=====

Originals

=====

**EFFECTS OF PLURONIC F-127 ON LOADING
OF FURA 2/AM INTO SINGLE SMOOTH
MUSCLE CELLS ISOLATED FROM
GUINEA PIG TAENIA COLI**

**Ikuo MARUYAMA, Tomohiko HASEGAWA, Toshinori YAMAMOTO
and Kazutaka MOMOSE**

Department of Pharmacology, School of Pharmaceutical Sciences, Showa University,
1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan

Accepted April 19, 1989

Abstract.....The effect of Pluronic F-127 (PF-127), a surfactant polyol, on the loading of fura 2/AM into smooth muscle cells isolated from guinea pig taenia coli was investigated. The presence of PF-127 during the loading of fura 2 acetoxymethyl ester (fura 2/AM), an intracellular Ca indicator, occasionally increased the incorporation of the dye into the cells. However, long time loading (over approximately 60 min) in the presence of PF-127 decreased the incorporation of fura 2. When the extracellular medium obtained from cells treated with DFP, then incubated with fura 2/AM in the presence of PF-127 was analyzed with excitation spectra, the fluorescence peaks shifted to longer wave lengths by addition of EGTA. However, the peak of the extracellular medium obtained from cells treated with DFP, then incubated without PF-127, did not shift. These results show that PF-127 affects the membranous permeability of the dye in single smooth muscle cells, permitting fura 2 hydrolyzed in the cytoplasm to leak out through the membrane.

Key words : Intracellular Ca, fura 2, Pluronic F 127, single smooth muscle cells, guinea pig taenia coli.

INTRODUCTION

Fluorescent calcium indicator fura 2 acetoxymethyl ester (fura 2/AM) has been available for determination of free calcium levels in smooth muscle cells such as blood vessels and uteri (Bitar et al., 1986 ; Sumimoto and Kuriyama, 1986 ; Williams and Fay, 1986). However, it has been difficult to determine the free calcium level in taenia coli of guinea pigs with use of fura 2/AM, since the dye could not be easily loaded in cells (Rink and Pozzan, 1985). Maruyama et al. (1987, 1988) reported that DFP greatly enhanced the incorporation of fura 2 into the cells. On the other hand, Pluronic F-127 (PF-127), which increases solubility of the dye molecules, has been used extensively by numerous investigators (Poenie et al., 1986 ; Cobbold and Rink, 1987 ; Ozaki et al., 1987 ; Himpens and Somlyo, 1988). However, the effect of PF-127 on the incorporation of fura 2 into isolated single smooth cells has not been reported.

In this paper, we examined the effect of PF-127 on loading fura 2/AM in single smooth muscle cells isolated from guinea pig taenia coli.

MATERIALS AND METHODS

Preparation of Fura 2-loaded Single Cells : Fura 2-loaded single cells were prepared according to a modified procedures of our method (Maruyama et al., 1988) as follows. Taenia coli were excised from male guinea pigs (Hartley) weighing 250–550 g. The tissues (approximately 120 mg) were immersed for 90 min in 50 ml of 0.18 mM Ca-Tyrode HEPES solution (137 mM NaCl, 2.7 mM KCl, 0.18 mM CaCl₂, 1.0 mM MgCl₂, 5.6 mM glucose, 4.2 mM HEPES, pH 7.4). Then, the tissues were minced and suspended in 2.0 ml of 0.18 mM Ca-Tyrode HEPES solution containing 1.0% bovine serum albumin. The minces were digested for 30 min in a mixture of highly purified collagenase (0.33 units/ml) and papain (11.25 units/ml), with gentle stirring. Then, the minces were washed as soon as possible by centrifugation at 37°C to finish the digestion. The centrifugation was terminated when it reach 110 xg. The sediment was resuspended in 2 ml of physiological salt solution (PSS, 130 mM NaCl, 4.8 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 1.0 mM Na₂HPO₄, 15 mM glucose, 10 mM HEPES : N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid, pH 7.4) containing Pluronic F 127 (3 µg/ml), 5 µM fura 2/AM, 0.1 mM Ep-475 : (+) -(2S, 3S)-[3- {(S)-3-methyl-1-(3-methylbutyl-carbamyl butylcarbamoyl) }] glycolic acid as papain inhibitor and 0.1% BSA, then incubated at 37°C for 1 hr. After the mince was washed twice, it was suspended in PSS (without bovine serum albumin) and the cells were gently dispersed by pipetting with a wide-bore pipette. The media used were aerated and all the experiments were carried out at 37°C.

