Analytical Behavior of Fura-2 and Its Determination of $[Ca^{2+}]_i$ in Lymphocytes Treated with Cefotaxime

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Abstract – The interaction of Fura-2 with Ca^{2+} is studied using steady fluorescence technique. The effect of pH on the spectra behavior of Fura-2 in the presence of Ca^{2+} is investigated, the excitation maxima (340 nm) and the isobestic point (360 nm) for the fluorescence spectra of Fura-2 depend on pH. At different temperatures the apparent dissociation constants (K_d) of Fura-2-Ca^{2+} complex are examined, K_d is found to decrease with increasing temperatures (20 ℃, 37 ℃, 50 ℃) and ΔH is calculated to be 21.16 kJ/mol by using the Van't Hoff equation at pH 7.4 for all the temperatures tested. The determination of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in lymphocyte is developed by using Fura-2 as a fluorescence probe in the presence of Cefotaxime at 37 ℃ and pH 7.4.

Key words – *Fura-2*; *pH*; *temperature*; $[Ca^{2+}]_i$; *cefotaxime*

Manuscript Number: 1674-8042(2010)04-0395-06

dio: 10.3969/j.issn.1674-8042.2010.04.21

1 Introduction

Calcium signals in cells of the immune system participate in several intracellular events[1]. Even small changes in $[Ca^{2+}]_i$ have an impact on cellular activities, including activation of calcineurin and protein kinases, ultimately can lead to alterations in cell function, lymphocyte proliferation, and programmed cell death[2-5]. Recently, numerous papers are involved in the measurement of $[Ca^{2+}]_i$ with fluorescent probes[6].

Since quin2 becomes an indicator to measure $[Ca^{2+}]_i$, a series of Ca^{2+} fluorescent probes have been synthesized for the detection purpose, including Indo-1, Fura-2, Fluo-3, and so on. Among these probes, Fura-2 exhibits unique ratiometric (λ_{ex1} = 340 nm, λ_{ex2} = 380 nm) fluorescence characteristics and is sensitive to the microenvironment. Upon the binding with Ca^{2+}, the excitation maxima of Fura-2 increases, the spectra suffer decrease at 380 nm and increase at 340 nm with a clear isobestic point at 360 nm, the ratio of the fluorescence excitation intensity at 340/380 nm will signal calcium[7]. So, Fura-2 has been widely used to determinate $[Ca^{2+}]_i$ in varied tissues such as mitochondrion and epithelium[8-9].

Cefotaxime (CEFA) as one of the β-lactams antibiotics (Fig. 1) has broad spectra activity against Gram-positive and Gram-negative bacteria[10-11] and is applied to treat infections in clinics and prevent bacterial contamination of tissue cultures[12]. Some cephalosporins have been reported to cause the oxidation of biological substances[13-14]. Hans et al.[15] provided penicillin that may induce allergic and in some cases also autoimmune diseases through reacting to T cells in an antigen-specific way. Stimulation of lymphocytes can induce a variety of cellular processes and changes in intracellular Ca^{2+} levels are known to be a triggering signal for many of these processes[4,6]. However, few studies is involved in the measurement of Ca^{2+} in the lymphocytes with the presence of CEFA. Although Fura-2 is an effective Ca^{2+} indicator, less research focused on the analytical behavior[16-18]. Grzegorz[7] figured out the effect of pH changes on Fura-2 at different concentrations of free Ca^{2+} and Dennis[19] reported the dependence of K_d (Fura-2-Ca^{2+}) on the temperature.

