Volume 309, number 2, 180–184 FEBS 11499 September 1992

Antibody to the inositol trisphosphate receptor blocks thimerosal-enhanced Ca^{2+}-induced Ca^{2+} release and Ca^{2+} oscillations in hamster eggs

Shun-ichi Miyazaki[a], Hideki Shirakawa[a], Ken Nakada[a], Yoshiko Honda[a], Michisuke Yuzaki[b], Shinji Nakade[c] and Katsuhiko Mikoshiba[d]

[a]*Department of Physiology, Tokyo Women's Medical College, Kawada-cho, Shinjuku-ku, Tokyo, Japan,* [b]*Department of Biochemistry, Jichi Medical School, Tochigi-ken, Japan,* [c]*Division of Regulation of Macromolecular Function, Institute for Protein Research, Osaka University, Suita, Japan* and [d]*Department of Molecular Neurobiology, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo, Japan*

Received 13 June 1992

The sulfhydryl reagent thimerosal enhanced the sensitivity of hamster eggs to injected inositol 1,4,5-trisphosphate ($InsP_3$) or Ca^{2+} to generate regenerative Ca^{2+} release from intracellular pools. A monoclonal antibody (mAb) to the $InsP_3$ receptor blocked both the $InsP_3$-induced Ca^{2+} release (IICR) and Ca^{2+}-induced Ca^{2+} release (CICR). The mAb also blocked Ca^{2+} oscillations induced by thimerosal. The results indicate that thimerosal enhances IICR sensitized by cytosolic Ca^{2+}, but not CICR from $InsP_3$-insensitive pools, and causes repetitive Ca^{2+} releases from $InsP_3$-sensitive pools.

Sulfhydryl reagent; Anti-$InsP_3$ receptor antibody; Ca^{2+}-induced Ca^{2+} release; $InsP_3$-induced Ca^{2+} release; Ca^{2+} oscillation; Hamster egg

1. INTRODUCTION

Intracellular Ca^{2+} release is a key mechanism for Ca^{2+}-dependent cellular processes in a wide variety of cells. Two major types of the mechanism are well known; IICR from $InsP_3$-sensitive Ca^{2+} pools mediated by the $InsP_3$ receptor/channel [1,2] and CICR from $InsP_3$-insensitive pools mediated by the ryanodine receptor/channel [3]. However, direct identification or clear discrimination of these mechanisms in intact non-muscle cells has been difficult, because precisely specific and potent pharmacological agents were not available. In muscle cells, ryanodine receptor-mediated CICR from the sarcoplasmic reticulum (SR) is sensitized by caffeine [4] and channels are open-locked [5] or blocked [6] by ryanodine. In hamster eggs, Ca^{2+} release is induced by injection or Ca^{2+} [7], but this CICR is caffeine- and ryanodine-insensitive [8] and is sensitized by thimerosal instead of caffeine [9]. Sulfhydryl (SH) reagents that oxidize the protein's SH groups can cause Ca^{2+} release [10] and an SH reagent-reactive 106 kDa CICR channel was identified from SR [11]. Thimerosal, therefore, is used for detecting CICR in caffeine-insensitive cells [12].

Moreover, thimerosal induces Ca^{2+} oscillations [9] similar to sperm-induced ones [13] in hamster eggs. Ca^{2+} oscillations occur in various cells in response to biological activators and several models have been proposed for the mechanisms [14]. A two-Ca^{2+} pool model [15,16] explains Ca^{2+} oscillations as a result of repeated CICR from $InsP_3$-insensitive pools which sequester Ca^{2+} ions mobilized from $InsP_3$-sensitive pools. In contrast, there are recent reports showing that SH reagents sensitize IICR [17,18] and can cause spontaneous Ca^{2+} release in permeabilized hepatocytes under the condition where only IICR is allowed to occur [17]. Furthermore, since a slight increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) can sensitize $InsP_3$ receptor/channels [19], CICR may be indistinguishable from Ca^{2+}-sensitized IICR.

