$ receptors in submandibular acini and of their activation by ATP and Bz-ATP. TCEt potentiated the increase of the $[Ca^{2+}]_i$ of acini in response to 1 mM ATP or 100 μM Bz-ATP. TCEt did not increase the sensitivity of the acini to the purines but rather potentiated the maximal response to these agonists. TCEt also potentiated the uptake of calcium in response to ATP. TCEt might thus favour the opening state of the non-specific channel formed by $P2X_7$ receptors. In order to test this hypothesis, we measured the uptake of barium. This divalent cation affects the fluorescence of fura-2 in a way similar to calcium. It has been shown that it can permeate through the channels formed by $P2X_7$ receptors (Wiley *et al*., 1993). But, at the opposite of calcium, barium is not a substrate for calcium-dependent ATPases and once inside the cell, it accumulates in the cytoplasm (Wiley *et al*., 1993). TCEt had no effect on the basal uptake of this ion or on its uptake in response to ATP or Bz-ATP. This result suggested that TCEt did not affect the opening state of the channel coupled to $P2X_7$ receptors. It is thus very likely that TCEt potentiated the increase of the $[Ca^{2+}]_i$ in response to $P2X_7$ receptors not by the activation of the channels formed by these receptors but rather by the inhibition of the uptake of calcium into intracellular pools. TCEt had also no effect on the formation by $P2X_7$ receptors of pores permeable to large ions since it did not affect the uptake of ethidium bromide in response to extracellular ATP. This result further confirmed that TCEt did not affect the activation of P2X receptors in salivary glands. According to Li *et al*. (1994), TCEt has no effect on the current evoked by the stimulation of purinergic receptors of the dorsal root ganglia. At the opposite, smaller molecules derived from ethanol inhibit these receptors by allosterically decreasing the affinity of the receptor for ATP. They interpreted these results as evidence that alcohols inhibit the function of ATP-gated ion channels by interacting with a hydrophobic pocket of circumscribed dimensions on the receptor protein. More recently Koles *et al*. (2000) reported that TCEt could inhibit the $P2X_3$ receptors expressed in HEK cells. Our results suggest that the salivary P2X receptors might lack the hydrophobic pocket through which TCEt could modulate their activation.

In conclusion, we have shown that TCEt deeply affects the handling of calcium by RSMG acini: it depletes intracellular stores of calcium probably by inhibiting thapsigargin-sensitive Ca^{2+}-ATPase, it inhibits ion movements at the plasma membrane level, it potentiates the increase of the $[Ca^{2+}]_i$ in response to purinergic agonists. TCEt has also an inhibitory effect on the activation of PPI–PLC by muscarinic, adrenergic and peptidergic agonists, as well as by aluminium fluoride. TCEt is a metabolite of trichlorethylene, a chemical widely used as a solvent, or as an intermediate in the production of chemicals such as polyvinyl chloride and pharmaceuticals or in the metal industry. The concentration of TCEt reached in bodily fluids after exposure to this compound remains always lower than the concentrations which we used in our study (Fisher *et al*., 1998). It is thus unlikely that the effects reported in this study can ever be observed after exposure to trichlorethylene except maybe in acute intoxication with this chemical. But TCEt is also the major metabolite of chloral hydrate. During anaesthesia with this drug, the concentration of TCEt can reach values as high as 5 mM. Norlen *et al*. (2000) have recently reported that chloral hydrate could affect the

acid secretion in anaesthetized animals and that this drug had a direct effect on gastric cells illustrating one of the short-comings of the use of this drug in the study of gastric acid secretion. In their study of 5-HT_3 receptors, Bentley & Barnes (1998) have shown that quipazine which is a partial agonist and which behaves as an antagonist in functional studies is a full agonist in rats anaesthetized with chloral hydrate. This discrepancy has been explained by a potentiation by TCEt of 5-HT_3 receptors (Parker *et al*., 1996). These two examples demonstrate that a direct effect of TCEt at the cellular level should not be overlooked. In line with these authors, we suggest that the results of studies of salivary secretion performed in intact animals anaesthetised with chloral hydrate should be interpreted with caution considering the major effects of TCEt on the handling of calcium by salivary acini.

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