Communications

The Bell-shaped Ca\(^{2+}\) Dependence of the Inositol 1,4,5-Trisphosphate-induced Ca\(^{2+}\) Release Is Modulated by Ca\(^{2+}\)/Calmodulin*

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Calmodulin inhibits inositol 1,4,5-trisphosphate (IP\(_3\)) binding to the IP\(_3\) receptor in both a Ca\(^{2+}\)-dependent and a Ca\(^{2+}\)-independent way. Because there are no functional data on the modulation of the IP\(_3\)-induced Ca\(^{2+}\) release by calmodulin at various Ca\(^{2+}\) concentrations, we have studied how cytosolic Ca\(^{2+}\) and Sr\(^{2+}\) interfere with the effects of calmodulin on the IP\(_3\)-induced Ca\(^{2+}\) release in permeabilized A7r5 cells. We now report that calmodulin inhibited Ca\(^{2+}\) release through the IP\(_3\) receptor with an IC\(_{50}\) of 4.6 \(\mu\)M if the cytosolic Ca\(^{2+}\) concentration was 0.3 \(\mu\)M or higher. This inhibition was particularly pronounced at low IP\(_3\) concentrations. In contrast, calmodulin did not affect IP\(_3\)-induced Ca\(^{2+}\) release if the cytosolic Ca\(^{2+}\) concentration was below 0.3 \(\mu\)M. Calmodulin also inhibited Ca\(^{2+}\) release through the IP\(_3\) receptor in the presence of at least 10 \(\mu\)M Sr\(^{2+}\). We conclude that cytosolic Ca\(^{2+}\) or Sr\(^{2+}\) are absolutely required for the calmodulin-induced inhibition of the IP\(_3\)-induced Ca\(^{2+}\) release and that this dependence represents the formation of the Ca\(^{2+}\)/calmodulin or Sr\(^{2+}\)/calmodulin complex.

Inositol 1,4,5-trisphosphate (IP\(_3\)) is a second messenger used by many cell types to release Ca\(^{2+}\) from internal stores (1). Cytosolic Ca\(^{2+}\) has a bell-shaped effect on the IP\(_3\) receptor (IP\(_3\)R), with low concentrations stimulating the release and high concentrations inhibiting it (2–5). Calmodulin, a Ca\(^{2+}\)-binding protein abundantly present in many cell types (6), binds to the modulatory region of the IP\(_3\)R in a Ca\(^{2+}\)-dependent way (7, 8). Calmodulin also interacts with the IP\(_3\)R in a Ca\(^{2+}\)-independent way (9–11), with one of the interaction sites located within the IP\(_3\)-binding domain (11). The findings that calmodulin inhibited IP\(_3\)-induced Ca\(^{2+}\) release in a medium containing 0.2 \(\mu\)M free Ca\(^{2+}\) and in addition inhibited [\(^{3}H\)IP\(_3\)] binding both in the absence and presence of cytosolic Ca\(^{2+}\) led to the proposal that the Ca\(^{2+}\)-independent binding of calmodulin was responsible for the regulation of the IP\(_3\)R (9).

Although the free cytosolic [Ca\(^{2+}\)] is a very important regulator of the IP\(_3\)R (2–5), there are no functional data showing how calmodulin modulates the Ca\(^{2+}\) release induced by IP\(_3\) at various Ca\(^{2+}\) concentrations. We have therefore studied how Ca\(^{2+}\) interferes with the effects of calmodulin on the IP\(_3\)-induced Ca\(^{2+}\) release in permeabilized A7r5 cells. All experiments were performed in the absence of Mg-ATP to avoid activation of the Ca\(^{2+}\)- and calmodulin-dependent protein kinase CaMKII that was reported to stimulate the IP\(_3\)R (12). We now report that calmodulin inhibited the IP\(_3\)-induced Ca\(^{2+}\) release if the free cytosolic [Ca\(^{2+}\)] was 0.3 \(\mu\)M or higher. This inhibition occurred with an IC\(_{50}\) of 4.6 \(\mu\)M and was particularly pronounced at low IP\(_3\) concentrations. Calmodulin did not affect the IP\(_3\)-induced Ca\(^{2+}\) release at lower Ca\(^{2+}\) concentrations. The effects of Ca\(^{2+}\) could be mimicked by Sr\(^{2+}\). We conclude that cytosolic Ca\(^{2+}\) or Sr\(^{2+}\) are absolutely required for the calmodulin-induced inhibition of the IP\(_3\)-induced Ca\(^{2+}\) release. As a consequence, the bell-shaped Ca\(^{2+}\) activation curve of the IP\(_3\)R becomes narrower in the presence of calmodulin.