Determination of Intensity of Fluorescence : The fluorescence of fura 2-loaded cells was measured in a Hitachi MPF-4 fluorescence spectrophotometer with an excitation wavelength at 340 nm (slit 4 nm) and emission at 510 nm (slit 10 nm). Isolated single

cells were suspended in the PSS (2×10^5 cells/ml) and placed in 1 cm square quartz cuvettes. The suspension was continuously stirred and intensity of the fluorescence was determined. The cells were treated with digitonin ($100 \mu\text{M}$) to lyse the cells and the suspension was mixed with calcium (1.0 mM) and fura 2/AM to obtain maximum intensity of the fluorescence. Then, EGTA (12.5 mM) was added in order to determine the minimum level of fluorescence since unchelated fura 2 had slight emission at 510 nm . In the present experiments, decrease in fluorescence intensity caused by adding EGTA represented the relative intracellular concentration of fura 2 resulting from the hydrolysis of fura 2/AM.

Contractile Response of Single Smooth Muscle Cells to Carbachol (CCh) : The cell suspension was divided into several tubes and the cells were incubated with various concentrations CCh for 10 sec at 37°C . The incubation was terminated by mixing with the same volume of 2% glutaraldehyde solution ($\text{pH } 7.4$). Then, the cells were transferred onto a slide glass and their dimensions were recorded with a computer (NEC-9801)-assisted light microscopic image analyzer to determine the degree of contraction.

Materials : The materials used were PF-127 (BASF Wyandotte Co.), fura 2/AM (Molecular Probes Co.), collagenase (Amano Pharmaceutical Co.), papain (Type III : Sigma Chemical Co.), diisopropylfluorophosphate (DFP : Sigma Chemical Co.), EGTA (Dojin Chemical Co.), and digitonin (Tokyo Chemical Co.). Ep-475 was kindly donated by the Research Laboratories of Taisho Pharmaceutical Co., Tokyo. Other chemicals used were of reagent grade.

RESULTS

Fig. 1 shows the effect of PF-127 on fura 2 loading in single smooth muscle cells. The resting level of the fluorescence intensity was lower when the dye was loaded on the single cells in the absence of PF-127 and the intensity was increased slightly by adding digitonin (Fig. 1A, left). The resting level was significantly increased when the dye was loaded in the presence of PF-127, since the detergent accelerated incorporation of fura 2/AM into the cells and the dye itself had emission of fluorescence at 510 without chelating Ca^{2+} (Fig. 1A, right). The intensity was increased slightly by adding digitonin and decreased significantly by EGTA. In some single cell preparations, presence of PF-127 did not increase the resting level of the fluorescence and the intensity was not increased by digitonin (Fig. 1B). When fura 2 was loaded in the presence of DFP, intensity of the fluorescence was significantly increased ($n=4$; $P<0.001$) and concentration of fura 2 in the single cells was estimated $70.8 \pm 1.8 \mu\text{M}$ (Fig. 1C, right).

Fig. 2 shows the time course of intensity of the fluorescence. The single cells were incubated with fura 2/AM in the presence of PF-127 for various periods and the intensity was determined. The resting level of the fluorescence was higher with longer incubation periods and it reached to maximum at 60 min. The difference

Fig. 1. Effect of PF-127 on fura 2 loading into single smooth muscle cells from taenia coli. Smooth muscle cells were incubated with fura 2/AM for 1 hr. Fura 2-loaded cells were lysed by adding digitonin (100 μ M). Then 30 mM Tris and 12.5 mM EGTA were added to the lysed cells. Excitation wavelength : 340 nm Emission wavelength : 510 nm. PF-127(-) : cells incubated with fura 2/AM in the absence of PF-127, PF-127(+): cells incubated with fura 2/AM in the presence of PF-127 (3 μ g/ml), DFP(-): cells no treated with DFP, DFP (+): cells treated with DFP.

between maximum fluorescence intensity was also increased. Over 60 min. incubation, then intensity became lower and the difference between maximum and minimum intensity was also decreased.

Fig. 3 shows the effect of either PF-127 or DFP, or both on fura 2 loading on the single smooth muscle cells. The resting level of the fluorescence in the cells treated without DFP or PF-127 was lower and the intensity was slightly increased by digitonin (Fig. 3A). The resting level was decreased significantly when the dye was incorporated in the presence of PF-127, and the intensity was slightly increased by digitonin (Fig. 3B). By treating the cells with DFP, the resting level was increased significantly and incorporation of fura 2/AM into the single cells was enhanced. The intensity was increased greatly by digitonin. The increase was approximately 2 times that in cells without the treatment with DFP (Fig. 3C). The intensity was decreased greatly by EGTA, since the Ca^{5+} -dependent fluorescence of the dye decreased as follows: DFP enhanced the incorporation of fura 2/AM and consequently the chelating of fura 2 with Ca^{2+} increased after hydrolysis of the ester. The fluorescence intensity increased in proportion to the chelating. Therefore, the intensity was decreased by EGTA. When DFP-treated cells were incubated with fura 2/AM in the presence of PF-127, the resting level of fluorescence in the cells was increased significantly (Fig. 3D). However, when the cells were treated with PF-127 and DFP, then incubated with the dye, the resting level of fluorescence intensity was greatly decreased (Fig. 3E). The increase in the intensity caused by adding digitonin was also decreased (Fig. 3E).