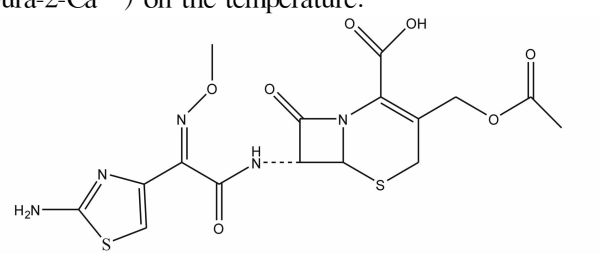

Fig. 1 Chemical structure of cefotaxime

In the studies, the analytical behavior of Fura-2 binding with Ca^{2+} is investigated based on the steady fluorescence technique. The effect of that factors including the temperature and pH on the process is involved in probing into the interaction between Fura-2 and Ca^{2+}. 37 ℃ and pH 7.4 are chosen to be the optimal conditions for measurement. Using the fluorescent probe, the effect

* **Received**: 2010-02-05
Project supported: This work was supported by the Key Project of National Nature Science Foundation of China (NO. 90813018) and the Returned Chinese Scholars Fund.
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of Cefotaxime on $[Ca^{2+}]_i$ and the mechanism in human peripheral lymphocytes are investigated. All these researches may provide a better understanding of the interaction of Fura-2 and Ca^{2+} and extend the analytical application.

2 Experiment

2.1 Apparatus

The fluorescence spectra are recorded on Varian Cary Eclipse luminescence spectrometer (USA). Excitation and emission bandwidths are both set at 10 nm. All pH values are measured with a pHS-3C digital pH meter (The Second Instrument Factory of Shanghai, China). L-500 type Centrifuge (The Xiangyi Instruments Factory of Hunan, China) is used to separate the lymphocyte cells.

2.2 Reagents

All the chemicals are of analytical-reagent grade, and all the water used is ultra pure water made in mol ultra water apparatus. Fura-2, Fura-2/AM, EGTA, TritonX-100 and N'-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid](HEPES) are all purchased from Sigma Chemical Company (St. Louis, MO, USA). Lymphocytes separation medium is obtained from the second reagent factory of Shanghai. Fetal calf serum is biochemistry regent produced in China. Cefotaxime is from the Aurobindo Tongling Datong Pharmaceutical CO. LTD., China. Simulating intracellular ionic composition buffer contains (in mM): 120 KCl, 20 NaCl, 1 $MgCl_2$, 10 Hepes, 1EGTA; pH 7.4. Na^+-medium contains (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 5.5 glucose, 10 Hepes; pH 7.4.

A stock standard solution of Fura-2, 1.2 mmolL^{-1}, is prepared by dissolving 1 mg Fura-2 in 1.0 mL ultra pure water, then loaded separately to 1.5 mL Centrifuge tube. A stock standard solution of Fura-2AM, 1.0 mmolL^{-1}, is prepared by dissolving 1 mg Fura-2AM in 1.0 mL DMSO, then loaded separately to 1.5 mL Centrifuge tube. All these stock solutions and working solutions are stored at −4 ℃.

2.3 Experimental methods

2.3.1 Excitation spectra of Fura-2-Ca^{2+}

Excitation spectra of Fura-2-Ca^{2+} (Fig. 2) are determined in simulating intracellular ionic composition buffer. When the effect of temperature is considered, it should be set at 20 ℃, 37 ℃, and 50 ℃ respectively. And the pH experiments are performed at the room temperature, pH equal to 5.7, 6.0, 6.5, 7.5 and 8.0 are chosen in the experiments. The fluorescence measurements are carried out on a Cary ecliptic fluorescence spectrophotometer (Varian, USA) with 510 emission wavelength.

2.3.2 Lymphocytes preparation

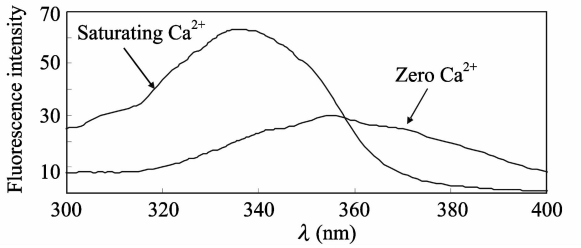

Fig. 2 Excitation spectra for Fura-2 in calibration buffers of varied $[Ca^{2+}]$. Excitation spectra of 1 μM Fura-2 was performed under simulating intracellular ionic composition buffer

Peripheral blood lymphocytes are obtained from healthy persons. The blood is diluted with the equal volume of 0.9 % NaCl solution and added to the lymphocytes separation medium, then is centrifuged at 2 500 rpm for 20 min. Removed the middle layer of lymphocytes, and washed lymphocytes with 0.9 % NaCl solution and Na^+-medium respectively. Cells are suspended in Na^+-medium.