EXPERIMENTAL PROCEDURES

\(^{45}\)Ca\(^{2+}\) fluxes were performed on saponin-permeabilized A7r5 cells from embryonic rat aorta (13). The nonmitochondriar Ca\(^{2+}\) stores were loaded for 50 min in 120 mM KCl, 30 mM imidazole-HCl (pH 6.8), 5 mM MgCl\(_2\), 5 mM ATP, 0.44 mM EGTA, 10 mM Na\(_2\)HPO\(_4\), and 150 mM free Ca\(^{2+}\) (25 \(\mu\)Ci ml\(^{-1}\)) and then washed once in 1 ml of efflux medium containing 120 mM KCl, 30 mM imidazole-HCl (pH 6.8), 4 \(\mu\)M thapsigargin, and, unless otherwise indicated, 1 mM BAPTA. The efflux medium was replaced every 2 min for 20 min. The additions of IP\(_3\), Ca\(^{2+}\), Sr\(^{2+}\), and calmodulin are indicated in the figures. The free [Ca\(^{2+}\)] was calculated with the CaBuf computer program using the following decimal logarithms of the association constants for ATP: H-ATP, 6.49; H-HATP, 4.11; Ca-ATP, 3.78; Ca-HATP, 1.98; Mg-ATP, 4.00; and Mg-HATP, 2.06 (14). The association constants for BAPTA were: H-BAPTA, 6.36; H-HBAPTA, 5.47; Ca-BAPTA, 6.97; and Sr-BAPTA, 5.13 (15). At the end of the experiment the \(^{45}\)Ca\(^{2+}\) remaining in the stores was released by incubation with 1 ml of a 2% SDS solution for 30 min.

Calmodulin from bovine brain (purity >99%) was obtained from Calbiochem (San Diego, CA) and dissolved as a 1 mM stock in 30 mM imidazole-HCl (pH 6.8). Control samples were treated with the same buffer.

RESULTS AND DISCUSSION

Effect of Calmodulin on IP\(_3\)-induced Ca\(^{2+}\) Release in the Presence of 0.3 \(\mu\)M Free Ca\(^{2+}\)—Permeabilized A7r5 cells loaded to equilibrium with \(^{45}\)Ca\(^{2+}\) slowly lost their accumulated \(^{45}\)Ca\(^{2+}\) during incubation in efflux medium containing 1 mM BAPTA and no added Ca\(^{2+}\). Thapsigargin (4 \(\mu\)M) was added to block the endoplasmic reticulum Ca\(^{2+}\) pumps during the additions of Ca\(^{2+}\). A short exposure to 1 \(\mu\)M IP\(_3\) and 0.3 \(\mu\)M free Ca\(^{2+}\) accelerated the rate of Ca\(^{2+}\) loss (Fig. 1a, closed circles). The release was less pronounced if 10 \(\mu\)M calmodulin was added together with the IP\(_3\) and Ca\(^{2+}\) (Fig. 1a, open circles). Addition of 0.3 \(\mu\)M free Ca\(^{2+}\) in the absence of IP\(_3\) by itself induced a small Ca\(^{2+}\) release (Fig. 1b), due to the exchange of labeled Ca\(^{2+}\) for unlabeled Ca\(^{2+}\) (3, 16). This release was identical in

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1 The abbreviations used are: IP\(_3\), inositol 1,4,5-trisphosphate; IP\(_3\)R, IP\(_3\) receptor; BAPTA, 1,2-bis(2-aminooxy)ethane-N,N,N',N''-tetraacetic acid.

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the presence (Fig. 1b, open circles) or absence (Fig. 1b, closed circles) of 10 μM calmodulin.

