

## Assessing physiological concentrations of endogenous substances *in situ* by inducing calcium oscillations *in vitro*. Case of liver<sup>1</sup>

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**KEY WORDS** calcium; hepatocytes; norepinephrine; vasopressins; adenosine triphosphate; rats

### ABSTRACT

**AIM:** To identify the physiological concentration ranges of norepinephrine (NE), vasopressin (VP), and ATP in the rat liver. **METHODS:** Rat hepatocytes were isolated by collagenase perfusion. Isolated cells were loaded with the fluorescent calcium indicator Fura-2 acetoxymethyl ester (Fura-2 AM). The effects of different concentrations of norepinephrine, vasopressin, and ATP on intracellular calcium concentration ( $[Ca^{2+}]_i$ ) increases in the freshly isolated rat hepatocytes were investigated.  $[Ca^{2+}]_i$  was measured by micro-fluorometry and recorded as fluorescence ratios ( $F_{340}/F_{380}$ ). **RESULTS:** NE, VP, and ATP induced increases in  $[Ca^{2+}]_i$  in a concentration-dependent manner. At lower concentrations,  $[Ca^{2+}]_i$  tended to show an oscillatory increase; with increasing concentrations,  $[Ca^{2+}]_i$  in more cells tended to show phasic or plateau increases. NE, VP, and ATP concentrations likely to induce an oscillatory  $[Ca^{2+}]_i$  response were 100 - 500 nmol/L, 50 - 100 pmol/L, and < 1  $\mu$ mol/L respectively. **CONCLUSION:** Physiological concentrations of NE, VP, and ATP are 100 - 500 nmol/L, 50 - 100 pmol/L, and < 1  $\mu$ mol/L respectively in the rat liver.

### INTRODUCTION

One of the most common and the earliest responses after stimulation of different cell types with neurotransmitters and hormones is an increase in the intracellular calcium concentration ( $[Ca^{2+}]_i$ ). At the single cell

level,  $[Ca^{2+}]_i$  increase occurs with the following general rule. At low or physiological concentrations, repeated  $[Ca^{2+}]_i$  spikes occur, ie,  $[Ca^{2+}]_i$  increasing to a certain peak magnitude, then decreasing to basal, pre-stimulated level, followed by subsequent identical spikes, with a certain frequency and amplitude. These repetitive spikes are also named calcium oscillations. With increasing agonist concentrations, the frequency and magnitude of the oscillations increases before repetitive spikes are gradually transformed into phasic, or plateau increase<sup>[1-3]</sup>. Based on such observations in the isolated pancreatic acinar cells and other cells, Habara and Kanno proposed the concept of physiological, pharmacological, and toxicological concentrations which, for the gut hormone cholecystokinin in the isolated pancreatic acinar cells, was < 10 pmol/L, 30 pmol/L - 1 nmol/L, and > 1 nmol/L respectively<sup>[1]</sup>. Since under physiological conditions,  $[Ca^{2+}]_i$  increases are by the form of oscillations, it should be conversely possible, to estimate the endogenous substances concentrations that cells are likely to encounter *in situ*, by determining what concentrations of these substances could induce calcium oscillations in isolated cells *in vitro*.

Therefore we used freshly isolated rat hepatocytes, which would be most closely related to the *in situ* situation in comparison with primary culture, to test a set of concentrations of norepinephrine (NE), vasopressin (VP), and ATP to estimate their possible physiological range of concentrations.

### MATERIALS AND METHODS

**Materials** Fura-2 AM was from Molecular Probes (Eugene, OR, USA). Norepinephrine,  $[Arg^8]$ -vasopressin, and adenosine 5'-triphosphate (ATP) were from Sigma (St Louis, MO, USA). Collagenase H was purchased from Boehringer Mannheim (Mannheim, Germany). Minimal Essential Medium (MEM) amino acids mixture was from GibcoBRL (Grand Island, NY, USA). Cell-Tak was obtained from Collaborative Biomedical Products (Bedford, MA, USA).

