Arch Pharm Res Vol 26, No 1, 64-69, 2003

Archives of Pharmacal Research
http://apr.psk.or.kr

Analysis of Vasopressin-Induced Ca^{2+} Increase in Rat Hepatocytes

Hyun-Sook Kim, Fumikazu Okajima[1], and Dong-Soon Im

Laboratory of Pharmacology, College of Pharmacy, Pusan National University, Busan, 609-735, Korea and [1]Laboratory of Signal Transduction, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi 371, Japan

(Received October 1, 2002)

To analyze vasopressin-induced Ca^{2+} increase in liver cells, rat hepatocytes were isolated and attached to collagen-coated cover slips. Using fura-2, a Ca^{2+}-sensing dye, changes in intracellular Ca^{2+} concentration by vasopressin were monitored. Results in this communication suggested that vasopressin-induced Ca^{2+} increase were composed of both Ca^{2+} release from internal Ca^{2+} stores and influx from the plasma membrane. The Ca^{2+} influx consisted of two distinguishable components. One was dependent on the presence of vasopressin and the other was not. SK&F96365 blocked vasopressin-induced Ca^{2+} influx in a dose-dependent manner. Vasopressin-induced Ca^{2+} release from internal stores diminished in a primary culture of hepatocytes according to the culture time. However, changes in vasopressin-induced Ca^{2+} influx across the plasma membrane differed from changes in the Ca^{2+} release from internal stores, suggesting two separate signalings from receptor activation to internal stores and to the plasma membrane.

Key words: Ca^{2+} influx, Hepatocytes, Vasopressin, G protein-coupled receptor, Capacitative Ca^{2+} entry, Calcium channels, Liver

INTRODUCTION

In hepatocytes, many processes including metabolism are regulated by hormones such as vasopressin, adrenaline and angiotensin II. The binding of these hormones to their receptors triggers coupling of $G_{q/11}$ type G proteins to phospholipase C_β (Rhee, 2001). The activation of phospholipase C produces second messengers, that is, inositol 1,4,5-trisphosphate (IP_3), diacylglycerol. IP_3 opens intracellular calcium channels in the endoplasmic reticulum, resulting in transient increase of cytosolic Ca^{2+} concentration (Burgess *et al.*, 1984).

The increase of intracellular Ca^{2+} is induced by the release of Ca^{2+} from intracellular Ca^{2+} stores and by influx of Ca^{2+} across the plasma membrane. The release of Ca^{2+} from internal stores by IP_3 empties the stores. The sensation of the emptying of the Ca^{2+} stores initiates a refilling process known as capacitative Ca^{2+} entry (or store-operated Ca^{2+} entry) (Putney *et al.*, 2001). The mechanisms of capacitative Ca^{2+} entry have been studied in many cell types, and several proposals have been made. The proposed mechanisms include a diffusible factor (CIF; calcium influx factor), the exocytosis model, Ca^{2+} regulation, and conformational coupling (Putney *et al.*, 2001). In hepatocytes, it was suggested that multiple channels were involved in Ca^{2+} homeostasis, specifically, lanthanide sensitive or non-sensitive channels, cAMP-regulated or Ca^{2+}-regulated ones and Mn^{2+} permeable or non-permeable ones (Barritt, 1999; Fernando and Barritt, 1995; Lenz and Kleineke, 1997).

In this communication, vasopressin Ca^{2+} increase was studied by using short-term and long-term primary cultured rat hepatocytes. Difference between the response changes in Ca^{2+} release from intracellular Ca^{2+} stores and in Ca^{2+} influx across the plasma membrane during primary culture were observed. Two distinguishable components of vasopressin Ca^{2+} influx were also observed.

