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Procaine-induced enhancement of fluid-phase endocytosis and inhibition of exocytosis in human skin fibroblasts

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Abstract

Local anaesthetics are often applied directly onto the skin, and for this reason the effect of some local anaesthetics upon morphology and cytoskeleton organisation in human skin fibroblasts was investigated. In this paper the authors report that procaine (*p*-aminobenzoic acid diethylamino-etyl ester hydrochloride) induced vacuolisation of cytoplasm and great enhancement of neutral red accumulation in human skin fibroblasts cultured in vitro. Procaine-induced vacuolisation of cell's cytoplasm was observed to be associated with the enhanced uptake and inhibited release of fluid taken by endocytosis. All these effects appeared fully reversible. The cell vacuolisation cannot be prevented by 3-methyadenine, brefeldine A, and cytochalasine D. On the other hand, nocodazole and caffeine prevent cytoplasm vacuolisation induced by procaine. These observations suggest that procaine-induced formation of great vacuoles is due to an impairment of membrane traffic between endosomes. The authors' results also demonstrate that neutral red uptake assay, if used as a cell viability test, must be interpreted with great caution.
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Keywords: Local anaesthetic; Procaine; Fluid-phase endocytosis; Exocytosis; Fibroblast; Skin, human; Neutral red uptake assay

1. Introduction

Local anaesthetics are primarily used to provide regional analgesia acting upon nerve cells. Many of them, such as procaine, lidocaine, and tetracaine, are usually applied topically onto the skin. In neurones they block voltage-gated Na^+ channels and inhibit Na^+ influx (Butterworth and Strichartz, 1990; Ragsdale et al., 1994). The action of these anaesthetics, however, is not limited to excitable cells. In other cells they cause, inter alia, changes in osmotic fragility (Constantinescu et al., 1987; Palek et al., 1977), inhibition of cell fusion (Coakley et al., 1983; Seravalli et al., 1984; Stygall et al., 1979), stimulation of plasma membrane expansion (Beresford and Fastier, 1980; Van Driessche et al., 1993), and increased membrane fluidity (Shimooka et al., 1992; Staffolani et al., 1993; Yun et al., 2002). In addition, they modify the activity of numerous enzymes including G-proteins (Hagelüken et al., 1994; Hollmann et al., 2001), protein kinases (Slater et al., 1993; Tan et al., 1999), phospholipases (Chen et al., 2002; Higuchi et al., 1983; Raucher and Sheetz, 2001), and cytochrome oxidase (Chazotte and Vanderkooi, 1981). Local anaesthetics have been reported to induce also vacuolisation in various cell types: in Hep-2 epithelial carcinoma cells (Finnin et al., 1969), foreskin fibroblasts (Henics and Wheatley, 1997), rat cardiomyocytes (Vitullo et al., 1993), alveolar macrophages (Miller and Foster, 1981), HeLa cells, chick vessel fibroblasts, and normal epidermal cells (Yang et al., 1965).

In the preceding paper, changes in human skin fibroblasts morphology and cytoskeleton organisation induced by local anaesthetics were described (Pierzchalska et al., 1998). Those changes, caused by procaine, lidocaine, and tetracaine, were accompanied by vacuolisation of cytoplasm. In spite of numerous reports of cell vacuolisation induced by a variety of agents, including local anaesthetics (Henics and Wheatley, 1999), little is known concerning the origin of these vacuoles and the relation between their appearance and endocytotic membrane traffic (cf. Discussion). This report contains the results of experiments describing the vacuolisation of cytoplasm in human skin fibroblasts induced by procaine and the effects of drugs known to affect the membrane traffic and intracellular calcium ion concentration.

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2. Materials and methods

2.1. Materials

Foetal calf serum and trypsin were obtained from Gibco (San Diego, CA, USA). Dulbecco's Modified Eagle Medium (DMEM), procaine hydrochloride (*p*-aminobenzoic acid diethylamino-etyl ester hydrochloride), tetracaine hydrochloride (4-[butylamino]benzoic acid 2-[dimethylamino]ethyl ester), lidocaine (2-diethylamino-N[2,6-dimethylphenyl]acetamide), trypsin, paraformaldehyde, triton X-100, EGTA, EDTA, neutral red, lucifer yellow, caffeine, brefeldin A, 3-methyladenine, cytochalasin D, and nocodazole were obtained from Sigma (St. Louis, USA). Penicillin, streptomycin, and neomycin were purchased from Polfa (Tarchomin, Poland); phosphate-buffered saline (PBS) from Wytwórnia Surowic i Szczepionek (Lublin, Poland). Culture flasks and 12-well plates were purchased from Corning NY, UK and glass coverslips from Chance (Warley, UK).

2.2. Cell culture

Human skin fibroblasts were obtained from skin grafts of healthy, adult donors and were cultured as previously described (Pierzchalska et al.1998) in DMEM containing 100 i.u./ml penicillin, 10 μg/ml streptomycin, and 10 μg/ml neomycin in the presence of 10% foetal calf serum. The cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

For the experiments, cells from 5 to 15 passages were used. The cells were plated at an initial cell density of 7×10^3 cells/cm^2 in 12-well plates with glass coverslips inserted into each well 24 h before the experiment.