Effect of PF-127 on fura 2 hydrolysis outside the single cells was examined as shown in Fig. 4. Single smooth muscle cells were incubated with fura 2/AM and the cell suspension was centrifuged to collect the supernatant. When EGTA was added to supernatants obtained from cell suspensions without both PF-127 and DFP (Fig. 4A) and from cells with PF-127 alone (Fig. 4B), the fluorescence intensity decreased and the fluorescence peaks shifted to longer wave length. In contrast, the peak of the supernatant obtained from cells treated with DFP alone did not shifted to longer wave lengths (Fig. 4C). But the peak of the specimen obtained from cells with PF-127 after treatment with DFP shifted to longer wave lengths (Fig. 4D). Also, when cells were treated previously with PF-127, followed by DFP, the supernatant peak did not shift (Fig. 4E).

Fig. 5 shows the effects of PF-127 on the contractile response of single cells to CCh. DFP-treated cells and cells without PF-127 were contracted dose-dependently by CCh. But PF-127-treated cells were not contracted dose-dependently by CCh.

The dye exclusion test with 1% trypan blue showed that more than of 75% the cells treated with DFP and 48% the cells treated with PF-127 loading remained viable (data not shown).

Fig. 2. Time course of incorporated fura 2/AM-dependent fluorescence intensity in the presence of PF-127. Smooth muscle cells were incubated with fura 2/AM. Fluorescence was measured at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. At each excitation wavelength, the fluorescence emission of fura 2 was increased when the dye chelated with the Ca^{2+} .

Fig. 3. The effect of the combination of PF-127 and DFP on fura 2 loading in single smooth muscle cells. Pluronic F-127 and DFP were used as shown in Fig. 1. Fluorescence was measured as described in the text. A : non-treated cells (intact cells), B : cells loaded with fura 2/AM in the presence of PF-127, C : DFP-treated cells, D : cells loaded with fura 2/AM in the presence of PF-127 after DFP treatment, E : cells treated with Pluronic F-127, followed with DFP.

Fig. 4. Effect of PF-127 on the excitation spectrum of the extracellular medium.

The extracellular medium was obtained by centrifugation of the loaded cell suspension. EGTA (12.5 mM) was added to the medium. A : non-treated cells (intact cells), B : cells loaded with fura 2/AM in the presence of PF-127, C : DFP-treated cells, D : cells loaded with fura 2/AM in the presence of PF-127 after DFP treatment, E : cells treated with PF-127, followed by DFP.

Fig. 5. Concentration-response curves of CCh on the contraction of cells treated with PF-127. ● non-treated cells (intact cells); □ PF-127-treated cells; ■ DFP-treated cells; ○ control cells (cells incubated without DFP and fura 2). Plots are means $\pm$ S.E. obtained from 4 experiments.

DISCUSSION

PF-127 helps to solubilize large dye molecules in physiological media and to increase the incorporation of dye (Himpens and Somlyo, 1988). In the present experiment, we found that the incorporation of fura 2 into the single cells of smooth muscle was dependent on solubility of the dye (Fig. 1A). The single cells lost their contractility within 1.5 hr after isolation, while that of the muscle is kept for a long time (Momose and Gomi, 1980). So, when single cells are used in physiological and pharmacological experiments, the dye must be incorporated into the cells as soon as possible. We reported that the cholinesterase inhibitor, DFP enhanced the loading of fura 2/AM into the smooth muscle cells significantly, since this reagent suppresses esterase activity outside the cells (Maruyama et al., 1987, 1989). Thus, the combination of PF-127 and DFP was expected to enhance the incorporation of fura 2/AM into the single smooth muscle cells. In some experiments, indeed, the incorporation was significantly increased by the use of this combination. It appears that the effect of the combination on the incorporation of the dye was due to the solubility of the dye in the medium being increased by PF-127 and to the DFP