METHODS AND MATERIALS

Materials

8-Arg-vasopresssin was purchased from Sigma Chemicals; fura 2-acetoxymethyl ester and *N*-2-hydoxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) were purchased from Dojindo (Tokyo, Japan); collagenase (type I) was purchased from Wako (Tokyo, Japan). The sources of all

Correspondence to: Dong-Soon Im, Laboratory of Pharmacology, College of Pharmacy, Pusan National University, San 30, Chang-Jun-dong, Keum-Jung-gu, Busan 609-735, Korea
E-mail: imds@pusan.ac.kr

other reagents were the same as described previously (Im *et al.*, 1997).

Isolation of hepatocytes

Hepatocytes were isolated from the liver of male rats of the Wister-derived Donryu strain (200-250 g) that had been fed *ad libitum* as described previously (Im *et al.*, 1997).

Measurement of intracellular Ca^{2+} concentration

Freshly prepared hepatocytes were suspended at a density of 1.92×10^5 cells/ml in the William's E medium (pH 7.4) containing 5% fetal calf serum, 1 nM insulin, 1 nM dexamethason, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate. They were maintained at a density of 4×10^4 cells/cm^2 on plastic Petri dishes each containing a collagen-coated cover glass at 37°C under 5% CO_2 and 95% air for 1.5 h. Fura 2-acetoxymethyl ester (final concentration: 5 μM) was then added to the medium. After further incubation for 30 min, the culture medium was washed two times with and changed to the Hepes-buffered medium (pH 7.4). The cover glass was removed and fixed on a sample chamber (SC-20 model, Nihonbunko, Japan) for perfusion method measurement. The sample chamber was set on a stage of a microscope (TMD300 model, Nikon, Japan). The Hepes-buffered medium which was warmed up in a 37°C water bath coursed through a plastic tube by a peristaltic pump (PST-103 model, Iwaki, Japan) at a rate of 1 ml/min. The medium ran through the sample chamber at the same rate. Cells were stimulated with vasopressin contained in a flowing medium. Measurements of fluorescence emission at 510 nM wavelength were collected every 0.1 sec from two excitation wavelengths (340 nM and 380 nM) (CAM-230 system, Nihonbunko, Japan). The ratio of fluorescence intensities at these two wavelengths was monitored as an estimate of cytoplasmic free Ca^{2+} concentration.

Data presentation

The representative traces for $[Ca^{2+}]_i$ were chosen out of 3-5 separate experiments and shown in the Fig. 1-4.

RESULTS

In our study, hepatocytes were attached to collagen-coated cover glasses, and the extracellular media were perfused continuously. Therefore the stimulation time of vasopressin and the present time of extracellular Ca^{2+} were controllable. Vasopressin mobilized Ca^{2+} in rat hepatocytes (Fig. 1a). The intracellular Ca^{2+} concentration increased immediately and robustly. After which, the increase plateaud for several minutes and then declined slowly. The significant Ca^{2+} rise disappeared about 10 min later from 1 min perfusion of 10^{-7} M vasopresssin. However the Ca^{2+} rise by the same concentration of vasopressin lasted longer if the agonist was continuously perfused (Fig. 1c). To distinguish Ca^{2+} influx from Ca^{2+} release, vasopresssin was perfused in Ca^{2+}-free media and Ca^{2+}-containing media were perfused after the recovery of $[Ca^{2+}]_i$ to base line. The first increase