2.3. Anaesthetic treatment

Procaine hydrochloride, lidocaine hydrochloride, and tetracaine hydrochloride were diluted in the culture medium from stock solutions in water to the working concentration of 2.5, 1, and 0.1 mM, respectively, directly before the experiments. Human skin fibroblasts were incubated with or without anaesthetic in DMEM with 10% foetal calf serum for different time periods (from 1 up to 96 h). Cells growing on glass coverslips inserted into 12-well plates were observed in an inverted Olympus IMT-2 microscope using phase contrast optics at a temperature of 37 °C.

In order to study the inhibition of anaesthetic-induced cytoplasm vacuolisation, procaine-containing medium was added after preincubation of cells in medium containing 20 μM brefeldin A, 10 mM 3-methyladenine, 2 μM cytochalasin D, 3 μM nocodazole or 10 mM caffeine.

To determine the reversibility of the vacuolisation process, cells were cultured for 24 h in medium containing 2.5 mM procaine. Cells were then washed with medium without procaine and incubated in the same medium for the next 72 h.

2.4. Determination of cell vacuolisation

Human skin fibroblasts were incubated with 2.5 mM procaine in DMEM with 10% foetal calf serum. At different time periods, cells were inspected for the cytoplasm vacuolisation under a Leitz Orthoplan microscope with phase contrast optics. To quantify the extent of vacuolisation, modified neutral red dye uptake assay was used (Papini et al., 1994). Cells in 96-well plates were incubated for 10 min at 37 °C with 0.1-ml freshly prepared 50 μg/ml neutral red in PBS containing 0.3% bovine serum albumin, and then washed three times with 0.2 ml of the same buffer. After addition of 0.1 ml of 70% ethanol in water containing 0.37% HCl, absorbance was measured with a Spectra MAX 250 microplate reader at 540 nm with subtraction of absorbance at 405 nm.

When needed, the cells were preincubated with 20 μM brefeldin A, 10 mM 3-methyladenine, 2 μM cytochalasin D, 3 μM nocodazole or 10 mM caffeine for 1 h before the addition of procaine, and the effect of those drugs on cytoplasm vacuolisation was determined as above.

2.5. Assay for endocytosis

Endocytosis was determined by fluorescent microscopy using fluorescent dyes neutral red and lucifer yellow as fluid-phase markers. Cells grown on coverslips under different experimental conditions were incubated with freshly prepared neutral red (50 μg/ml) or lucifer yellow (1 mg/ml) in DMEM containing 10% foetal calf serum. After different times of incubation, endocytosis was stopped by washing three times with ice-cold PBS, after which the cells were washed twice in the medium without fluorescent dye at 37 °C. The intensity of fluorescence of individual cells was measured using a Leitz Orthoplan microscope working in an epifluorescence mode equipped with a photomultiplier attachment (for neutral red-excitation wavelength 541 nm, emission wavelength 640 nm, and for lucifer yellow-excitation wavelength 428 nm, emission wavelength 535 nm).

At least 300 randomly selected cells were analysed for each value measured and all experiments in the given experimental conditions were at least three times repeated. Mean and standard deviation for each parameter were calculated. The statistical significance was determined with the nonparametric Mann–Whitney U test, with $P < 0.05$ considered significant.

2.6. Assay for exocytosis

Efflux of fluid-phase endocytosis marker from preloaded cells was used to determine exocytosis (Giocondi et al., 1995). Cells grown on coverslips were cultured in DMEM containing 10% foetal calf serum and 2.5 mM procaine or in the same medium without procaine. Following 24-h incubation, cells were loaded with 50 μg/ml neutral red in cultured medium for 5 min. Cells were then washed three times with

PBS and neutral red solution was replaced with the same type of medium as before staining (with or without procaine). After different time periods of incubation, cells were washed three times with PBS and fluorescence intensity of neutral red stored in vacuoles in single cells was measured as in Section 2.5.

In order to study the inhibition of procaine-induced changes in the exocytosis, cells preloaded with neutral red were washed with PBS and anaesthetic-containing medium was replaced with the same medium containing 10 mM caffeine. After different times of incubation the effect of caffeine on neutral red releasing kinetics was determined as above.

2.7. Microphotography

Microphotographs were taken under a Leitz Orthoplan microscope with an epifluorescence and phase-contrast optics, equipped with the Nikon FX-35DX camera or under an inverted Olympus IMT-2 microscope with phase-contrast optics equipped with the Olympus OM-4 Ti camera. High-sensitivity Kodak TMAX 3200 films or Fomapan 100 films were used. Some microphotographs were taken under a BIORAD MRC 1024 laser scanning confocal microscope.