2.3.3 The loading of Fura-2AM

To measure $[Ca^{2+}]_i$, the cells (1×10^8/mL) are incubated for 40 min at 37 ℃ with 2 μM acetoxymethyl ester form of Fura-2 (Fura-2AM) and fetal call serum in Na^+-medium. Then lymphocytes are washed twice with Na^+-medium, and finally resuspended in 3 mL buffer Na^+-free medium at pH 7.4.

2.3.4 Intracellular Ca^{2+} fluorescence measurements

Measurement of $[Ca^{2+}]_i$ in Fura-2AM-loaded human peripheral lymphocytes is performed in a Varian Cary Ecliptic luminescence spectrometer, together with an Intracellular Biochemistry Ratio Application software(Varian) to allow data captured in the form of Microsoft Excel spreadsheets. Fluorescence is measured by using a 340/380 nm excitation ratio under 510 nm emission wavelength. R_{min} is estimated by treating cells with 50 μL 0.8 M EGTA; R_{max} is estimated by treating cells with 50 μL of 0.1% Triton X-100. S_f/S_b is the ratio of fluorescence intensities after excitation at 380 nm, for Ca^{2+}-free medium and Ca^{2+}-saturated medium, respectively.

The cells are placed in a quartz cuvetle, stirred at a constant temperature of 37 ℃. The data is calculated as mean ±SEM for three to five separate experiments.

3 Results and discussion

3.1 Spectral characteristics of Fura-2 binding to Ca^{2+}

The fluorescence excitation spectra of Fura-2 and Fura-2-Ca^{2+} are shown in Fig. 2. As can be seen, binding to Ca^{2+} causes the increase of fluorescence intensity of Fura-2, and the excitation intensity suffers the decrease at 380 nm and increase at 340 nm, a clear isobestic point is located at 360 nm. All of these spectral changes indicate the formation of Fura-2-Ca^{2+} complex.

3.1.1 Effects of temperatures on the binding of Fura-2 to Ca^{2+}

The effects of temperatures (20 ℃, 37 ℃, 50 ℃) on the excitation spectra of Fura-2-Ca^{2+} are shown in Fig. 3.