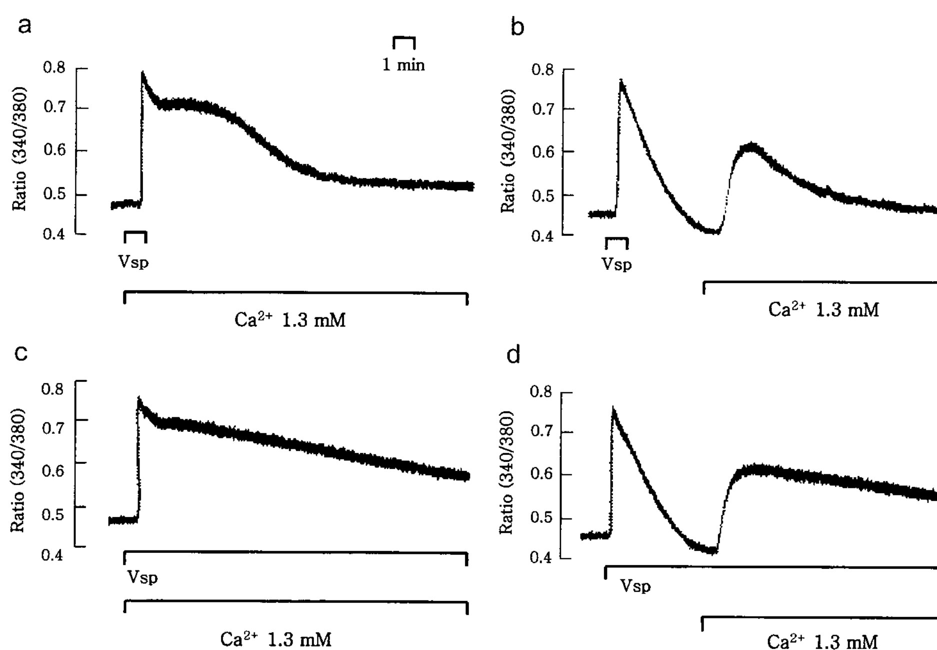

Fig. 1. Vasopressin-induced Ca^{2+} influxes in 1.5 h cultured rat hepatocytes. Vasopressin 10^{-7} M was delivered to hepatocytes for 1 min (a and b) or 15 min (c and d). Ca^{2+}-free Hepes-buffered media were perfused for 3 min before the vasopressin stimulation. 1.3 mM $CaCl_2$-containing media were perfused for the time indicated in the figures. Intracellular Ca^{2+} increases were shown by ratio changes of fluorescence at 340 nM and 380 nM.

of cytosolic Ca^{2+} concentration was observed by the release of Ca^{2+} from internal stores (Fig. 1b). The second rise was due to Ca^{2+} influx across the plasma membrane after readdition of extracellular Ca^{2+}. The Ca^{2+} influx was observed both by a one-minute stimulation and by sustained stimulation of vasopressin (Fig. 1b and 1d). The Ca^{2+} influx was observed by a one-minute stimulation of vasopressin, which quickly declined (Fig. 1b). However, the sustained perfusion of the agonist caused the Ca^{2+} influx to decrease more slowly (Fig. 1d).

These measurements indicate that the magnitude of the Ca^{2+} influx changes depend on the readdition time of extracellular Ca^{2+}. The later the extracellular Ca^{2+} was added, the smaller the Ca^{2+} influx was observed (Fig. 2b-e). Usually, capacitative Ca^{2+} influx was measured in the present of agonist by readdition of extracellular Ca^{2+} by others (Fernando and Barritt, 1994; Lenz and Kleineke, 1997). The Ca^{2+} influx observed after readdition of extracellular Ca^{2+}, that is usually thought as capacitative Ca^{2+} entry, have to be observed same magnitute independent to readdition time. However, in our measuring system, agonist was washed out by perfusion of extracellular media. Therefore we could dilute the effect of agonist stimulation by changing the readdition time. The major portion of Ca^{2+} influx observed in Ca^{2+} readdition just after the Ca^{2+} mobilization from internal stores disappeared following to the washing out (Fig. 2b-e). This observation suggests a new method to discreminate agonist-induced Ca^{2+} influx and capacitative Ca^{2+} entry. The small portion of Ca^{2+} influx observed even up to 5.5 min after vasopressin stimulation may be independent of the readdition time, implying capacitative Ca^{2+} entry (Fig. 2e). Without vasopressin stimulation, Ca^{2+} influx was not observed (Fig. 2e).