3. Results

3.1. Effect of procaine on cytoplasm vacuolisation in human skin fibroblasts

In the primary experiments it was observed that among the three local anaesthetics tested (2.5 mM procaine, 1 mM lidocaine, and 0.1 mM tetracaine) procaine induced the strongest changes in cytoplasm vacuolisation in human skin fibroblasts. One-hour incubation of human skin fibroblasts in the presence of procaine induced formation of vacuoles

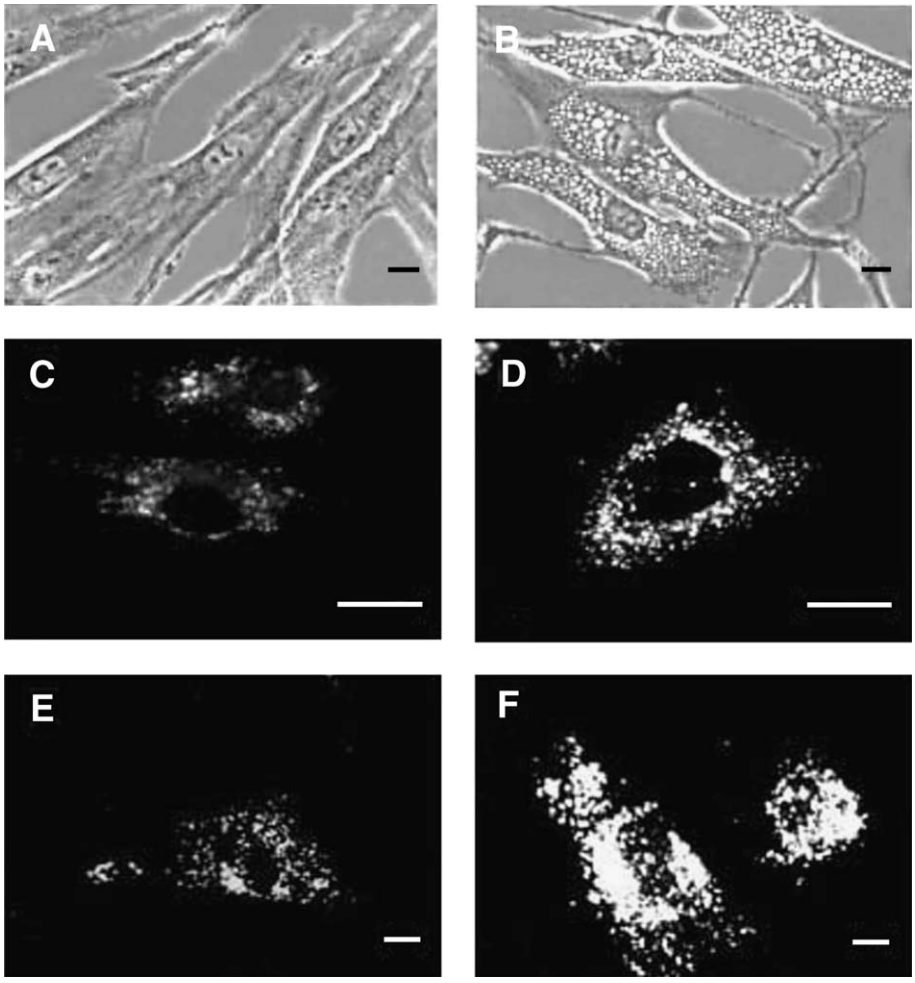

Fig. 1. Effect of procaine on cytoplasm vacuolisation of human skin fibroblasts. Cells were grown on glass coverslips in MEM supplemented with 10% foetal calf serum without (A, C, E) or with 2.5 mM procaine (B, D, F) for 1 h. Neutral red (C, D) or lucifer yellow (E, F) was present in the culture medium in the concentration 50 μg/ml or 1 mg/ml, for 30 min, respectively. Microphotographs were taken under an inverted Olympus IMT-2 microscope with phase-contrast optics (A, B) or with an epifluorescence (C, D) or under a BIORAD MRC 1024 laser scanning confocal microscope (E, F). Scale bar = 10 μm.

localised mainly in the perinuclear region of cytoplasm (Figs. 1B and 2C,D). During the next hours of incubation the number and size of vacuoles increased and vacuoles were dispersed throughout the cytoplasm (Fig. 2E and F). The procaine-induced vacuole formation appeared fully reversible and, after removal of the anaesthetic from the medium, the vacuoles disappeared (Fig. 2G and H). As shown in Fig. 1C and D, a fluorescent dye, neutral red, which stains acidic intracellular organelles (Cover et al., 1991), was accumulated into procaine-induced vacuoles. The incubation of human skin fibroblasts in medium containing both 2.5 mM procaine and lucifer yellow (1 mg/ml)—a commonly used marker of fluid phase endocytosis—revealed that vacuoles induced by anaesthetic had collected extracellular fluid with lucifer yellow (Fig. 1E and F). Fluorescence intensity of lucifer yellow accumulated in procaine-induced vacuoles varied among vacuoles; usually large vacuoles were less stained while small ones were brighter (data not shown).