We recently found that one of the mAbs to the $InsP_3$ receptor, 18A10, which recognizes the epitope close to the Ca^{2+} channel region at the COOH terminus of the receptor protein [20], is a highly useful tool as a specific functional inhibitor of IICR, as demonstrated in cerebellar microsomes [21] and hamster eggs [8]. This mAb completely blocks Ca^{2+} waves and Ca^{2+} oscillations at fertilization of hamster eggs [8]. In the present study, using 18A10, we examined which type of Ca^{2+} release is enhanced by thimerosal and is responsible for Ca^{2+} oscillations in hamster eggs. We found that it is IICR.

2. MATERIALS AND METHODS

Mature hamster eggs freed from the surrounding zona pellucida were used, as in previous works [13]. Eggs were transferred to a drop of modified Krebs-Ringer solution in a dish placed on an inverted microscope. The mAb 18A10, which was prepared as described previously [1], was injected into eggs through a micropipette by air pressure,

Correspondence address: S. Miyazaki, Department of Physiology, Tokyo Women's Medical College, 8-1 Kawada-cho, Shinjuku-ku, Tokyo, 162, Japan. Fax: (81) (3) 5296 7414.

Published by Elsevier Science Publishers B.V.

together with the Ca^{2+}-sensitive dye fura 2 (Molecular Probes Inc., OR, USA). Another mAb, 4C11, which recognizes the NH_2 terminus of the $InsP_3$ receptor [20], was used as a control. The volume injected was estimated from fluorescence of fura 2. Fifty to ninety minutes later, thimerosal (sodium ethylmercurithiosalicylate) was applied to the medium and D-myo-$InsP_3$ (Boehringer Mannheim Biochemicals, Mannheim, Germany) or Ca^{2+} was injected into the egg through a pipette by current pulses. Relative doses of $InsP_3$ or Ca^{2+} were represented by the magnitude of injection pulses (nA × s). For measurement of $[Ca^{2+}]_i$, images with UV light of 340 nm wave length (F_{340}) (practically, through a narrow band-pass filter of 340 ± 10 nm) were accumulated during 0.5 s intervals every 2 or 5 s (4 or 10 s in some cases) using an image processor (Argus-100, Hamamatsu Photonics, Hamamatsu, Japan). Images with 360 nm light (F_{360}) were taken before and after the record of F_{340}, and data were processed to calculate the ratio F_{340}/F_{360}, assuming a linear degradation of F_{340}. Spatial distribution of $[Ca^{2+}]_i$ was analysed at three areas in the egg (inset of Fig. 3). Experiments were done at 32°C. Further details have been described elsewhere [8].

3. RESULTS

Thimerosal causes Ca^{2+} oscillations (Fig. 1), as have been demonstrated by hyperpolarizing responses in the membrane potential [9]. Upon application of thimerosal (final concentration, 200 μM), a gradual increase in $[Ca^{2+}]_i$ began 2–3 min later. The rate of rise was progressively enhanced and then the first Ca^{2+} transient (referred to as 'spike' below) was generated 4–6 min later (Fig. 1a, solid line). The spike reached the peak of 500–700 nM and decayed to a slightly higher level than the original basal level. $[Ca^{2+}]_i$ gradually increased again, leading to the next spike. The interval between spikes was about 5 min and fairly constant. Each Ca^{2+} spike

