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Effect of DFP on loading of fura 2/AM and quin 2/AM into single smooth muscle cells prepared from guinea pig taenia coli

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The effect of diisopropyl fluorophosphate (DFP), a potent cholinesterase (ChE) inhibitor, on loading quin 2 acetoxymethyl ester (quin 2/AM) and fura 2/AM into smooth muscle cells isolated from guinea pig taenia coli was investigated spectrofluorometrically. The presence of DFP during the loading permitted the incorporation of quin 2 into the cells, so that it became possible to measure intracellular Ca^{2+} concentrations using the ester of this dye. Also, DFP significantly enhanced the incorporation of fura 2 into the cells. These results indicate that loading of quin 2/AM and fura 2/AM into the smooth muscle cells may depend on the suppression of ChE or various serine protease activities outside cells.

Quin 2; Fura 2; Diisopropyl fluorophosphate; (Single smooth muscle cell, Guinea pig taenia coli)

1. INTRODUCTION

Guinea pig taenia coli and the single isolated cells have been used extensively as physiological and pharmacological models for studying the contractile mechanism of mammalian smooth muscle [1–7]. However, it was impossible to determine Ca in the cells by intracellular Ca indicators, such as quin 2 [8], although cells of other tissues from the same animals and most mammalian cells are readily loaded with these dyes [9–12]. Therefore, it appears that these cells may not have the appropriate cytoplasmic enzymes which hydrolyze the ester of the dyes [9].

We found that a large amount of cholinesterase (ChE) exists outside the cells. Therefore, there was a possibility that the ester of these dyes may be hydrolyzed at the surface of a cell, to prevent the dye from penetrating the cell membrane. Recently, a new fluorescent Ca indicator, fura 2/AM, with a higher sensitivity than quin 2 was synthesized [13]. In the present study, we examined the effect of diisopropyl fluorophosphate (DFP) on quin 2 and fura 2 content of smooth muscle cells after loading guinea pig taenia coli with the acetoxymethyl esters of their compounds.

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2. MATERIALS AND METHODS

2.1. *Materials*

Quin 2/AM, 2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-[bis-(carboxymethyl)amino]quinoline, tetraacetoxymethyl ester was obtained from Dojin Chemical Co., fura 2/AM, 1-(2-(5′-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy)-2-(2′-amino-5′-methylphenoxy) ethane-*N,N,N′,N′*-tetraacetic acid, pentaacetoxy-

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methyl ester from Molecular Probes and DFP from Sigma.

2.2. *Preparation of fura 2 loaded single cells*

Single cells were prepared from guinea pig taenia coli according to our method in [14]. In the procedures, DFP (final 8 mM) was added to the suspension of minces which was then immediately diluted 16-fold. The minces were washed as soon as possible at 37°C with centrifugation. The centrifugation was terminated when it reached 110 × *g*. The precipitate was resuspended in 2 ml of physiological salt solution (PSS, 130 mM NaCl, 4.8 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM Na_2HPO_4, 15 mM glucose, 10 mM Hepes, pH 7.4) containing 5 μM fura 2/AM, 0.1 mM EP-475:(+)-(2*S*,3*S*)-[3-{(*S*)-3-methyl-1-(3-methylbutyl-carbamyl butylcarbamoyl)}]glycilic acid as a papain inhibitor and 0.1% BSA, then incubated at 37°C for 1 h. After washing the minces twice, they were suspended in PSS (without bovine serum albumin) and the cells were gently dispersed by pipetting with a wide-bored pipette. The media used were aerated and all the experiments were carried out at 37°C. The cells were measured at concentrations of 2×10^5 cells/ml and stirred continuously.

2.3. *Preparation of quin 2 loaded single cells*

Quin 2 loaded cells were prepared as described in section 2.2 except for the period of incubation and the use of quin 2/AM instead of fura 2. The cells were incubated for 3 h in a medium containing 50 μM quin 2/AM.

3. RESULTS AND DISCUSSION

Fig.1 shows the effect of DFP on quin 2 loading in smooth muscle cells of the taenia coli. The resting level of fluorescence intensity in cells without treatment of DFP was low and was little changed by adding digitonin (fig.1a). By treating the cells with DFP, however, the resting level was increased significantly. The fluorescence in cells treated with DFP was increased by adding digitonin, since quin 2 in the cytoplasm chelated with the Ca added in the medium (fig.1b). The fluorescence seen after the addition of EGTA was higher after treatment with DFP than without, since DFP enhanced the incorporation of this dye

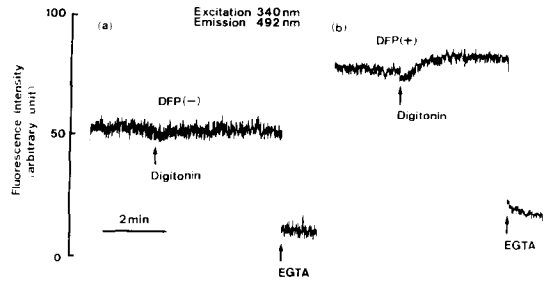

Fig.1. Effect of DFP on quin 2 loading into smooth muscle cells isolated from guinea pig taenia coli. Fura 2 loaded cells were lysed by addition of digitonin (100 μM). Then, 5 mM EGTA was added to the lysed cells. (a) Untreated cells, (b) cells treated with DFP.

which has a slight fluorescence before chelating with Ca^{2+}. These results show that quin 2/AM can be loaded into the taenia coli cells with the aid of DFP and that the cells have the appropriate cytoplasmic esterase for hydrolyzing quin 2/AM, although it had appeared that the cells did not have such enzymes. It seems that the poor response seen after digitonin treatment of DFP-treated then quin 2 loaded cells is due to the lesser incorporation of quin 2 into the cells than that of fura 2 and to the lower fluorescence of quin 2 than that of fura 2.