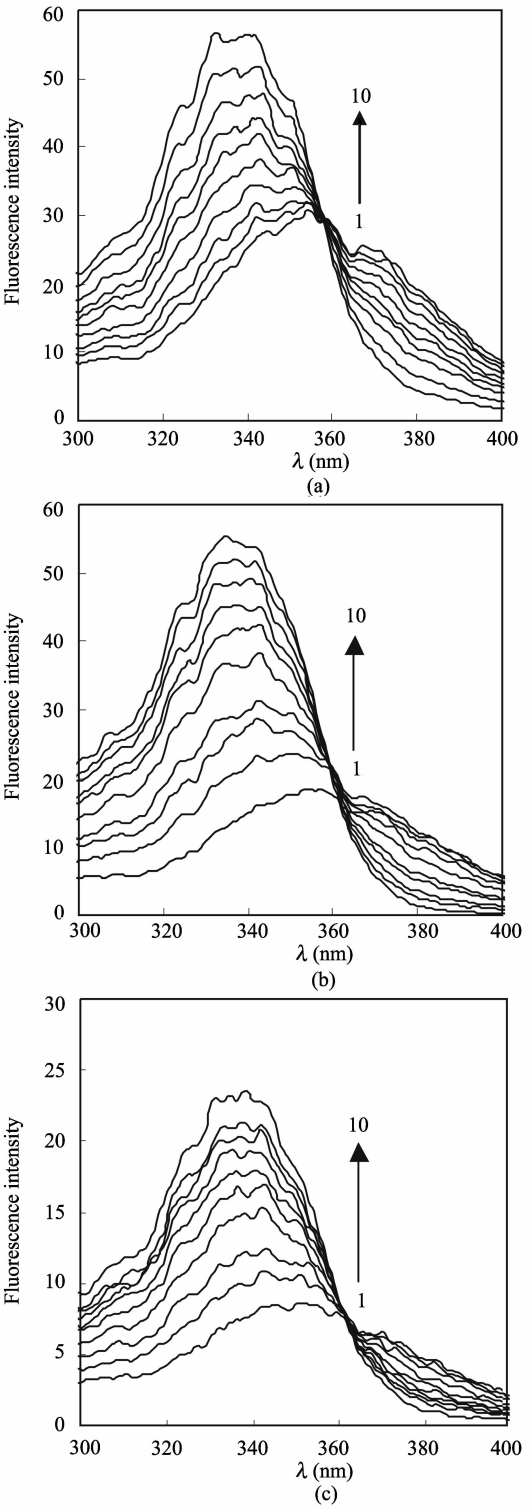

Fig. 3 Temperature effect on excitation spectra of Fura-2-Ca^{2+}, Ca^{2+} concentration in base pairs: $0, 2.6\times10^{-6}, 8.0\times10^{-6}, 2.4\times10^{-5}, 3.2\times10^{-5}, 7.2\times10^{-5}, 9.6\times10^{-5}, 2.6\times10^{-4}, 5.0\times10^{-4}, 8.0\times10^{-4}$ M: (a) 20 ℃, (b) 37 ℃ (c) 50 ℃

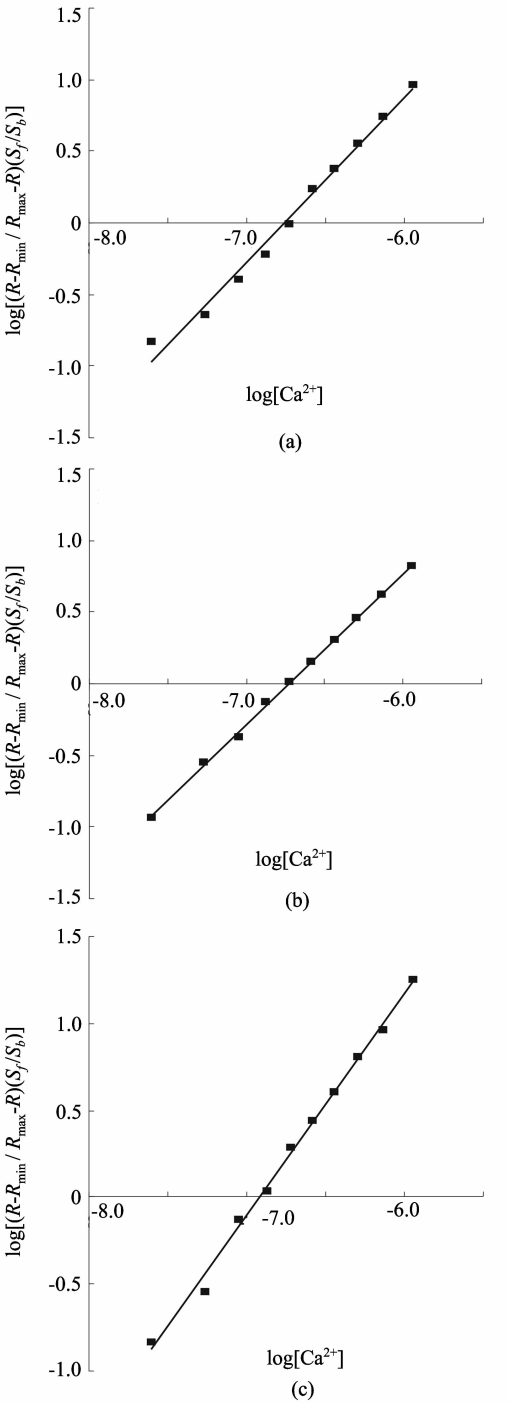

Fig. 4 Temperature effect on the K_d of Fura-2-Ca^{2+} complex. The logK_4 for Ca^{2+} binding to Fura-2 is determined by the intercept with the x-axis in plot. Three separate titration with 10 different Ca^{2+} concentrations were performed at: (a) 20 ℃, (b) 37 ℃, (c) 50 ℃