3.2. Effect of procaine upon the uptake of neutral red in human skin fibroblasts

Figs. 2 and 3A present the results of observations and measurements concerning the time dependence of vacuolisation of human skin fibroblasts induced by procaine and the procaine-stimulated uptake of neutral red. The cells pretreated with procaine (2.5 mM) for 24 to 96 h and then treated with 50 μg/ml neutral red for 5 min accumulated almost four times more neutral red than control cells incubated in the control medium. This effect of procaine was clear already after 1-h preincubation of the cells in the presence of procaine (Fig. 3A). When the cells after the preincubation in the presence of procaine (for 24 h) were then incubated for 72 h in the control medium, the stimulation of neutral red uptake almost disappeared (Figs. 2G,H and 3A).

The kinetics of the procaine stimulated uptake of neutral red by human skin fibroblasts was determined in the cells incubated in the control medium with 50 μg/ml of neutral red in the absence of procaine and in the medium supplemented with 2.5 mM procaine. At 30-min intervals for 210 min the relative fluorescence of neutral red per single cell was measured in 300 cells in at least three separate experiments for each experimental point, as described in Materials and methods. The results presented in Fig. 3B show that procaine accelerated and more than three times increased the neutral red uptake by single cells already within 120 min of cell exposure to procaine.

3.3. Effect of procaine on membrane traffic during endocytosis

In the next experiments the authors investigated the effect of some inhibitors, known to affect the membrane traffic during endocytosis, upon the procaine-stimulated uptake of neutral red and vacuolisation of cytoplasm in human skin fibroblasts. The effects were examined for: brefeldin A—an inhibitor of secretion (Misumi et al., 1986) which also affects the early-to-late endosome transi-

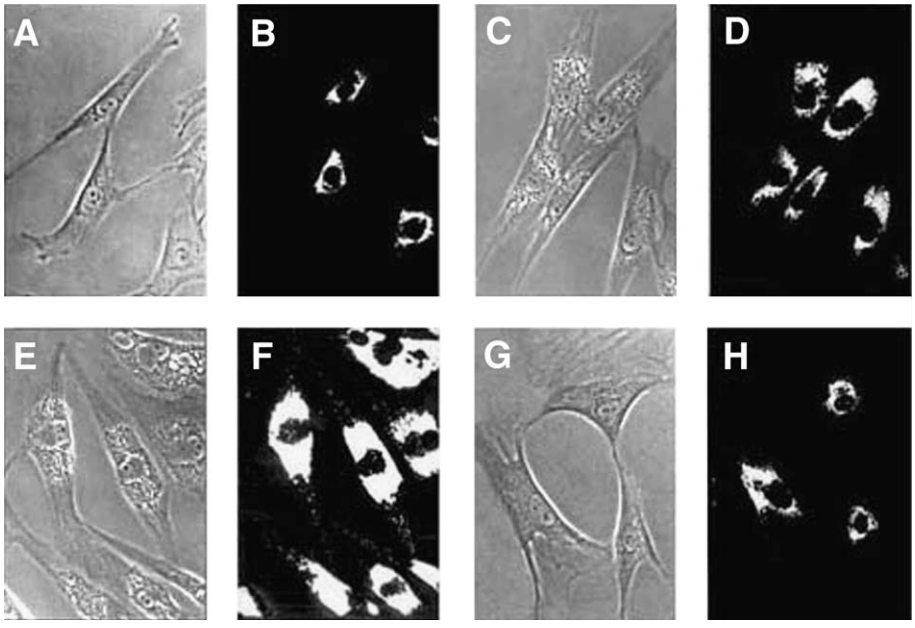

Fig. 2. Effect of procaine upon neutral red uptake in human skin fibroblasts. Cells were grown on glass coverslips in MEM supplemented with 10% foetal calf serum (control medium) (A, B) or with the same medium containing 2.5 mM procaine for 24 h (C, D), 96 h (E, F) or 24 h with the anaesthetic and then 72 h in the control medium (G, H). Then cells were incubated with 50 μg/ml neutral red for 5 min. Microphotographs were taken under an inverted Olympus IMT-2 microscopy with phase-contrast optics (A, C, E, G) or with epifluorescent (B, D, F, H). Scale bar = 10 μm.