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**Isolation of rat hepatocytes** Male Sprague-Dawley rats (150 – 400 g) were obtained from either Institute of Zoology, Chinese Academy of Sciences, or Beijing Weitonglihua Experimental Animals Center (SPF, Certificate No SCXK 11-00-0008). Hepatocytes were isolated as reported previously<sup>(4)</sup>. Briefly, rat was killed by cervical dislocation, the portal vein was cannulated and perfused with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -deficient buffer (with no  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  addition, plus egtazic acid 0.5 mmol/L) at 20 mL/min for a total volume of 100 mL, followed by standard buffer ( $\text{Ca}^{2+}$  2.5 mmol/L,  $\text{Mg}^{2+}$  1.13 mmol/L) containing collagenase 0.2 g/L at 15 mL/min (total volume 70 mL). Standard buffer had the following composition (mmol/L): NaCl 118, KCl 4.7,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  2.5,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  1.13,  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  1, *D*-glucose 5.6, *N*-2-hydroxyethylpiperazine-10'- $\alpha$ -ethanesulfonic acid (HEPES) 10, bovine serum albumin (BSA) 2 g/L, MEM amino acid mixture ( $\times 50$ ), *L*-glutamine 2, sodium pyruvate 2, pH was adjusted to 7.4 with NaOH 4 mol/L. The buffer was oxygenated with 100 %  $\text{O}_2$  for half an hour before use. After perfusion with collagenase, the caudate and the right lobes were dissected out, cut into pieces with a scalpel blade and incubated in the collagenase-containing buffer (5 mL) for an additional 2 min in a shaking water-bath (37 °C). The digested tissue was dispersed through a plastic pipette, filtered through a nylon mesh (150 mesh), centrifuged and washed once with standard buffer containing BSA 3 g/L, then washed 3 more times with standard buffer.

**Measurement of  $[\text{Ca}^{2+}]_i$**  Isolated hepatocytes were loaded with Fura-2 AM at 5  $\mu\text{mol/L}$  in a shaking water-bath (50 min<sup>-1</sup>, 37 °C, 30 min). Fura-2-loaded cells were attached to Cell-Tak-treated cover-slip in a Sykus-Moore chamber. Attached cells were perfused with standard buffer at 1 mL/min on stage of an inverted Olympus fluorescence microscope (IX70) coupled to a calcium measurement system (M40, PTI). Excitation wavelengths were 340 nm/380 nm, and emitted fluorescence was measured at 510 nm. Calcium concentration was expressed as fluorescence ratios ( $F_{340}/F_{380}$ ). The high speed change of excitation wavelengths was achieved with a stepping motor driven monochrometer. Stimulating chemicals were introduced by changing the buffer, and dead-time was corrected for all recordings shown. In control experiments, it was confirmed that no spontaneous  $[\text{Ca}^{2+}]_i$  increase was observed in unstimulated hepatocytes.

## RESULTS

### Norepinephrine-induced $[\text{Ca}^{2+}]_i$ increases

NE-induced calcium concentration increases in a concentration-dependent manner. NE 50 nmol/L induced a few calcium spikes in a limited number of cells, whereas no response was observed in some other cells. The frequency of the induced calcium spikes was low (Fig 1A,  $n = 5$ ). The lack of response in some cells was probably due to a lower sensitivity of the cell to NE, because later addition of higher concentrations to the same cell induced marked calcium increase (data not shown). NE 100 nmol/L induced calcium oscillations in 8 out of 11 cells (Fig 1B), the remaining cells (3 out of 11) showed a phasic increase. Calcium oscillations occurred only after a brief period of phasic increase when NE 200 nmol/L was used (Fig 1C,  $n = 4$ ). At 500 nmol/L, NE induced calcium oscillations in 5 out of 9 cells, the other 4 showed a phasic increase (Fig 1D). At 1  $\mu\text{mol/L}$ , NE induced phasic increase in all cells, and the increase tended to be a plateau (Fig 1E,  $n = 3$ ).

### Vasopressin-induced $[\text{Ca}^{2+}]_i$ increases

VP 50 pmol/L triggered calcium oscillations in 5 out of 6 cells (Fig 2A), and even at this low concentration, one cell showed a phasic increase (data not shown). VP 100 pmol/L elicited calcium oscillations in 6 out of 10 cells (Fig 2B), the other 4 showed a phasic increase (data not shown). At 500 pmol/L, VP caused phasic increase in 5 out of 6 cells (Fig 2C) and only one cell showed calcium oscillations (data not shown). At the even higher concentration of 1 nmol/L, all cells examined showed phasic increase (Fig 2D).