With the concentration of Ca^{2+} increasing, the fluorescence spectra of Fura-2 show the enhancement in the fluorescence intensity, and the maxima excitation shifts toward 340 nm and the isobestic point is at 360 nm under different temperature conditions. Temperature can affect the intensity of excitation peak wavelength of Fura-2 binding with Ca^{2+} but not the location of the peak. So Fura-2 is a useful tool to determine the Ca^{2+} in a wide range of temperatures.

The apparent dissociation constant (K_d) is the vital conversion parameter linking the fluorescence signal to ion concentration. It is sensitive to many factors including pH, temperature, ionic strength and buffer solution[7,16]. K_d is calculated by using the equation[1]

$$[Ca^{2+}] = K_d \times (R - R_{min})/(R_{max} - R) \times S_f/S_b .$$

Where $[Ca^{2+}]$ is the concentration of Ca^{2+} in simulating intracellular ionic composition buffer, R is the observed fluorescence ratio(F340/F380, R_{min} is the dye fluorescence ratio in a virtually Ca^{2+}-free medium; R_{max} is the maximum fluorescence ratio of Ca^{2+}-saturated with dye. S_f/S_b is the ratio of fluorescence intensities at 380 nm, for Ca^{2+}-free medium and Ca^{2+}-saturated medium, respectively[7].

The K_d of the Fura-2-Ca^{2+} complex at different temperatures are determined from Hill plots, where intercept with x-axis equals Log K_d. The plot yields a straight line, showing a 1∶1 stoichiometry for the Fura-2-Ca^{2+} complex at the three temperatures (Fig.4). The K_d is calculated to be 272 nM, 185 nM, and 120 nM at 20 ℃, 37 ℃, and 50 ℃, respectively. In addition, the increase of the temperature can decrease the fluorescence quantum yield, so the fluorescence intensity declines with the increase of the temperature, as can be seen from Fig. 4. These results correspond well to the reports by Shuttleworth et al and Dennis et al[17-19]. ΔH in these processes is calculated to be 21.16 kJ/mol by using the Van't Hoff isochore equation which demonstrates that the formation of Fura-2-Ca^{2+} complex is endothermic reaction, so the increase of the temperature makes Fura-2 more easier bind to Ca^{2+}, the Fura-2-Ca^{2+} is more hard to be resolved. However, 37 ℃ is more closed to the temperature of normal physiological conditions of the human body, so 37 ℃ is the optimal condition for determination.

3.1.2 Effect of pH on the binding of Fura-2 to Ca^{2+}

The changes of excitation spectra of Fura-2-Ca^{2+} induced by different pH are clearly observed in Fig. 5. The same phenomenon as the first set of experiments happens from the pH of 5.7 to 7.5, that the maxima excitation shifts to 340 nm and the isobestic point is at 360 nm for all pH conditions with increasing Ca^{2+}. However, under pH 8.0 the fluorescence of Fura-2 decreases with increase of Ca^{2+} while, when the concentration of Ca^{2+} is 7.20×10^{-5} mol/L, a shift of the peak obviously appeares which fluorescence intensity increases with the increasing concentration of Ca^{2+} (Fig.5(e)). Maybe at pH 8.5 serious reactions exist.

$$Ca^{2+} + 2OH^- \rightleftharpoons Ca(OH)^+ + OH^- \rightleftharpoons Ca(OH)_2 \downarrow .$$

These reactions are reversible. When the concentration of Ca^{2+} is lower, as soon as entering the solution, Ca^{2+} can react with OH^-, followed by the decrease of the fluorescence intensity and the peak location remains unchanged. When the concentration of Ca^{2+} is 7.20×10^{-5} mol/L, it has extra free Ca^{2+} to bind with Fura-2, leading to the increase of fluorescence intensity and the blue shift of the peak location. However, this mechanism remains uncertain very much and needs more deep researches to confirm.