Hepatocytes are non-excitable cells; There are no voltage-dependent Ca^{2+} channels. Ca^{2+} influx was not blocked by diltiazem. Inversion of the membrane potential by 40 mM KCl in an extracellular buffer did not evoke Ca^{2+} influx in hepatocytes (data not shown). SK&F96365, a Ca^{2+} channel blocker of receptor-mediated Ca^{2+} entry in non-excitable cells, blocked the vasopressin-induced Ca^{2+} entry in hepatocytes in the previous reports (Fernando and Barritt, 1994; Lenz and Kleineke, 1997). We tested the effect of SK&F96365 on the vasopressin-induced Ca^{2+} entry in the current measuring system. It blocked the Ca^{2+} influx in a dose dependent manner (Fig. 3) (Merritt *et al.*, 1990).

Adrenergic responses in hepatocytes were switched from α-receptor dominant to β-receptor dominant during liver regeneration *in vivo* and primary culture *in vitro* (Itoh *et al.*, 1984; Kajiyama and Ui, 1994; Okajima and Ui, 1984). The decrease of α-adrenoceptor-mediated response during primary culture happened simultaneously with a total decrease of Gq protein-mediated responses, that is, vasopresssin-mediated and angiotension II-mediated ones (Kajiyama and Ui, 1994; Kajiyama and Ui, 1998). The decrease of vasopressin-mediated Ca^{2+} release from internal stores during primary culture was reproduced in this report (Fig. 4). A significant decrease was observed in 8 h-cultured hepatocytes (compare the first Ca^{2+} rise in Fig. 4b to the one in Fig. 4c). Interestingly the decrease of vasopressin-induced Ca^{2+} influx did not happen in the up to 8 h-cultured haptocytes. The magnitudes of Ca^{2+} influxes between 4 h- and 8 h-cultured hepatocytes were the same. In 24 h-cultured hepatocytes, vasopressin-induced Ca^{2+} influx was significantly diminished. The responses to sphingosine 1-

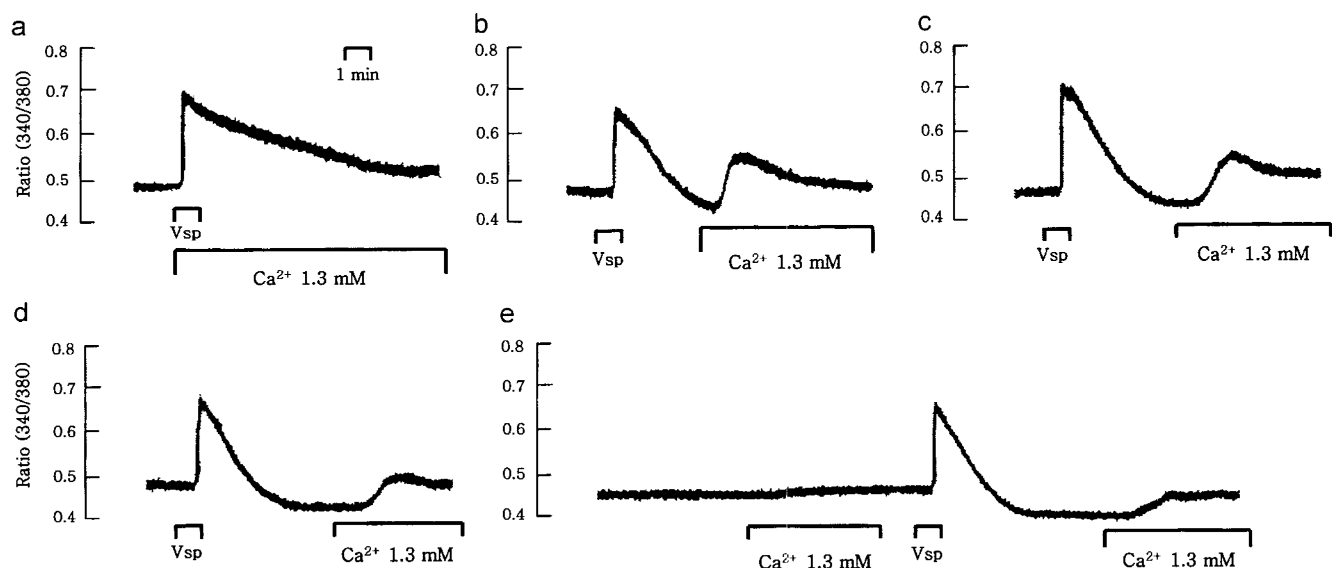

Fig. 2. Decrease of Ca^{2+} influx depending on the addition time. Vasopressin 10^{-7} M was delivered to hepatocytes for 1 min as indicated in the figure. Calcium-containing media were added 2.5(b), 3.5(c), 4.5(d), and 5.5(e) min later from the vasopressin stimulation.