inhibition of the hydrolysis of fura 2/AM while it was permitting membranes. However, in other experiments, the incorporation was decreased significantly by the use of this combination. In addition, PF-127 alone sometimes decreased the incorporation of the dye less than it was decreased without PF-127, while in most of the experiments the reagent alone increased the incorporation. To determine why the conflicting effects of PF-127 occurred, we measured the fluorescence intensity in the cells after the long-time incubation with PF-127 containing fura 2/AM. The incorporation into the cells increased with the passage of time up to 1 hr, but it decreased after that. It appeared that these variations might be due to the fura 2 incorporated by PF-127 being leaked outside the membrane. To determine the influence of PF-127 on the membrane, we examined cells that had been treated previously with the compound. We found that the incorporation of the dye into the cells was greatly decreased by the pretreatment with PF-127. So apparently the PF-127 had some effect on the membrane. Also when suspensions after incubation with fura 2/AM were centrifuged and the supernatants were analyzed by fluorescence, we found free fura 2 outside the cells where it cannot be hydrolyzed. This shows that free-fura 2 hydrolyzed in the cytoplasm was leaked outside the cells.

Considering these results, it is clear that PF-127 affects the membrane of single cells, resulting in leakage of the free-fura 2 accumulated in the cytoplasm. So it is to be emphasized that should not be used to load fura 2/AM into single smooth muscle cells. And probably we should pay more attention to its use, when it is used to load the cells in smooth muscles.

On the other hand, because DFP significantly increased the incorporation of fura 2/AM into the smooth muscle cells, it shortened the incubation time (Maruyama et al., 1987, 1988). Thus the viability of these cells was well preserved. In addition, DFP did not affect the cell contractility (Maruyama et al., 1989). So it appears that DFP may be the most effective substance for loading fura 2/AM into smooth muscle cells. Experiments are in progress to examine the changes in the intracellular concentration of Ca^{2+} by various muscarinic agonists such as CCh, using cells treated with DFP.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Hideaki Karaki (Department of Veterinary Pharmacology, Faculty of Agriculture, University of Tokyo) for pertinent suggestions.

REFERENCES

- Bitar, K. N., Bradford, P., Putney, J. W., et al. (1986): Cytosolic calcium during contraction of isolated mammalian gastric muscle cells. *Science*, **232**, 1143–1145.

- Cobbold, P. H. and Rink, T. (1987) : Fluorescence and bioluminescence measurement of cytoplasmic free calcium. *Biochem. J.*, **248**, 313–328.
- Himpens, B. and Somlyo, A. P. (1988) : Free-calcium and force transients during depolarization and pharmacomechanical coupling in guinea-pig smooth muscle. *J. Physiol.*, **395**, 507–530.
- Maruyama, I., Oyamada, H., Hasegawa, T., et al. (1987) : Effect of DFP on loading of fura 2/AM and quin 2/AM into single smooth muscle cells prepared from guinea pig taenia coli. *FEBS LETTER*, **220**, 89–92.
- Maruyama, I., Oyamada, H., Hasegawa, T., et al. (1989) : Loading of fura-2/AM with an aid of DFP on single smooth muscle cells prepared from guinea pig taenia coli. *J. Pharmacol. Methods*, **21**, 1–12.
- Maruyama, I., Yoshida, T., Nakayama, M., et al. (1988) : Improvement of a procedure for preparing single smooth muscle cells from guinea pig taenia coli by purified collagenase and papain. *J. Pharmacol. Methods*, **19**, 155–164.
- Momose, K. and Gomi, Y. (1980) : Studies of isolated smooth muscle cells VI/Dispersion procedures for acetylcholine-sensitive smooth muscle cells of guinea pig. *Jap. J. Smooth Muscle Res.*, **16**, 29–36.
- Ozaki, H., Sato, K. and Karaki, H. (1987) : Simultaneous recordings of calcium signals and mechanical activity using fluorescent dye fura 2 in isolated strips of vascular smooth muscle. *Jpn. J. Pharmacol.*, **45**, 429–433.
- Poenie, M., Alderton, J., Steinhardt, R., et al. (1986) : Calcium rises abruptly and briefly throughout the cell at the onset of anaphase. *Science*, **233**, 886–889.
- Rink, T. J. and Pozzan, T. (1985) : Using quin 2 in cell suspensions. *Cell Calcium*, **6**, 133–144.
- Sumimoto, K. and Kuriyama, H. (1986) : Mobilization of free Ca^{2+} measured during contraction-relaxation cycles in smooth muscle cells of the porcine coronary artery using quin 2. *Pflügers Arch*, **406**, 173–80.
- Williams, D. A. and Fay, F. S. (1986) : Calcium transients and resting levels in isolated smooth muscle cells as monitored with quin 2. *Amer. J. Physiol.*, **250**, C779.