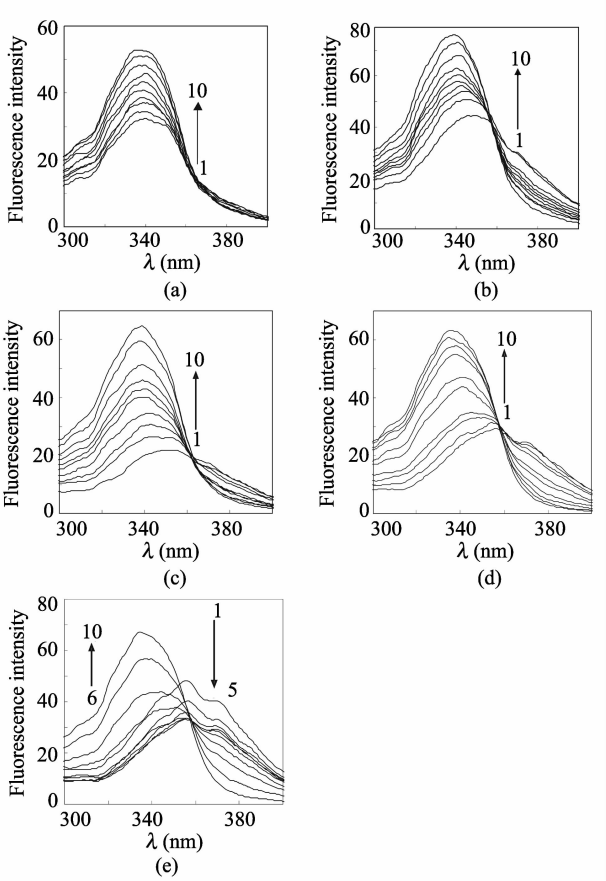

Fig.5 Effect of pH on excitation spectra of Fura-2-Ca^{2+}: (a) pH=5.05, (b) pH=6.05, (c) pH=6.56, (d) pH=7.50, (e) pH=8.05, with increasing Ca^{2+} concentrations: 0, 2.6×10^{-6}, 8.0×10^{-6}, 2.4×10^{-5}, 3.2×10^{-5}, 7.2×10^{-5}, 9.6×10^{-5}, 2.6×10^{-4}, 5.0×10^{-4}, 8.0×10^{-4} M

These results demonstrate that Fura-2 is well suited to monitoring rapid changes in $[Ca^{2+}]$ from tie pH of 5.7 to 7.5. Similarly, pH 7.4 is more closed to the normal physiological conditions, so pH 7.4 is selected to be the most suitable pH.

3.2 Intracellular Ca^{2+} fluorescence measurements

The measurements of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in Fura-2AM-loaded human peripheral lymphocytes are performed in a Varian Cary Ecliptic luminescence spectrometer. Fluorescence is measured by using a 340/380 nm excitation ratio and a 510 nm emission wavelength. The fluorescence ratio F_{340}/F_{380} is converted into $[Ca^{2+}]_i$ values by using equation (2) as Grynkiewicz et al. reported[7]

$$[Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times S_f/S_b . \quad (2)$$

Where R is the observed fluorescence ratio, R_{min} is the dye fluorescence ratio in a virtually Ca^{2+}-free medium, and

estimated by treating cells with 50 μL 0.8 M EGTA; R_{max} is the maximum fluorescence ratio of Ca^{2+}-saturated with dye, and estimated by treating cells with 50 μL of 0.1% Triton X-100. S_f/S_b is the ratio of fluorescence intensity after excitation at 380 nm, for Ca^{2+}-free medium and Ca^{2+}-saturated medium, respectively. In the experiments, the basal $[Ca^{2+}]_i$ in resting human peripheral lymphocytes is about 100 nM.

3.2.1 Effect of Cefotaxime on $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ in lymphocytes are measured in a wide range of concentrations of Cefotaxime (1～500 μM). All of the significant effects of Cefotaxime on intracellular Ca^{2+} concentration in lymphocytes are observed clearly (Fig.6). The figure shows different concentrations of Cefotaxime have different effects on the $[Ca^{2+}]_i$, assuming that Cefotaxime regularizes $[Ca^{2+}]_i$ via different mechanisms at different concentrations.