### ATP-induced $[\text{Ca}^{2+}]_i$ responses

ATP-induced calcium increases similarly in a concentration-dependent manner. At lower concentrations (0.5 and 1  $\mu\text{mol/L}$ ), ATP induced oscillatory increases in 4 out of 11 cells; the other 7 cells showed phasic increase. At a higher concentration (10  $\mu\text{mol/L}$ ), calcium increase triggered by ATP was either phasic or plateau in all cells examined (Fig 3C,  $n = 4$ ).

## DISCUSSION

The study showed that at NE 100 – 500 nmol/L, most of the cells showed oscillatory response; but at NE 1  $\mu\text{mol/L}$ , all cells showed plateau increase. With VP 50 – 100 pmol/L, most cells showed oscillatory response; at 500 – 1000 pmol/L, nearly all cells showed plateau

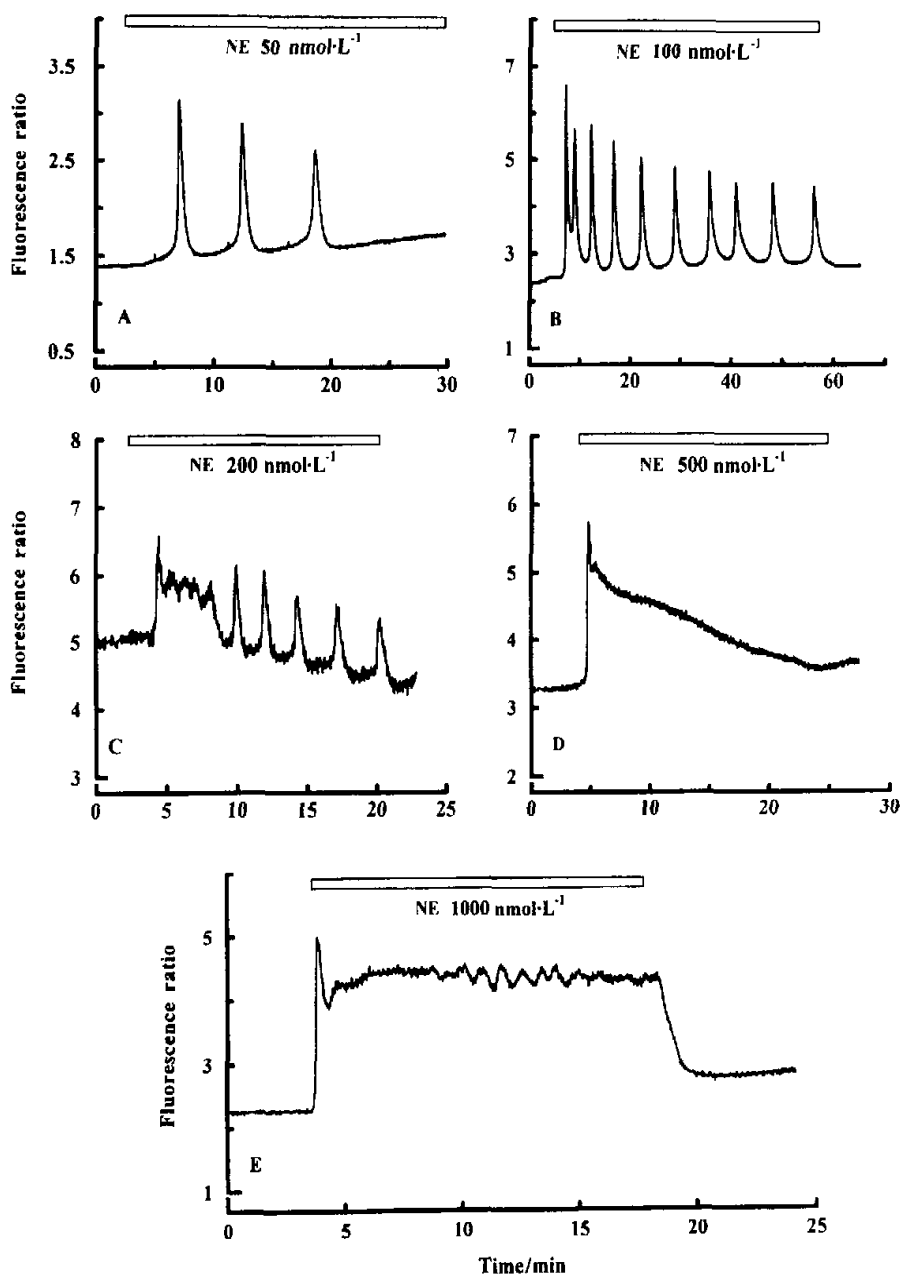


Fig 1. NE-induced increases in  $[Ca^{2+}]_i$  in the freshly isolated rat hepatocytes. NE was added as indicated by the horizontal bars. Note the concentration-dependent transformation of the different patterns of  $[Ca^{2+}]_i$  increase. Each trace is representative of a number of similar traces.