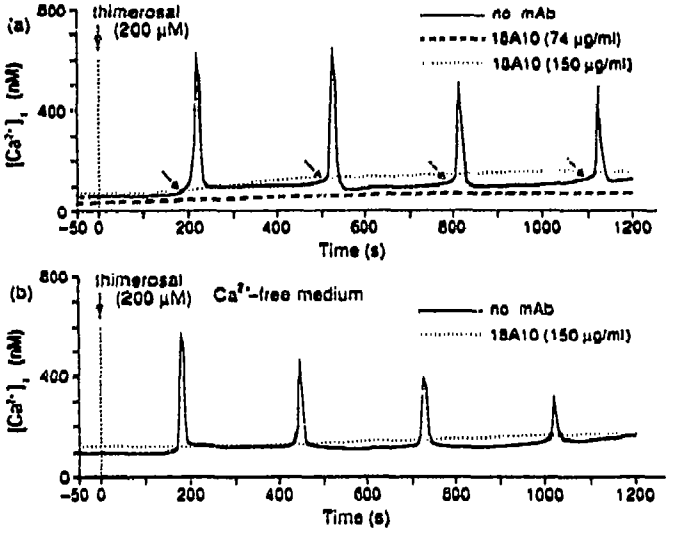

Fig. 1. Thimerosal-induced Ca^{2+} oscillations in unfertilized hamster eggs and block of the Ca^{2+} oscillations by 18A10 in normal medium (a) and Ca^{2+}-free medium (b). Thimerosal was applied to the bathing medium at the arrow (zero time). $[Ca^{2+}]_i$ was averaged in the whole egg. Real peak $[Ca^{2+}]_i$ of spikes might be missed during the sampling interval of 5 s (a) or 10 s (b) used for long-term recording of $[Ca^{2+}]_i$ with imaging.

was preceded by an 'augmenting Ca^{2+} rise' (thin arrows in Fig. 1a) and their transition occurs at $[Ca^{2+}]_i$ between 100 and 150 nM. Ca^{2+} spikes progressively declined in amplitude and disappeared within 20 min in most cases. The basal $[Ca^{2+}]_i$ became gradually higher. All these patterns of the rise in $[Ca^{2+}]_i$ were observed in Ca^{2+}-free (plus 1 mM EGTA) medium (Fig. 1b, solid line), indicating that Ca^{2+} oscillations are due to intracellular Ca^{2+} release.

The thimerosal-induced Ca^{2+} oscillations were completely blocked in eggs injected with 18A10 (70–170 μg/ml, n=30) (Fig. 1a) whereas 4C11 had no inhibitory effect (150–270 μg/ml, n=11) (data not shown). In 18A10-treated eggs, basal $[Ca^{2+}]_i$ gradually increased without any oscillation in the presence of thimerosal. The magnitude was variable from cell to cell without relation to the dose of 18A10; in some cells the elevated $[Ca^{2+}]_i$ level was about 100 nM (Fig. 1a, broken line) and in some other cells it was even higher than the level at which Ca^{2+} spikes were generated in mAb-untreated eggs (Fig. 1a, dotted line). The elevation of basal $[Ca^{2+}]_i$ was recognized in Ca^{2+}-free medium (Fig. 1b, dotted line).

We have previously shown that injection of $InsP_3$ into hamster eggs induces regenerative, propagating Ca^{2+} release [8]. The threshold injection pulse to elicit the regenerative response was 1 nA × 1 s in control eggs (Fig.2a) (0.9–1.2 nA × 1 s, n=15). After the regenerative Ca^{2+} release, the response to the same injection pulse was much smaller and gradually increased with time (Fig. 2a). It takes more than 2 min to produce a full response after the previous $InsP_3$ injection, probably corresponding to the time for refilling Ca^{2+} pools [22]. When $InsP_3$ was injected immediately after a spontaneous Ca^{2+} spike induced by thimerosal (filled circle in Fig. 2b), the Ca^{2+} rise was quite small even with 3 nA × 1 s pulses. It is probable that IICR is involved in the spike and IICR pools have been almost empty. In contrast, when $InsP_3$ was injected about 2 min after the spontaneous spike (before the next augmenting Ca^{2+} rise appeared), an injection pulse of only 0.2 nA × 1 s was enough to induce regenerative Ca^{2+} release (n=5) (open circle in Fig. 2b) and the rise in $[Ca^{2+}]_i$ was greater than in control medium. Thus, IICR is remarkably sensitized by thimerosal. After the $InsP_3$-evoked Ca^{2+} spike, the Ca^{2+} rise in response to a 3 nA × 1 s pulse became much smaller again (Fig. 2b, right). In eggs injected with 18A10 (150 μg/ml per egg), the Ca^{2+} rise with a 3 nA × 1 s pulse was almost blocked and even a 5 nA pulse caused a quite small response (Fig. 2c), indicating that thimerosal-enhanced IICR is mediated by the $InsP_3$ receptor. The mAb 4C11 (200–270 μg/ml) did not inhibit thimerosal-enhanced IICR (n=6).