Fluorescence of fura 2 is approx. 30-times brighter than quin 2. Because of this, one can use much less chelator to load cells [15–17]. We also considered the possibility that treatment with DFP might be effective in fura 2 loading into the taenia coli cells. Fig.2 shows this effect of DFP on fura 2 loading into the smooth muscle cells. The resting level of fluorescence intensity in the cells was increased significantly by DFP. Each fluorescence intensity was increased by the addition of digitonin (fig.2a). In this case, there was a small increment of fluorescence intensity in cells without treatment with DFP. But the increment in cells treated with DFP was approx. 3-times that in cells without treatment. These results show that DFP enhances fura 2 loading into the cells.

From these results we considered the possibility that the ester of these dyes might be hydrolyzed outside the cells. To clarify if hydrolysis of fura 2/AM took place outside the cells, the excitation spectra of the media were analyzed after incubation of cells with fura 2/AM (fig.2b). A peak obtained from a medium after treatment with DFP

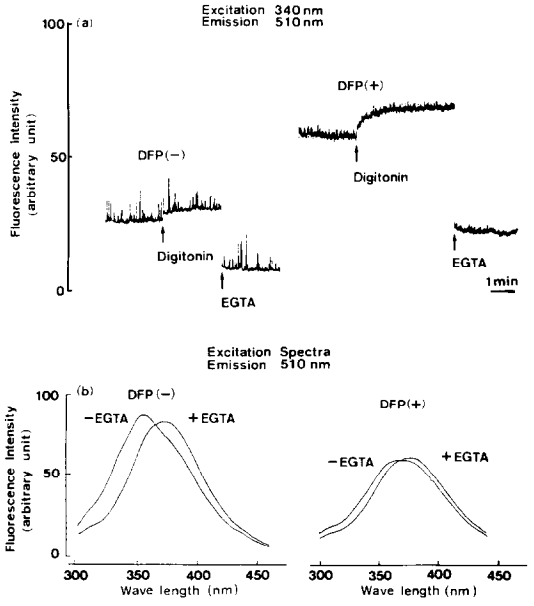

Fig.2. Effect of DFP on fura 2 hydrolysis and the loading into smooth muscle cells from taenia coli. DFP(−), cells without DFP treatment; DFP(+), cells treated with DFP. (a) Effect of DFP on fura 2 loading into cells. Digitonin and EGTA were used under conditions similar to those in fig.1. (b) Effect of DFP on excitation spectrum of extracellular medium. The extracellular medium was obtained by centrifugation of loaded cell suspension. EGTA (5 mM) was added to the medium. Emission wavelength, 510 nm.

was significantly lower than that of an untreated medium. When EGTA was added to these incubation media, the peak obtained from the medium with DFP treatment shifted to longer wavelength only slightly, while the one obtained without the treatment shifted significantly. This shows that fura 2, resulting from hydrolysis of fura 2/AM, existed in incubation media from cell suspensions without DFP treatment.

We found that a large amount of ChE exists outside the cells (not shown). Also, when quin 2/AM and fura 2/AM were incubated with ChE preparation, their esters were hydrolyzed with the passage of time (not shown). It is well known that DFP inhibits ChE. Therefore, from our fluorescence analysis, it can be concluded that if the cells were not treated with a ChE inhibitor, most of the dyes were hydrolyzed principally by ChE outside the cells. In this condition the dyes cannot penetrate the cell membrane. However, it is well known that DFP also inhibits various serine proteases which have serine residues in the active site. Therefore, the possibility that serine protease may also be related to the hydrolysis of these dyes is not excluded. In the present study, both dyes were sufficiently loaded into cells with the aid of a ChE inhibitor, and hydrolyzed in the cytoplasm. It is apparent that the mechanism for loading dyes such as quin 2/AM and fura 2/AM into cells in the guinea pig taenia coli is different from that for cells of other tissues from the same animal, and from most mammalian cells.

In the present study, the dyes were loaded into the minces after enzymatic digestion, since the viability of these cells was well preserved. Also it is essential to determine whether DFP treatment affects various physiological properties. We confirmed that DFP treatment did not affect the contractility of cells and that loaded fura 2 did not affect the contractility of the cells (a detailed report will be presented subsequently). Therefore, it will be possible to examine the contraction of cells and the behavior of intracellular Ca simultaneously in the same cells. Experiments are in progress to examine the changes in intracellular concentration of Ca by various muscarinic agonists such as CCh using cells treated with DFP.

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