increase. With ATP  $1 \mu\text{mol/L}$ , a number of cells were oscillatory; but when ATP was increased to  $10 \mu\text{mol/L}$ , all cells showed phasic or plateau increase. Therefore NE  $100 - 500 \text{ nmol/L}$ , VP  $50 - 100 \text{ pmol/L}$ , ATP  $< 1 \mu\text{mol/L}$  induced in most cells oscillatory increases in  $[Ca^{2+}]_i$ , and these were the physiological concentrations

of NE, VP, and ATP. It was under these concentrations that the hepatocytes *in situ* were to function consistently over long periods of time.

It was obvious that hepatocytes showed some difference in the sensitivity to NE, VP, and ATP. This was consistent with works reported previously<sup>(4,5)</sup>. But

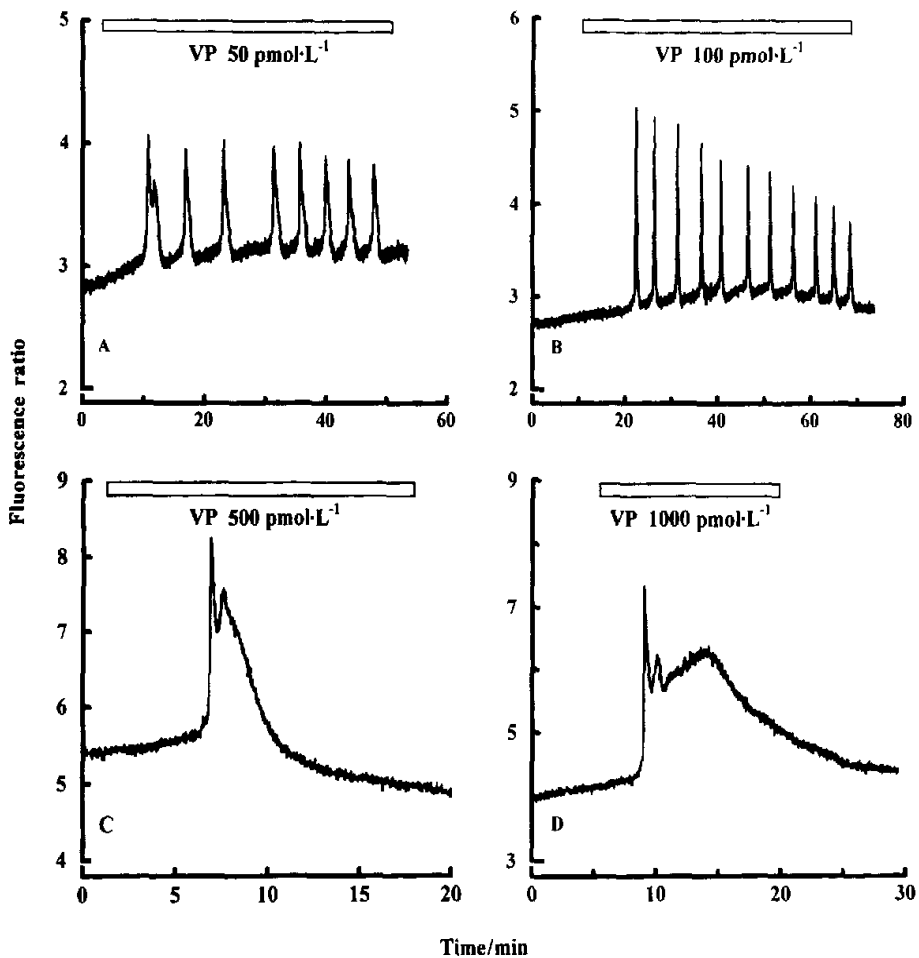


Fig 2. Vasopressin-induced increases in  $[Ca^{2+}]_i$  in the freshly isolated rat hepatocytes. Vasopressin was added as indicated by the horizontal bars. Note the concentration-dependent transformation of the different patterns of  $[Ca^{2+}]_i$  increase. Each trace is representative of a number of similar traces.

this heterogeneity was not an obstacle to our present work.