Injection of Ca^{2+} causes a non-linearly augmented increase in $[Ca^{2+}]_i$ [7,8]. The critical injection pulse of Ca^{2+} was 1–1.5 nA × 2 s in control eggs (n=12). In Fig. 3, Ca^{2+} was injected at the left margin of the egg and

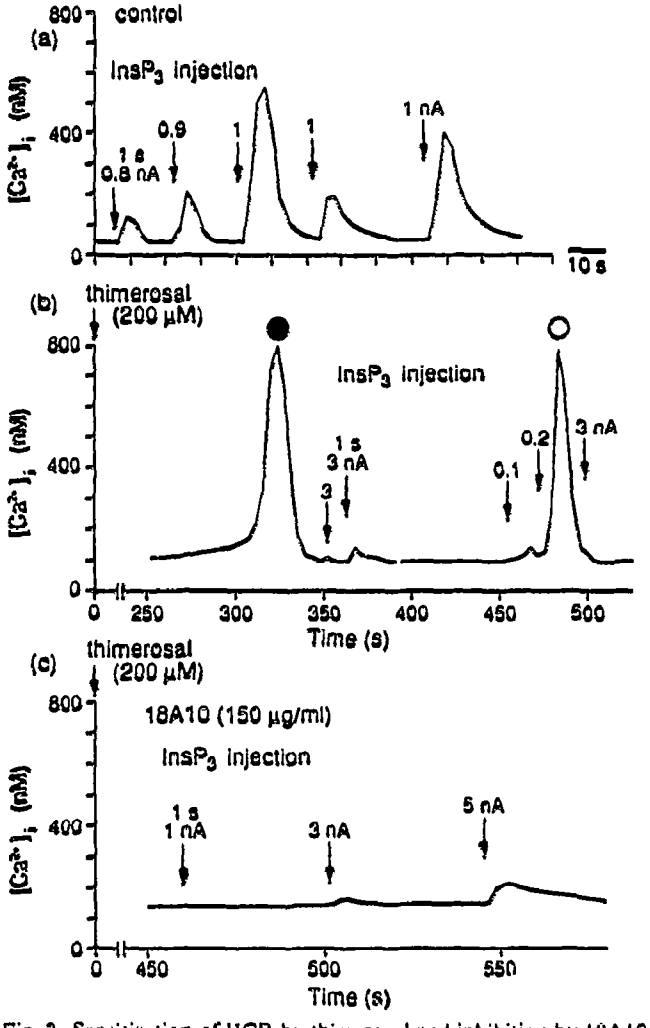

Fig. 2. Sensitization of IICR by thimerosal and inhibition by 18A10. (a) $InsP_3$ injections (at arrows) into a control egg with negative current pulses indicated. (b) $InsP_3$ injections immediately after a spontaneous Ca^{2+} spike (filled circle) and 2 min after the spike. Thimerosal was applied at the zero time. (c) $InsP_3$ injections into an 18A10-treated egg 5 min after application of thimerosal. $[Ca^{2+}]_i$ was averaged in the whole egg. To avoid leakage of $InsP_3$ (190 μM in the pipette), bucking current (positive DC current of 1.2 nA) was continuously applied except when $InsP_3$ was injected. Sampling interval of $[Ca^{2+}]_i$ measurement was 2 s in (a) and (c) and 4 s in (b).