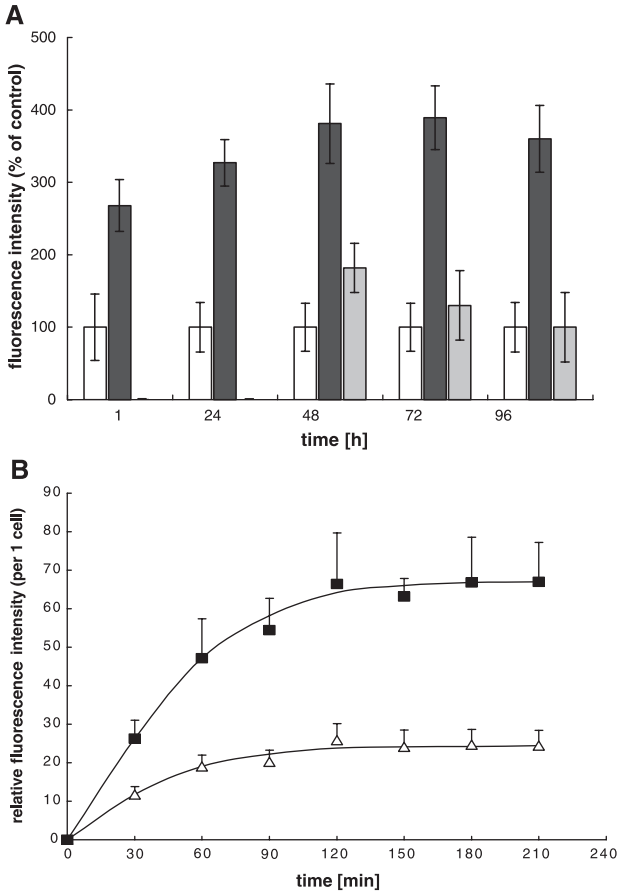

Fig. 3. Effect of procaine upon neutral red uptake (A) and on the rate of fluid-phase endocytosis (B) in human skin fibroblasts. (A) Cells were cultured in 96-well plates in MEM supplemented with 10% foetal calf serum (open columns) or with the same medium containing 2.5 mM procaine (dark grey columns) for 1, 24, 48, 72, and 96 h, respectively, or 24 h with the anaesthetic and then in control medium (light grey columns) up to the total time of incubation 48, 72, and 96 h, respectively. Subsequently, cell vacuolisation was determined by using the neutral red uptake test (as described in Materials and methods). The mean fluorescence of neutral red uptake by single cells for each of the experimental conditions is presented as percentage of control. At least 300 randomly selected cells were calculated for each experimental point. The results presented are the means of at least three separate experiments. (B) Cells were cultured in MEM supplemented with 10% foetal calf serum and 50 μg/ml neutral red with (squares) or without (triangles) 2.5 mM procaine for 210 min. The fluid-phase endocytotic activity was determined (as described in Materials and methods) at 30-min intervals for each of the experimental conditions. The values of each experimental point were calculated as mean (± S.D.) of at least 300 randomly selected cells. The results presented are the means of at least three separate experiments.

tion (Pelham, 1991), 3-methyladenine—an inhibitor of autophagocytosis (Seglen and Bohley, 1992) and transport between late endosomes and lysosomes in fibroblasts (Punnenen et al., 1994), cytochalasin D—an F-actin cytoskeleton destabilising agent which was reported to block the formation of early endosomes (Jackman et al., 1994) and postulated to affect the receptor-mediated endocytosis (Lamaze et al., 1997), and nocodazole, which causes destruction of microtubules required for membrane traffic from early endosomes to late endosomes, and changes in the cell surface properties (Burgess and Kelly, 1987; Gruenberg et al., 1989). The results presented in Table 1 show that only brefeldin A significantly inhibits neutral red uptake in human skin fibroblast cultured in control medium (DMEM with 10% foetal calf serum). Procaine accelerated and nearly 2.5 times increased the neutral red uptake by both control and brefeldin A-treated cells. The procaine-stimulated uptake of neutral red was insignificantly inhibited by 3-methyladenine and cytochalasin D. The procaine-stimulated uptake of neutral red and cytoplasm vacuolisation in fibroblasts were clearly prevented only by nocodazole (Fig. 4 and Table 1).

Procaine has been suggested as inhibiting the intracellular ryanodine receptor-like Ca^{2+} channel (Kasai and Ide, 1996). Since caffeine is an activator of this channel (Kasai and Ide, 1996; Larini et al., 1995), its effect on procaine-induced cell vacuolisation and neutral red accumulation was examined in the following experiments. It was observed that 1 h of human skin fibroblasts incubation in the caffeine-supplemented medium (10 mM) did not affect the cell morphology (Fig. 4E). Nevertheless, such 1-h preincubation of these cells in the presence of caffeine significantly inhibited the procaine-induced cell vacuolisation and the uptake of neutral red by human skin fibroblasts in the presence of procaine (Fig. 4F, Table 1). In the presence of 10 mM caffeine, procaine did not stimulate the uptake and accumulation of neutral red in human skin fibroblasts (Table 1).

Table 1
Effect of some drugs affecting membrane traffic on procaine-induced cytoplasm vacuolisation of human skin fibroblasts

Neutral red quantity [μg/10^6 cells ± S.D.]		
	DMEM + 10% serum	DMEM + 10% serum + 2.5 mM procaine
Control	27.36 ± 2.15	70.75 ± 5.18 (*)
Brefeldin A (20 μM)	19.50 ± 0.93 (*)	46.46 ± 2.93 (*)
3-methyladenine (10 mM)	31.04 ± 0.86	59.13 ± 2.48 (*)
Cytochalasin D (2 μM)	28.25 ± 2.62	54.13 ± 3.92 (*)
Nocodazole (2 μM)	29.42 ± 2.40	31.50 ± 3.24 (#)
Caffeine (10 mM)	28.00 ± 2.86	29.68 ± 3.34 (#)

Cells were cultured in 96-well plates in MEM supplemented with 10% foetal calf serum (control) and in the same medium with 20 μM brefeldin A, 10 mM 3-methyladenine, 2 μM cytochalasin D, 3 μM nocodazole or 10 mM caffeine, respectively. After 1 h incubation the procaine was added to the concentration of 2.5 mM for 1 h, after which cells were incubated with 0.5 mg/ml neutral red for 10 min. Neutral red uptake was determined as described in Materials and methods. The results presented are the means of at least three separate experiments. All values were compared with those for cells treated neither with used inhibitors nor with procaine (control). Values significantly different from control are indicated by (*). Cells treated with procaine in the presence of inhibitors were compared to cells treated with procaine alone. Values significantly different from that for procaine-treated cells are indicated by (#).