Ca2+ release was always measured in the absence of Mg-ATP. Moreover, because there were six wash steps between the loading of the stores in the presence of Mg-ATP and the challenge with IP3, all residual Mg-ATP should have been effectively removed. The involvement of the Ca2+- and calmodulin-dependent protein kinase CaMKII in the observed inhibition seems therefore unlikely. In addition, we have also tested the effect of the CaMKII inhibitor KN62. Calmodulin (10 μM) inhibited the Ca2+ release induced by 1 μM IP3 and 0.3 μM free Ca2+ by 54 ± 3% in the absence of KN62 and by 51 ± 4% (n = 3) in the presence of 10 μM KN62. These findings exclude the involvement of CaMKII in the inhibition of the IP3R by calmodulin.

Ca2+ ] Dependence of the Effect of Calmodulin on IP3-induced Ca2+ Release—Similar experiments to those illustrated in Fig. 1 were performed at several free Ca2+ concentrations (Fig. 2a). Calmodulin (10 μM) did not inhibit the IP3R in the absence of added Ca2+ or in the presence of low free Ca2+ concentrations (0.03 or 0.1 μM). The same concentration of calmodulin, however, strongly inhibited the IP3R at higher free Ca2+ concentrations (0.3 and 1 μM).

Because the calmodulin used was lyophilized from a dialysis buffer containing 30 μM Ca2+, four Ca2+ ions were bound to each molecule of calmodulin. We calculated that the addition of 10 μM calmodulin and its contaminating 40 μM Ca2+ to the efflux medium containing 1 mM BAPTA and 650 μM total Ca2+ could therefore have slightly increased the free [Ca2+] from 0.30 to 0.36 μM. Although unlikely, we wanted to exclude that the inhibitory effect of calmodulin could be caused by this small change in free [Ca2+]. Addition of 40 μM Ca2+ in the absence of...
calmodulin inhibited the Ca\textsuperscript{2+} release induced by 1 \mu M IP\textsubscript{3} and 0.3 \mu M free Ca\textsuperscript{2+} by only 4.3 \pm 2.2\% (n = 3). We have also tested the effect of calmodulin in an efflux medium containing 6 mM BAPTA instead of 1 mM BAPTA. The addition of 10 \mu M calmodulin to efflux medium containing 6 mM BAPTA and 3.897 mM total Ca\textsuperscript{2+} increased the calculated free [Ca\textsuperscript{2+}] from 0.30 to only 0.31 \mu M. Fig. 2b shows that under these conditions of strong Ca\textsuperscript{2+} buffering, 10 \mu M calmodulin still inhibited the IP\textsubscript{3}R in the presence of 0.3 and 0.6 \mu M free Ca\textsuperscript{2+}. The same concentration of calmodulin had again no effect on the IP3-induced Ca\textsuperscript{2+} release at lower free Ca\textsuperscript{2+} concentrations. Fig. 2 also illustrates that cytosolic Ca\textsuperscript{2+} exerted its biphasic effect on the IP\textsubscript{3}R both in the presence and in the absence of calmodulin. In the presence of calmodulin, the inhibition occurred at lower Ca\textsuperscript{2+} concentrations. As a consequence, the bell-shaped Ca\textsuperscript{2+} activation curve of the IP\textsubscript{3}R became narrower in the presence of calmodulin.

The [Ca\textsuperscript{2+}]\textsuperscript{-dependence of the IP\textsubscript{3}}-induced Ca\textsuperscript{2+} release was markedly different when Ca\textsuperscript{2+} was buffered with 1 mM (Fig. 2a) or 6 mM BAPTA (Fig. 2b). Both the stimulatory and inhibitory effects of Ca\textsuperscript{2+} were more pronounced at the higher concentration of BAPTA. It is possible that this difference is caused by the postulated local [Ca\textsuperscript{2+}] rise in the vicinity of the IP\textsubscript{3}Rs as a result of the passive Ca\textsuperscript{2+} leak from the stores (17, 18). This [Ca\textsuperscript{2+}]\textsuperscript{-rise will be less pronounced in the presence of 6 mM BAPTA, thereby reducing the IP\textsubscript{3}-induced Ca\textsuperscript{2+} release at lower free Ca\textsuperscript{2+} concentrations. As a consequence the stimulatory effect of elevating the [Ca\textsuperscript{2+}] was more pronounced in the presence of 6 mM BAPTA. An alternative possibility could be that the IP\textsubscript{3}R at low free Ca\textsuperscript{2+} concentrations is inhibited by the Ca\textsuperscript{2+}-free form of BAPTA, which is the predominant form of the chelator under these conditions (19, 20). Such inhibition would be more pronounced at 6 mM BAPTA, which could again explain why the release in the absence of Ca\textsuperscript{2+} was reduced at the higher concentration of BAPTA. However, we have previously shown that this inhibitory effect was relatively small in A7r5 cells (21).