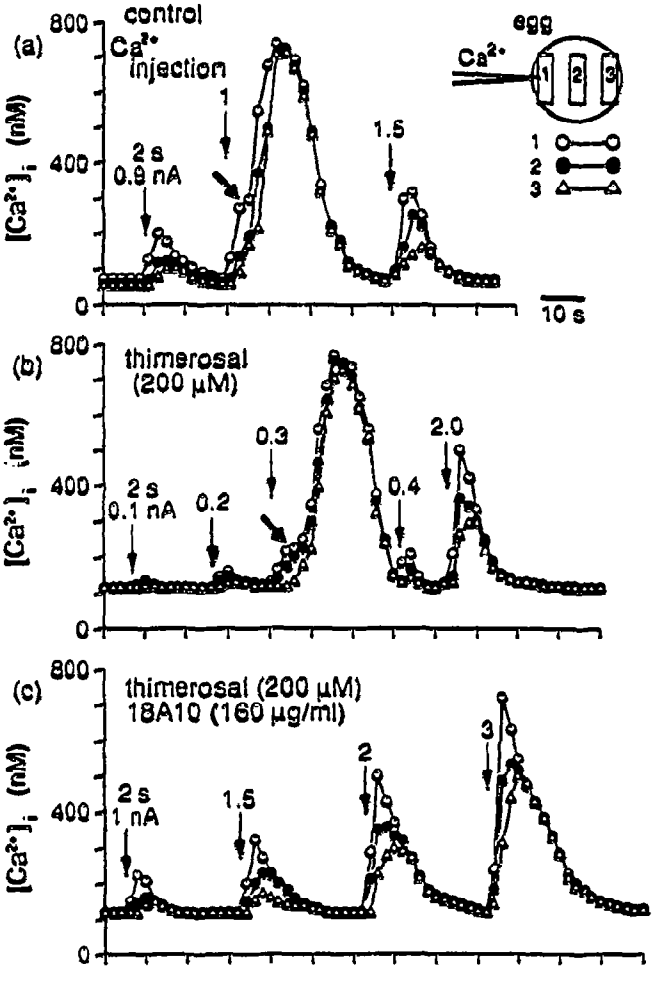

Fig. 3. The rise in $[Ca^{2+}]_i$ upon injection of Ca^{2+} in the absence of thimerosal and mAb (a), in the presence of thimerosal (b) and in the presence of thimerosal and 18A10 (c). Detailed explanations are in the text. Inset shows positions of the injection pipette and areas in which $[Ca^{2+}]_i$ was measured, together with symbols in the graph. To avoid leakage of Ca^{2+} (20 mM $CaCl_2$ in the pipette), negative DC current of 1.2 nA was continuously applied except when Ca^{2+} was injected.

$[Ca^{2+}]_i$ was measured at the injection site, center and opposite side (see inset). With a subthreshold pulse (0.9 nA × 2 s), the rise in $[Ca^{2+}]_i$ was the largest at the injection site (Fig. 3a); injected Ca^{2+} ions probably diffuse toward the opposite side. With the threshold pulse (1 nA × 2 s), peak $[Ca^{2+}]_i$ reached 700 nM in the whole egg. An inflexion was seen at about 300 nM in the rising phase of $[Ca^{2+}]_i$ at the injection site (thick arrow in Fig. 3a), apparently corresponding to the threshold $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ increased 4 s later at the opposite side of the egg than at the injection site whereas peak $[Ca^{2+}]_i$ was identical (Fig. 3a), indicating that the Ca^{2+} rise is due to propagating Ca^{2+} release rather than diffusion of Ca^{2+}.

After the regenerative Ca^{2+} release, the same or even greater injection of Ca^{2+} failed to induce the next regenerative response. This refractory period is about 2 min, when examined in hyper-polarizing responses [7]. These findings indicate CICR.

Clear sensitization of CICR by thimerosal was found, when Ca^{2+} was injected at the interval between spontaneous Ca^{2+} spikes in the presence of thimerosal. The regenerative Ca^{2+} release was produced by 0.1–0.3 nA × 2 s pulses (n=5) (0.3 nA in Fig. 3b) and the apparent threshold $[Ca^{2+}]_i$ was lower than in control eggs (about 200 nM in Fig. 3b, thick arrow).

This thimerosal-enhanced CICR was blocked by 18A10 (120–160 μg/ml, n=8). As shown in Fig. 3c, peak $[Ca^{2+}]_i$ was always highest at the Ca^{2+} injection site and increased with increasing doses of Ca^{2+} in an approxi-

mately linear manner, up to 700 nM at the injection site; the rise in $[Ca^{2+}]_i$ is due to injected Ca^{2+} per se and its diffusion. Thus, the apparent CICR is mediated by the $InsP_3$ receptor.