Other works reported in the literature supported the observations found in the present study. For example, Combettes *et al*<sup>[6]</sup> found that in the freshly isolated rat hepatocytes, NE 100 nmol/L induced regular calcium oscillations. It was interesting to note that with the synthetic  $\alpha$ -receptor agonist, phenylephrine, much higher concentrations were required to induce calcium oscillations in the freshly isolated rat hepatocytes, ie, 0.5 – 10  $\mu\text{mol/L}$ <sup>[7,8]</sup>. Rooney *et al*<sup>[9]</sup> reported that in the primary cultured rat hepatocytes phenylephrine 10 – 50  $\mu\text{mol/L}$  induced oscillatory increases in  $[Ca^{2+}]_i$ . From the studies above, we could easily see that after primary culture, hepatocytes became less sensitive to phenylephrine.

Combettes *et al*<sup>[6]</sup> found that in the freshly isolated rat hepatocytes VP 0.1 nmol/L induced calcium oscillation, whereas at 0.5 nmol/L, oscillations was observed over a plateau and at 10 nmol/L, a plateau increase was observed. Other workers reported that in the freshly isolated rat hepatocytes, VP 0.1 – 1.5 nmol/L induced calcium oscillations<sup>[7,8]</sup>. In the present work, 50 – 100  $\text{pmol/L}$  induced calcium oscillations, the concentration to induce phasic or plateau increase was 0.5 nmol/L. In the primary cultured rat hepatocytes, Rooney *et al*<sup>[9]</sup> reported that VP 0.5 – 1.0 nmol/L induced calcium oscillations, whereas at 3 – 5 nmol/L phasic or plateau increase was observed. Takemura *et al*<sup>[10]</sup> found that in the primary cultured rat hepatocytes VP 0.1 nmol/L had no effect, VP 1 nmol/L was required to induce calcium oscillations, whereas VP 10