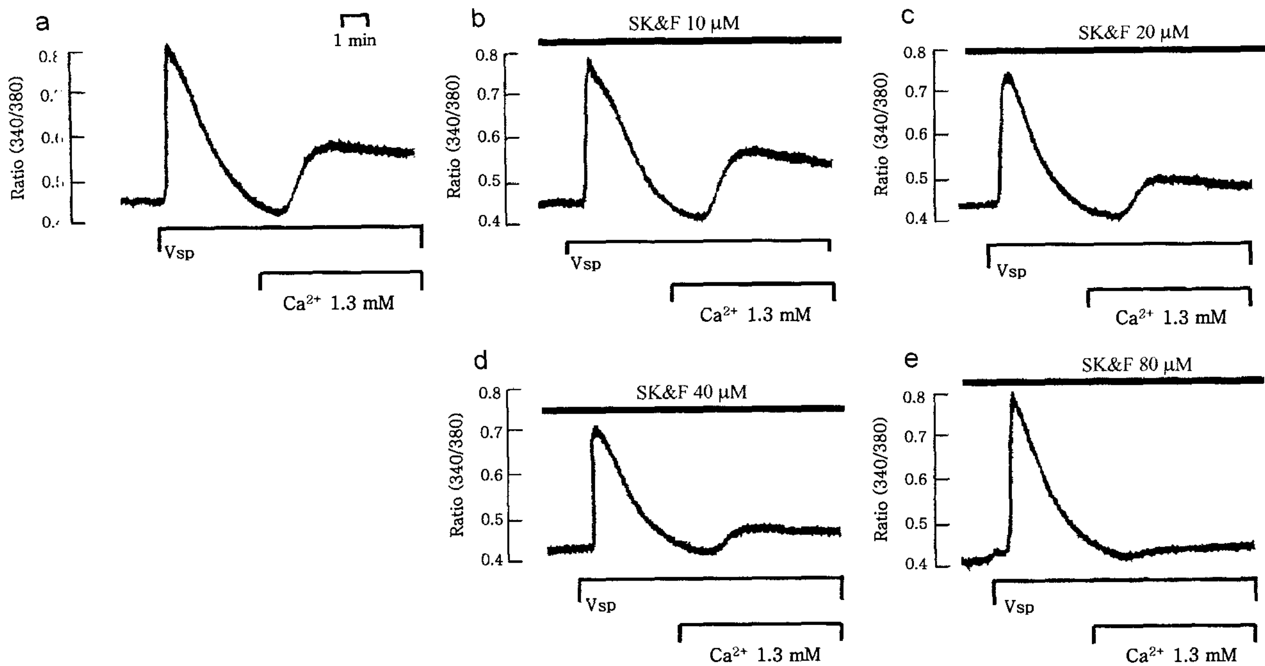

Fig. 3. SK&F96365 inhibition of vasopressin Ca^{2+} influx. Vasopressin and extracellular Ca^{2+} presence were indicated in the figure. SK&F96365 compound were present throughout the measurements at the concentration of 10 μM (b), 20 μM (c), 40 μM (d) and 80 μM (e).