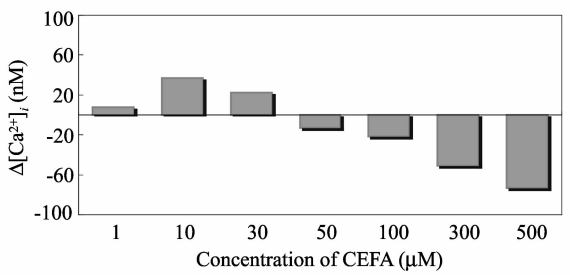

Fig.6 The effect of different concentrations of CEFA on $\Delta[Ca^{2+}]_i$ in lymphocytes

3.2.2 Effect of low CEFA concentration on $[Ca^{2+}]_i$

In the low CEFA concentration, drugs of different concentrations which lead to different extent increase of $[Ca^{2+}]_i$, among which 10 μM can cause the highest increase, followed by 30 μM and the 1 μM lowest possible because the CEFA is too little to effect on the $[Ca^{2+}]_i$. These observations demonstrate that low-concentration CEFA induces the accumulation of cellular Ca^{2+}, and as a result, Ca^{2+} is released through phosphatidylinositol transduction pathway, a cross-membrane message transduction style, because of the weak membrane infiltration ability of CEFA which can't allow the CEFA through the membrane into the inside of the cell. The process is that the $-NH_2$ of CEFA has interaction with $-COOH$ of G protein-linked receptor, the G protein-linked receptor makes PIP_2 become into IP_3 which opens the channel of ER membrane, making Ca^{2+} flux form ER, then lead to the increase $[Ca^{2+}]_i$. Meanwhile, it doesn't regularize $[Ca^{2+}]_i$ via the single mechanism, it also activates the Ca^{2+}-ATPase and membrane Ca^{2+} channels. So when the concentration of CEFA reaches to 30 μM, its increase is less than that of 10 μM CEFA. Overall, in the low-concentration CEFA, the two kinds of mechanism both exist, and the phosphatidylinositol transduction pathway is the prominent way.

3.2.3 Effect of high CEFA concentration on $[Ca^{2+}]_i$

The high-concentration CEFA (50 μM～500 μM) induces dose-dependent decreases in $[Ca^{2+}]_i$ and some restoration occurs as follows. These results suggest that high concentrations of Cefotaxime may stimulate the decline of $[Ca^{2+}]_i$ via lymphocytes membrane Ca^{2+} channels and Ca^{2+}-ATPase. The restoration may be caused by releasing Ca^{2+} from ER. The process involves various mechanisms and the membrane Ca^{2+} channels and Ca^{2+}-ATPase are the prominent ways.

4 Conclusion

The temperature and pH affect the analytical behavior of Fura-2 binding with Ca^{2+}. The temperature exhibits significant effects on K_d for Fura-2-Ca^{2+}, the increasing temperature induces the decrease of K_d, which can be explained by ΔH (21.16 kJ/mol), indicated that the formation of Fura-2-Ca^{2+} complex is endothermic reaction, the increase of temperature leads to the hard resolution of Fura-2-Ca^{2+}. Meanwhile, the ability of Fura-2 to shift its peak excitation wavelength upon binding of Ca^{2+} is not affected by the changes in the temperatures between 20 ℃ and 50 ℃. Thus, Fura-2 is a suitable tool for measurements of Ca^{2+} in a wide temperature range. However, the changes in pH affect the excitation spectra of Fura-2, hence Fura-2 provides a valuable probe into the physiological pH conditions. The effect of the temperature and pH on the interaction of Fura- and Ca^{2+} clearly recognizes the analytical behavior of Fura-2. The CEFA regulates calcium homeostasis in lymphocytes over a wide range of concentrations that may result in physiological changes during the application of CEFA as a β-lactams antibiotics in the wide cases of immune deficiency treatments.

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