4. DISCUSSION

We demonstrated here that 18A10 blocksboththimerosal-induced Ca^{2+} oscillations and thimerosal-enhanced CICR as well as IICR in hamster eggs. The specificity of 18A10 has been ascertained by immunostaining of blotted proteins from hamster eggs, showing that 18A10 reacts with a single band of the 250 kDa $InsP_3$ receptor protein [8] without cross-reaction with the 106 kDa CICR channel. If it is present in hamster eggs. The ryanodine receptor is not detected in hamster eggs with an antibody to the ryanodune receptor [8]. 18A10 reacts with the epitope close to the Ca^{2+} channel region [21] and suppresses Ca^{2+} release induced by the injection of $InsP_3$ in a non-competative manner [8]. Therefore, thimerosal-enhanced Ca^{2+} release is IICR (from $InsP_3$-sensitive pools), but not CICR from $InsP_3$-insensitive pools.

The apparent CICR is thought to be Ca^{2+}-sensitized IICR. Is has been shown that IICR in skinned smooth muscle fibers is enhanched by increasing $[Ca^{2+}]_i$ between 0 and 300 nM and is inhibited by $[Ca^{2+}])_i$ higher than 300 nM [23]. The similar effect has been found in microsomal vesicles [24] and the maximum probability of opening of $InsP_3$-gated Ca^{2+} channels in lipid bilayers occurs at $[Ca^{2+}]$ of 200 nM [19]. Sensitization of IICR by Ca^{2+} will allow a regenerative Ca^{2+} release by a positive feedback loop between Ca^{2+} and IICR. Correspondingly, regenerative and propagating Ca^{2+} release was induced when $[Ca^{2+}]_i$ was elevated to about 300 nM by injected Ca^{2+} (Fig. 3a). Thimerosal lowered this threshold $[Ca^{2+}]_i$ level (Fig. 3b). Thus, thimerosal further sensitizes Ca^{2+}-sensitized IICR, so that IICR is induced by much smaller amounts of $InsP_3$ (Fig. 2b) and, moreover, spontaneous Ca^{2+} spikes are elicited (probably by endogenous $InsP_3$) when the augmenting Ca^{2+} rise reaches a certain level between 100 and 150 nM (Fig. 1). Sensitization of $InsP_3$ receptors by SH group oxidization has been suggested in hepatocytes with oxidized glutathion (GSSG) [17] or *tert*-butyl hydroperoxide (TBHP) [18] which oxidizes glutathion (GSH) to GSSG. Ca^{2+}-dependent activation of phospholipase C (PLC) leading to further production of $InsP_3$ can be another candidate for the regenerative process in IICR [25,26]. This may occur in the cell's cortex, if it is present, but evidence suggests that Ca^{2+} release takes place even in the deep cytoplasm of the hamster egg [27]; Ca^{2+}-sensitized IICR is more likely. TBHP can induce Ca^{2+} oscillations without any requirement for PLC activation [18]. Although the precise mechanism of sensitization remains to be elucidated, thimerosal is not the specific probe to identify CICR.

Ca^{2+} oscillations can occur in fertilized hamster eggs, based only on IICR [8]. The present findings with thimerosal support the idea of a single Ca^{2+} pool model, instead of a two-pool model [15,16], based on Ca^{2+}-sensitized IICR [8]. A slight elevation of basal $[Ca^{2+}]_i$ is commonly observed during both sperm-induced [8] and thimerosal-induced Ca^{2+} oscillations. The rise in basal $[Ca^{2+}]_i$ caused by thimerosal was resistant to 18A10 and removal of external Ca^{2+} (Fig. 1b). An inhibition of Ca^{2+} pumps, as has been demonstrated in hepatocytes treated with TBHP [28,29], may account for this elevation of $[Ca^{2+}]_i$. Inhibition of Ca^{2+} sequestration into Ca^{2+} pools may account for progressive attenuation of Ca^{2+} release as well (Fig. 1). On the other hand, the elevation of basal $[Ca^{2+}]_i$ by sperm depends on continuous Ca^{2+} influx due to increased Ca^{2+} permeability of the plasma membrane [7,22]. The elevated $[Ca^{2+}]_i$ supports the generation of Ca^{2+} oscillations by sensitizing IICR [17], although it alone does not induce repeated Ca^{2+} releases in unfertilized hamster eggs [7]. It is conceivable that, at a slightly higher level of basal $[Ca^{2+}]_i$, Ca^{2+} oscillations can be generated by endogenous $InsP_3$ when IICR is further sensitized by thimerosal or generated by low but persistent supply of $InsP_3$ [22,30] when sperm stimulates production of $InsP_3$.