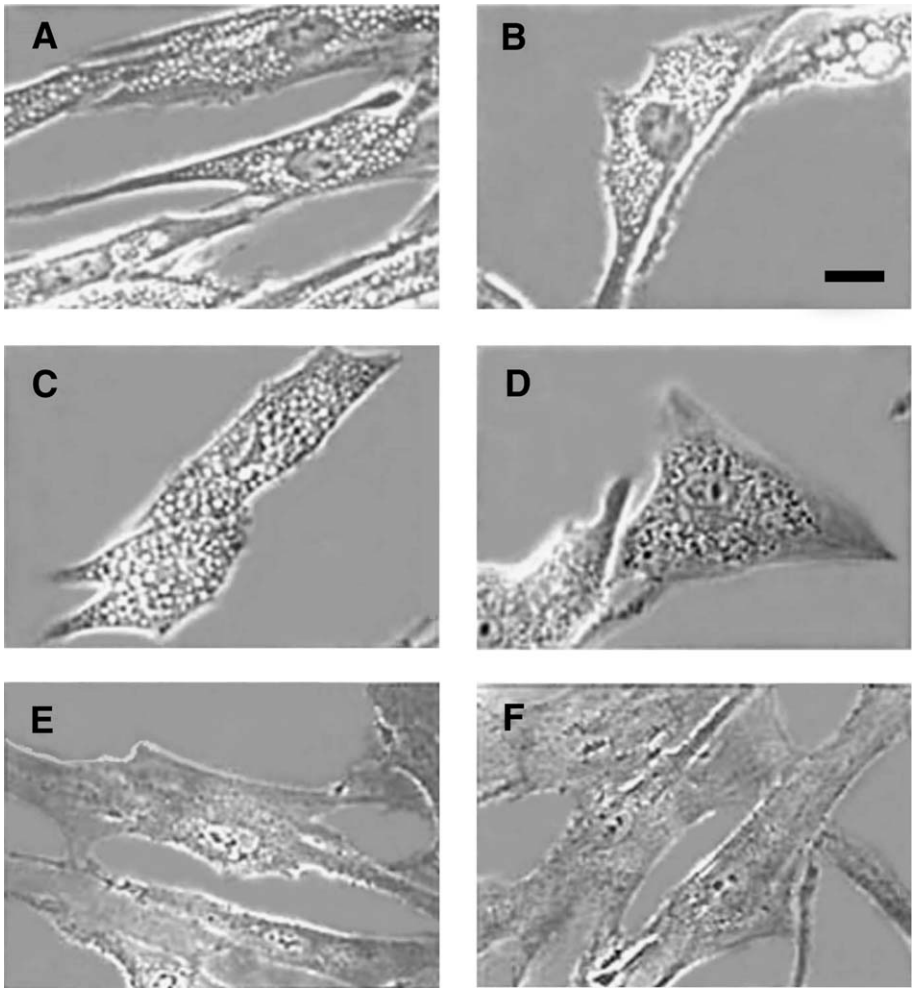

Fig. 4. Effect of some drugs affecting membrane traffic on procaine-induced cytoplasm vacuolisation of human skin fibroblasts. Cells were grown on glass coverslips in MEM supplemented with 10% foetal calf serum with 20 μM brefeldin A (A), 10 mM 3-methyladenine (B), 2 μM cytochalasin D (C), 3 μM nocodazole (D) or 10 mM caffeine (E, F) respectively, for 1 h; after which procaine was added (A, B, C, D, F) to the concentration of 2.5 mM for 1 h. Microphotographs were taken under an inverted Olympus IMT-2 microscopy with phase-contrast optics. Scale bar = 10 μm.

3.4. Effect of procaine on dye exocytosis in human skin fibroblasts

The amount of neutral red accumulated in the vacuolised human skin fibroblasts depends on the balance between the rate of the dye uptake and the rate of its extrusion. Therefore, in the next experiments the rate of neutral red release from pre-loaded cells in the absence and presence of procaine was measured. It was observed that the human skin fibroblasts preincubated for 24 h in the presence of 2.5 mM procaine accumulated over three times more neutral red during 5-min exposure to the dye than the cells preincubated in the control medium without procaine. As shown in Fig. 5, removal of neutral red and procaine from the medium caused the fibroblasts to lose the dye relatively fast. In both cases, in cells exposed (white squares in Fig. 5) and unexposed (white triangles in Fig. 5) to procaine the amount of accumulated neutral red decreased about 50% during the first 6 h of cell incubation in the absence of neutral red and procaine. However, if the cells remained exposed to procaine after the neutral red removal, the release of neutral red from the cells was completely inhibited. The cells contained the same amount of neutral red 24 h after the neutral red removal from the medium as directly after neutral red loading.