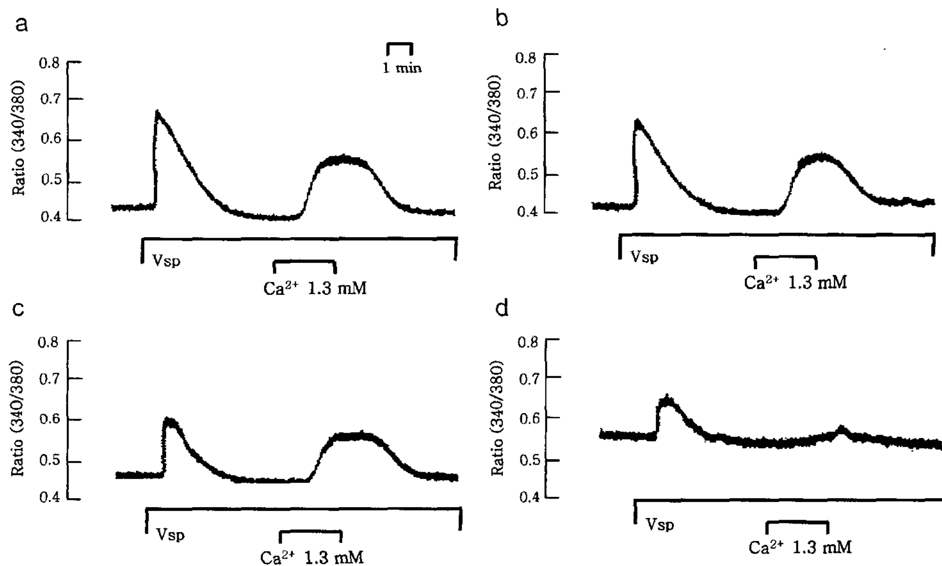

Fig. 4. Changes of Ca^{2+} release and Ca^{2+}influx during primary culture. Vasopressin and extracellular Ca^{2+} were delivered as indicated in the figure. The cultured hepatocytes for 1.5 (a), 4 h (b), 8 h (c), and 24 h (d) were used for the Ca^{2+} measurement.

phosphate, including Ca^{2+} increase, were enhanced during the primary culture, suggesting that 24 h-cultured hepatocytes are healthy and intact in terms of responsiveness (Im *et al.*, 1997).

DISCUSSION

The major findings from this research on vasopressin-induced Ca^{2+} increase, which used rat hepatocytes attached to cover glasses by delivering agonist in the flowing media, are three fold: First, the Ca^{2+} influx was two components. That is, one is dependent on the agonist presence. The other is not. Second, the Ca^{2+} influx was blocked by SK&F96365. Third, the release of Ca^{2+} from internal stores and the influx of Ca^{2+} across the plasma membrane by vasopressin are modulated differently during the primary culture of hepatocytes.

The sensation of emptying of the Ca^{2+} stores is a trigger

signal for the Ca^{2+} influx (Barritt, 1999; Putney *et al.*, 2001). The precise mechanisms are not known yet. In this study, vasopressin was delivered by perfusion. After one-min stimulation of vasopressin, vasopressin in the extracellular media was washed out. The washing effect was observed clearly when the Ca^{2+} rise caused by the one-minute stimulation was compared to the rise caused by sustained stimulation. The dependence of the Ca^{2+} influx on the readdition time might mean that this portion of the Ca^{2+} influx was induced by the presence of vasopressin in the extracellular media. Another possible explanation would be that a diffusible factor like CIF was involved (Randriamampita and Tsien, 1993). That is, if a diffusible factor, which mediated Ca^{2+} influx, was present transiently and disappeared over time, the Ca^{2+} influx would decrease depending on the readdition time. The present study, however, was not able to distinguish between the two possibilities. The small portion of the Ca^{2+} influx independent of the readdition time was interesting. This result might imply that the signal for this portion of the Ca^{2+} influx is present persistently until the influx occurs. That is, the trigger signal might be negatively regulated by the Ca^{2+} influx. One of the models for capacitative Ca^{2+} entry is the conformational change of the IP_3 receptor, which couples to the capacitative Ca^{2+} channel in the plasma membrane (Berridge, 1995; Boulay *et al.*, 1999; Irvine, 1990; Kiselyov *et al.*, 1999; Kiselyov *et al.*, 1998; Zubov *et al.*, 1999). When the Ca^{2+} store is empty, IP_3 receptor changes its conformation and triggers the channel opening. Then, if the store is refilled, the conformation of the IP_3 receptor reverses and the channels are closed (Berridge, 1995; Boulay *et al.*, 1999; Irvine, 1990; Kiselyov *et al.*, 1999; Kiselyov *et al.*, 1998; Zubov *et al.*, 1999). Considering the small portion of Ca^{2+} entry in this study, together with the conformational change model, the small portion of Ca^{2+} entry could be capacitative Ca^{2+} entry.