Acknowledgements: This study was supported by Grants-in-Aid for General Scientific Research and for Scientific Research on Priority Areas to S.M. and by a Grant for Specially Promoted Research to K.M., from the Japan Ministry of Education, Science and Culture.

REFERENCES

[1] Maeda, N., Niinobe, M., Nakahira, K. and Mikoshiba, K. (1988) J. Neurochem. 51, 1724–1730.
[2] Supattapone, S., Worley, P.F., Baraban, J.M. and Snyder, S.H. (1988) J. Biol. Chem. 263, 1530–1534.
[3] Takeshima, H. et al. (1989) Nature 339, 439–445.
[4] Endo, M. (1976) Physiol. Rev. 57, 71–108.
[5] Fleischer, S., Ogunbunmi, E.M., Dixson, M.C. and Fleer, E.A.M. (1985) Proc. Natl. Acad. Sci. USA 82, 7256–7259.
[6] McGrew, S.G. et al. (1989) Biochem. 28, 1686–1691.
[7] Igusa, Y. and Miyazaki, S. (1983) J. Physiol. 340, 611–632.
[8] Miyazaki, S. et al. (1992) Science, in press.
[9] Swann, K. (1991) FEBS Lett. 278, 175–178.
[10] Zaidi, N.F. et al. (1989) J. Biol. Chem. 265, 21725–21736.
[11] Zaidi, N.F. et al. (1989) J. Biol. Chem. 264, 21737–21747.
[12] Islam, Md.S., Rorsman, P. and Berggren, P-O (1992) FEBS Lett. 296, 287–291.
[13] Miyazaki, S. et al. (1986) Dev. Biol. 118, 259–267.
[14] Berridge, M.J., Cobbold, P.H. and Cuthbertson, K.S.R. (1988) Philos. Trans. R. Soc. B 320, 325–343.
[15] Berridge, M.J. and Galione, A. (1988) FASEB J. 2, 3074–3082.
[16] Berridge, M.J. (1991) Cell Calcium 12, 63–72.
[17] Missiaen, L., Taylor, C.W. and Berridge, M.J. (1991) Nature 352, 241–244.
[18] Rooney, T.A., Renard, D.C., Sass, E.J. and Thomas, A.P. (1991) J. Biol. Chem. 266, 12272–12282.
[19] Bezprozvanny, I., Watras, J. and Ehrlich, B.E. (1991) Nature 351, 751–756.
[20] Furuichi, T. et al. (1989) Nature 342, 32–38.
[21] Nakade, S., Maeda, N. and Mikoshiba, K. (1991) Biochem. J. 277, 125–131.
[22] Miyazaki, S. (1991) Cell Calcium 12, 205–216.

[23] Iino, M. (1990) J. Gen. Physiol. 95, 1103–1122.
[24] Finch, E.A., Turner, T.J. and Goldin, S.M. (1991) Science 252, 443–446.
[25] Swann, K. and Whitaker, M.J. (1986) J. Cell Biol. 103, 2333–2342.
[26] Harootunian, A.T., Kao, J.P.Y., Paranjape, S. and Tsien, R.Y. (1991) Science 251, 75–78.
[27] Igusa, Y. and Miyazaki, S. (1986) J. Physiol. 377, 193–205.
[28] Bellomo, G., Mirabelli, F., Richelmi, P. and Orrenius, S. (1983) FEBS Lett. 163, 136–139.
[29] Jones, D.P., Thor, H., Smith, M.T., Jewell, S.A. and Orrenius, S. (1983) J. Biol. Chem. 258, 6390–6393.
[30] Miyazaki, S. (1988). J. Cell Biol. 106, 345–353.