Since caffeine was found to prevent the procaine-induced cell vacuolisation and neutral red uptake in human skin fibroblasts, the authors tested the effect of caffeine on procaine-induced inhibition of neutral red release from the cells preloaded with the dye. The human skin fibroblasts preloaded with neutral red in the presence of procaine were incubated in neutral red-free media and the dye release from the cells was measured. The results are presented in Fig. 5. In the absence of procaine, caffeine slightly (statistically insignificantly) accelerated the release of neutral red. The addition of caffeine to procaine-containing medium resulted in some restoration of neutral red release from human skin fibroblasts but did not fully abolish the inhibitory effect of

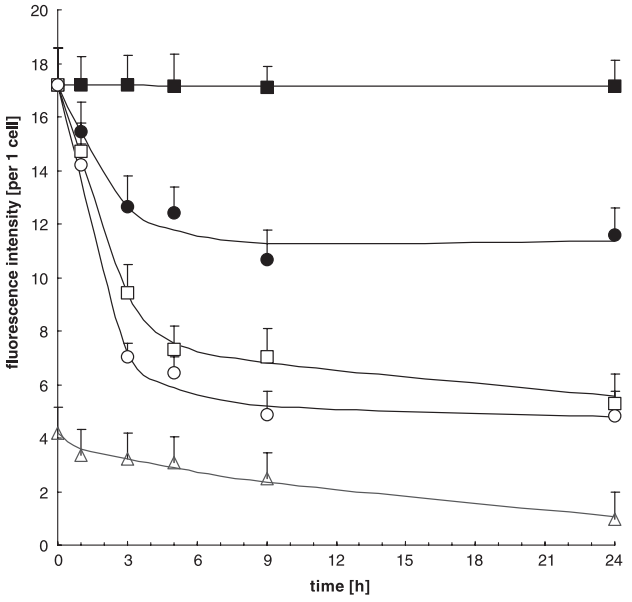

Fig. 5. Procaine-induced inhibition of fluid-phase exocytosis of human skin fibroblasts. Cells were cultured in MEM supplemented with 10% foetal calf serum (triangles) or with the same medium containing 2.5 mM procaine (squares) for 24 h, after which cells were stained with 50 μg/ml neutral red for 5 min. Subsequently, cells were incubated in MEM with 10% foetal calf serum (white triangles and white squares) in the same medium with 2.5 mM procaine (black squares), in MEM with 10% foetal calf serum and 10 mM caffeine without (white circles) or with the 2.5 mM procaine (black circles). The fluorescence intensity was determined for different time periods up to 24 h for each of the experimental conditions and is presented as mean and standard deviations of at least 300 randomly selected cells. The results presented are the means of at least three separate experiments.

procaine on neutral red release from the dye pre-loaded cells.

4. Discussion

Local anaesthetics, and in particular procaine, are often applied directly onto the skin topically to relieve a pain. Its doses are used usually much higher (0.5–1% procaine) (Morris and Whish, 1984; Strichartz and Berde, 1994) than those used in the authors' experiments. They report in this communication that procaine, at concentration 2.5 mM, induced vacuolisation of cytoplasm and great enhancement of neutral red accumulation in human skin fibroblasts cultured in vitro. All these effects appeared fully reversible. The great vacuoles appeared in the perinuclear cell region and then spread over the entire cytoplasm in the human skin fibroblasts exposed to procaine. This has no effect on cell viability and after the removal of procaine the vacuoles disappeared within 72 h.

Kimura et al. (1981) reported that intradermal injection of lidocaine or procaine caused the formation of vacuoles in epidermal cells. Bupivicaine, lignocaine, and cocaine caused vacuolisation of cytoplasm in neurones after their injection around spinal cords in rats (Bahar et al., 1984). Chloroquine and other weak bases induced vacuoles in rat embryo fibroblasts (Wibo and Poole 1974) and in mouse peritoneal macrophages (Ohkuma and Poole, 1981). Although the vacuolisation induced by local anaesthetics had been observed, the origin of these vacuoles and their relation to endo- and exocytosis were not elucidated. The present authors observed that lucifer yellow and neutral red, the dyes commonly used in research on fluid phase endocytosis, accumulated in the procaine-induced vacuoles in human skin fibroblasts. The vacuoles containing these dyes had been earlier described in HeLa cells in which vacuolisation was induced by vac A bacterial toxin secreted by pathogenic strains of *Helicobacter pylori* (Montecucco et al., 1999; Papini et al., 1994, 1996). The origin of vacuoles induced by procaine seems to correspond to that of the great vacuoles described in the HeLa cells. In neither case can the cell vacuolisation be prevented by 3-methyadenine, an inhibitor of autophagy (Caro et al., 1988; Punnenen et al., 1994), and cytochalasine D, an F-actin cytoskeleton destabilising agent affecting the first stages of endocytosis (Durrbach et al., 1996). Nor did brefeldine A, an inhibitor of membrane traffic among the Golgi apparatus and endoplasmic reticulum (Misumi et al., 1986), prevent the cytoplasm vacuolisation in human skin fibroblasts caused by procaine. In both cases, however, as observed by Papini et al. (1994, 1997) and in the present authors' experiments, nocodazole, which interferes with microtubules and the transport among early and late endosomes (Gruenberg et al., 1989), prevents the cytoplasm vacuolisation induced by Vac A toxin in HeLa cells and by procaine in human skin fibroblasts, respectively.