Another observation was SK&F96365 inhibition of Ca^{2+} influx. The inhibition of Ca^{2+} influx in hepatocytes by SK&F96365 has been reported previously in a different measuring system (Fernando and Barritt, 1994). The major portion of the Ca^{2+} influx, which is dependent on the readdition time was mediated by SKF96365-sensitive Ca^{2+} channels. The small portion of the Ca^{2+} influx independent of the readdition time was also blocked by the high concentration of SK&F96365.

It is well known that the liver regenerates after 70% hepatectomy. The experimental model system of liver regeneration was the primary culture of hepatocytes, because, during the primary culture, hepatocytes move from a differentiated state to a proliferating state (Okajima and Ui, 1984). One of the phenomena was the switching of adrenergic responses from α-receptor one to β-receptor one (Itoh *et al.*, 1984; Kajiyama and Ui, 1994; Okajima and Ui, 1984). Ui and his colleagues demonstrated that not only α-adrenoceptor-mediated signaling but also other Gq protein-coupled receptor-mediated signaling weakened during the primary culture (Kajiyama and Ui, 1994; Kajiyama and Ui, 1998). In our study, the decrease of Ca^{2+} release from internal stores by vasopressin was observed from 8 h-cultured hepatocytes. However, the vasopressin Ca^{2+} influx did not diminish in the 8 h-cultured hepatocytes. This implies that the signaling cascade for the Ca^{2+} influx is different to the signaling for the Ca^{2+} release. Despite the fact that the Ca^{2+} release by vasopressin decreased, the vasopressin Ca^{2+} influx was intact, suggesting that the signal for the influx originated from the vasopressin receptor activation contributed mainly and the signal from the sensation of the Ca^{2+} stores emptying contributed neglitably. As described above, the small portion of Ca^{2+} entry might be the capacitative Ca^{2+} entry triggered by store emptying. This observation, taken together with the dependence of Ca^{2+} influx on the readdition time, indicates that vasopressin Ca^{2+} influx in hepatocytes may be induced by receptor activation via unknown mechanisms, which are temporally present or need sustained receptor occupancy by vasopressin.

ACKNOWLEDGEMENT

This work was supported by the Pusan National University Research Grant.

REFERENCES

Barritt, G. J., Receptor-activated Ca2+ inflow in animal cells: a variety of pathways tailored to meet different intracellular Ca2+ signalling requirements. *Biochem. J.*, 337 (Pt 2), 153-169 (1999).

Berridge, M. J., Capacitative calcium entry. *Biochem. J.*, 312 (Pt 1), 1-11 (1995).

Boulay, G., Brown, D. M., Qin, N., Jiang, M., Dietrich, A., Zhu, M. X., Chen, Z., Birnbaumer, M., Mikoshiba K., Birnbaumer, L., Modulation of Ca(2+) entry by polypeptides of the inositol 1,4,5-trisphosphate receptor (IP3R) that bind transient receptor potential (TRP): evidence for roles of TRP and IP3R in store depletion-activated Ca(2+) entry. *Proc. Natl. Acad. Sci. USA*, 96, 14955-14960 (1999).

Burgess, G. M., Godfrey, P. P., McKinney, J. S., Berridge, M. J., Irvine, R. F., Putney, J. W., Jr., The second messenger linking receptor activation to internal Ca release in liver. *Nature*, 309, 63-66 (1984).