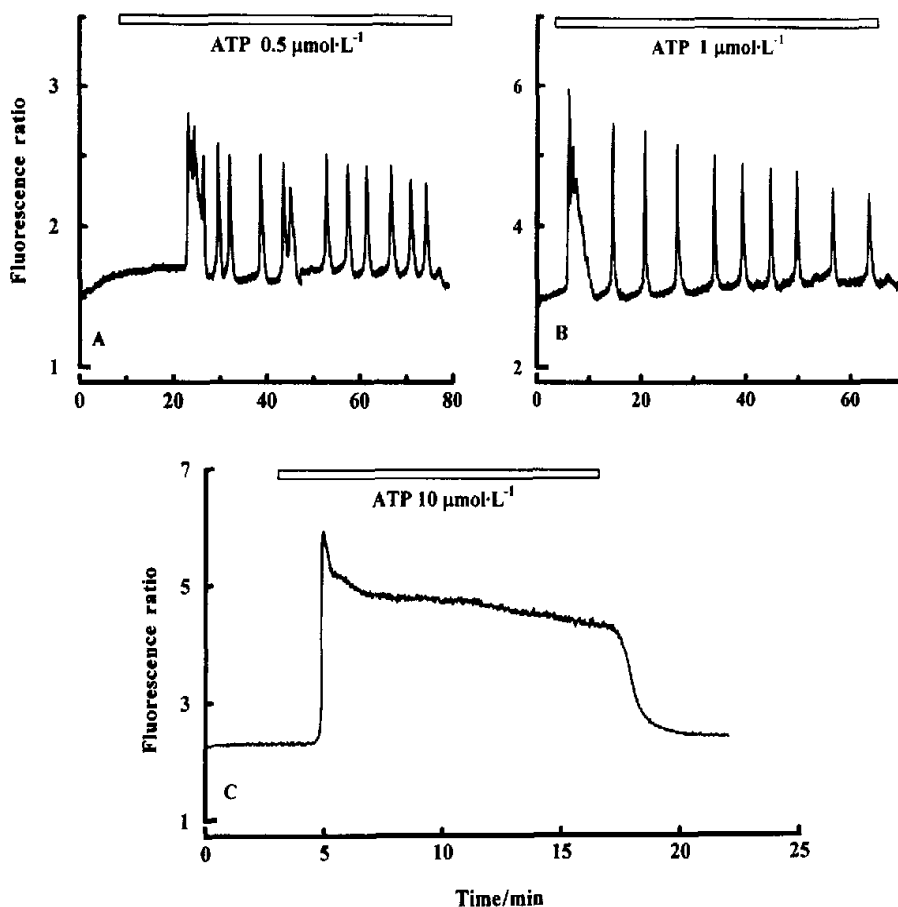


Fig 3. ATP-induced increases in  $[Ca^{2+}]_i$  in the freshly isolated rat hepatocytes. ATP was added as indicated by the horizontal bars. Note the concentration-dependent transformation of the different patterns of  $[Ca^{2+}]_i$  increase. Each trace is representative of a number of similar traces.

nmol/L induced plateau  $[Ca^{2+}]_i$  increases. Thus it could be obviously seen that primary cultured rat hepatocytes were much less responsive to VP than the freshly isolated hepatocytes.

Kummer *et al*<sup>(11)</sup> reported that in the freshly isolated rat hepatocytes ATP 1.2  $\mu\text{mol/L}$  induced calcium oscillations. Also in the freshly isolated rat hepatocytes, Kawanishi *et al*<sup>(8)</sup> found that ATP 0.5  $\mu\text{mol/L}$  induced calcium oscillations. These were similar to the concentrations obtained in the present work.

The physiological concentrations of NE, VP, and ATP that the hepatocytes were likely to encounter *in situ* were difficult to measure directly. For lack of a more suitable yardstick, NE and VP concentrations in the plasma were a useful point by which to judge the relevance of the data obtained in this work. Plasma NE concentration has been found to be in the range of 2.8 –

15 nmol/L<sup>(12-14)</sup>, and plasma VP of 1.3 – 18 pmol/L<sup>(15-19)</sup>. These indicated that the physiological concentrations of NE and VP identified in the present work were probably correct. These concentrations induced oscillatory responses which, by frequency modulation and amplitude modulation, could encode complex signals and ensure specificity of signaling.

In sum, NE 100 – 500 nmol/L, VP 50 – 100 pmol/L, ATP < 1  $\mu\text{mol/L}$  induced oscillatory increases in  $[Ca^{2+}]_i$ . These are the concentrations of NE, VP, and ATP that the hepatocytes are likely to encounter *in situ*, i.e., the physiological concentrations in the liver. Only the freshly isolated hepatocytes could be used to gauge the physiological concentrations, because primary cultured cells generally become much less sensitive to identical stimuli, possibly due to down-regulation of relevant receptors. Calcium oscillations induced *in vitro* might

also be used to identify the physiological concentrations of endogenous calcium-mobilizing molecules in other types of cells.

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利用体外诱发的钙振荡估测在体内源性物质的生理浓度。肝脏中的情况<sup>1</sup>

R96 A

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**关键词** 钙; 肝细胞; 去甲肾上腺素; 加压素; 腺苷三磷酸; 大鼠

**目的:** 研究大鼠肝中去甲肾上腺素、加压素、ATP 的生理浓度。 **方法:** 肝细胞通过胶原水解酶灌注肝脏得到。分离的肝细胞加载钙离子荧光指示探针 Fura-2, 通过显微荧光术测量胞内钙浓度的变化, 以荧光比例 ( $F_{340}/F_{380}$ ) 表示, 分别检测不同浓度范围的去甲肾上腺素、加压素、ATP 对单个肝细胞胞浆钙离子浓度升高的作用。 **结果:** 去甲肾上腺素、加压素、ATP 以剂量依赖的方式诱导胞内钙离子浓度的增加。较低浓度刺激, 胞内钙浓度呈振荡性增加; 随着刺激物浓度的增加, 更多的细胞胞内钙离子浓度呈现缓慢或高台样增加。去甲肾上腺素、加压素、ATP 诱发钙振荡的最佳浓度分别是 100-500 nmol/L、50-100 pmol/L 和  $<1 \mu\text{mol/L}$ 。 **结论:** 在大鼠肝脏中, 去甲肾上腺素、加压素、ATP 的生理性浓度分别在 100-500 nmol/L、50-100 pmol/L 和  $<1 \mu\text{mol/L}$  范围。 (责任编辑 韩向晖)