The tested inhibitors that prevented procaine-induced vacuolisation of cytoplasm also inhibited the enhancement of neutral red uptake and accumulation in procaine-treated human skin fibroblasts. This demonstrates that the content of great vacuoles appearing in the procaine-exposed human skin fibroblasts originates from the intensified fluid phase endocytosis and some disturbances in the membrane traffic between endosomes. The suggestion is supported by the results of quantitative measurements of an uptake and release of neutral red, a fluorescent dye commonly used as a marker of fluid phase endocytosis (Antal et al., 1995; Pl—ytycz et al., 1992; Ricci et al., 2002). The human skin fibroblasts preincubated in the presence of procaine for 1 h and then exposed to neutral red accumulated more than twice the dye than the cells preincubated in the control medium (cf. Fig. 3A). When procaine and neutral red were administered simultaneously in the culture medium the cells accumulated the dye faster and up to three times more than the cells exposed to neutral red in the control medium (cf. Fig. 3B). This accelerated and intensified uptake of neutral red by procaine-treated human skin fibroblasts was inhibited by nocodazole but not by cytochalasin D, brefeldin A, or 3-methyladenine (cf. Table 1).

In the control medium, nocodazole, cytochalasin B, and 3-methyladenine did not inhibit neutral red accumulation,

but brefeldin A decreased it significantly. Brefeldin A was shown to impair not only the Golgi apparatus but also the endosomal system, mainly by inhibiting heterotyping fusion (Lippincott-Schwartz et al., 1991; Pelham, 1991). The authors' results suggest that this drug, although it changes the endocytosis in the control conditions, cannot overcome the stimulatory effect of procaine on vacuolisation. It indicates that vacuoles formed in procaine-containing medium originate rather from homotypic fusion of some membrane vesicles or their swelling. Nocodazole—a microtubule disintegrating agent—did not change the fluid phase marker (the neutral red) uptake in control conditions, which is consistent with other results (Hamm-Alvarez et al., 1996; Sokolova et al., 1998). However, it inhibited the procaine-induced enhancement of neutral red uptake and vacuolisation of fibroblasts (cf. Table 1). It was also shown by others that in nocodazole-treated HeLa cells, endocytic pathway is blocked on the level of endosomal carrier vesicle—the compartment intermediate between early and late endosomes (Bayer et al., 1998). In the case of cells incubated with nocodazole and procaine simultaneously, the vacuoles were not formed, which suggests that they originate from late endosomes.

Caffeine, which is known to affect intracellular calcium and activate intracellular ryanodine receptor-like Ca^{2+} channel (Larini et al., 1995; Mc Pherson and Campbell, 1993), inhibited the human skin fibroblasts vacuolisation in the presence of procaine. Caffeine also counterbalanced the inhibitory effect of procaine on neutral red exocytosis. It is known that intracellular calcium concentration influences the process of membrane fusion by affecting SNAREs proteins (Luzio et al., 2001; Scott et al., in press). It is likely that, in the presence of procaine, the fusion of intracellular membranes is modified because of the altered calcium metabolism. The authors' results suggest that the procaine-induced formation of great vacuoles is associated with an impairment of membrane fusion. This suggestion is confirmed by the results of measurements showing the inhibition of neutral red release from the dye-preloaded human skin fibroblasts by procaine and the decrease of this inhibition by caffeine.

The results presented here show that procaine exerts an extensive effect on skin fibroblasts. It induces strong but reversible vacuolisation of cytoplasm associated with the enhanced uptake and inhibited release of fluid taken by endocytosis. (Berridge and Galione, 1988; Bolton and Imaizumi, 1996).

The accumulation of neutral red has been postulated to correlate with the number of cells per culture (Putnam et al., 2002) and with cell viability (Pape, 1997). The measurements of neutral red accumulation by cells, estimated often per culture vessel or well, has been frequently used for testing cytotoxic effects of various agents upon cells in culture in vitro (Andrisano et al., 2001; Lee et al., 2000; Moreno, 2000; Salvadori et al., 2001). The authors' results demonstrate that the accumulation of neutral red by human skin fibroblasts can substantially increase without any changes in cell number or viability. This clearly shows that the neutral red accumulation assays, if used, must be interpreted with great caution and the dye accumulation should not be considered as a direct marker of cell number or viability.

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