Fernando, K. C., Barritt, G. J., Characterisation of the inhibition of the hepatocyte receptor-activated Ca2+ inflow system by gadolinium and SK&F 96365. *Biochim. Biophys. Acta*, 1222, 383-389 (1994).

Fernando, K. C., Barritt, G. J., Characterisation of the divalent cation channels of the hepatocyte plasma membrane

receptor-activated Ca2+ inflow system using lanthanide ions. *Biochim. Biophys. Acta*, 1268, 97-106 (1995).

Im, D. S., Fujioka, T., Katada, T., Kondo, Y., Ui, M., Okajima, F., Characterization of sphingosine 1-phosphate-induced actions and its signaling pathways in rat hepatocytes. *Am. J. Physiol.*, 272, G1091-1099 (1997).

Irvine, R. F., 'Quantal' Ca2+ release and the control of Ca2+ entry by inositol phosphates--a possible mechanism. *FEBS Lett.*, 263, 5-9 (1990).

Itoh, H., Okajima, F., Ui, M., Conversion of adrenergic mechanism from an alpha- to a beta-type during primary culture of rat hepatocytes. Accompanying decreases in the function of the inhibitory guanine nucleotide regulatory component of adenylate cyclase identified as the substrate of islet-activating protein. *J. Biol. Chem.*, 259, 15464-15473 (1984).

Kajiyama, Y., Ui, M., Switching from alpha 1- to beta-subtypes in adrenergic response during primary culture of adult-rat hepatocytes as affected by the cell-to-cell interaction through plasma membranes. *Biochem. J.*, 303 (Pt 1), 313-321 (1994).

Kajiyama. Y., Ui. M., Differential mitogenic actions of alpha 1- and beta-adrenergic agonists on rat hepatocytes. *Cell Signal*, 10, 241-251 (1998).

Kiselyov K., Mignery, G. A., Zhu, M. X., Muallem, S., The N-terminal domain of the IP3 receptor gates store-operated hTrp3 channels. *Mol. Cell*, 4, 423-429 (1999).

Kiselyov K., Xu, X., Mozhayeva, G., Kuo, T., Pessah, I., Mignery, G. Zhu, X., Birnbaumer, L., Muallem, S., Functional interaction between InsP3 receptors and store-operated Htrp3 channels. *Nature*, 396, 478-482 (1998).

Lenz, T., Kleineke, J. W., Hormone-induced rise in cytosolic Ca2+ in axolotl hepatocytes: properties of the Ca2+ influx channel. *Am. J. Physiol.*, 273, C1526-1532 (1997).

Merritt, J. E., Armstrong, W. P., Benham, C. D., Hallam, T. J., Jacob, R., Jaxa-Chamiec, A., Leigh, B. K., McCarthy, S. A., Moores, K. E., Rink, T. J., SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem. J.*, 271, 515-522 (1990).

Okajima, F., Ui, M., Predominance of beta-adrenergic over alpha-adrenergic receptor functions involved in phosphorylase activation in liver cells of cholestatic rats. *Arch. Biochem. Biophys.*, 230, 640-651 (1984).

Putney, J. W., Jr., Broad, L. M., Braun, F. J., Lievremont, J. P., Bird, G. S., Mechanisms of capacitative calcium entry. *J. Cell Sci.*, 114, 2223-2229 (2001).

Randriamampita, C., Tsien, R. Y., Emptying of intracellular Ca2+ stores releases a novel small messenger that stimulates Ca2+ influx. *Nature*, 364, 809-814 (1993).

Rhee, S. G., Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.*, 70, 281-312 (2001).

Zubov, A. I., Kaznacheeva, E. V., Nikolaev, A. V., Alexeenko, V. A., Kiselyov, K., Muallem, S., Mozhayeva, G. N., Regulation of the miniature plasma membrane Ca(2+) channel I(min) by inositol 1,4,5-trisphosphate receptors. *J. Biol. Chem.*, 274, 25983-25985 (1999).