**The Inhibition of the IP\textsubscript{3}R by Calmodulin Is Dose-dependent**—Fig. 3a illustrates the Ca\textsuperscript{2+} release induced by 1 \mu M IP\textsubscript{3} and 0.3 \mu M free Ca\textsuperscript{2+} in the presence of various concentrations of calmodulin. Calmodulin inhibited the IP\textsubscript{3}R with an IC\textsubscript{50} of 4.6 \mu M and a Hill-coefficient of 1.0, which is consistent with a single interaction with no evidence for cooperativity between the subunits of the IP\textsubscript{3}R tetramer.

**Inhibitory Effect of Calmodulin on the Ca\textsuperscript{2+} Release Induced by Various IP\textsubscript{3} Concentrations**—Fig. 3b shows the Ca\textsuperscript{2+} release as a function of the [IP\textsubscript{3}] in the absence (closed circles) and presence (open circles) of 10 \mu M calmodulin in a medium containing 0.3 \mu M free Ca\textsuperscript{2+}. IP\textsubscript{3} stimulated the IP\textsubscript{3}R with an EC\textsubscript{50} of 0.25 \mu M IP\textsubscript{3} in the absence of calmodulin and with an EC\textsubscript{50} of 2.9 \mu M IP\textsubscript{3} in the presence of calmodulin. Calmodulin not only increased the EC\textsubscript{50} for IP\textsubscript{3}-induced Ca\textsuperscript{2+} release but also decreased the maximal Ca\textsuperscript{2+} release induced by high IP\textsubscript{3} concentrations. Interestingly, the inhibition was relatively more pronounced at lower IP\textsubscript{3} concentrations, e.g. 10 \mu M calmodulin caused an 82\% inhibition of the Ca\textsuperscript{2+} release induced by 0.5 \mu M IP\textsubscript{3}, whereas in the presence of 300 \mu M IP\textsubscript{3} was only inhibited by 20\%.

**[Sr\textsuperscript{2+}]\textsuperscript{-Dependence of the Effect of Calmodulin on the IP\textsubscript{3}R induced Ca\textsuperscript{2+} Release**—The inhibitory effects of calmodulin were clearly dependent on the presence of Ca\textsuperscript{2+}. To discriminate whether calmodulin acted by potentiating the inhibitory effects of Ca\textsuperscript{2+} or whether the requirement for Ca\textsuperscript{2+} to see the inhibition by calmodulin reflected the formation of Ca\textsuperscript{2+}/calmodulin, we have studied the effect of calmodulin in the presence of various Sr\textsuperscript{2+} concentrations. Sr\textsuperscript{2+} is only 3-fold less potent than Ca\textsuperscript{2+} in activating the liver IP\textsubscript{3}R but is 600-fold less potent in inhibiting it (22). A similar effect was observed in A7r5 cells, where Sr\textsuperscript{2+} up to 100 \mu M induced a concentration-dependent decrease in the EC\textsubscript{50} for IP\textsubscript{3}-induced Ca\textsuperscript{2+} release, whereas Ca\textsuperscript{2+} induced a biphasic effect with low Ca\textsuperscript{2+} concentrations decreasing the EC\textsubscript{50} and higher Ca\textsuperscript{2+} concentrations increasing it (23). Fig. 4 shows the Ca\textsuperscript{2+} release induced by 1 \mu M IP\textsubscript{3} in the presence of increasing Sr\textsuperscript{2+} concentrations. The closed bars confirm that Sr\textsuperscript{2+} activated the IP\textsubscript{3}R and that no significant inhibition was observed at 30 \mu M Sr\textsuperscript{2+}. The hatched bars show the effect of 10 \mu M calmodulin. Calmodulin did not inhibit the IP\textsubscript{3}R in the absence of added Sr\textsuperscript{2+} or in the presence of low free Sr\textsuperscript{2+} concentrations (1 or 3 \mu M). The same concentration of calmodulin, however, strongly inhibited the IP\textsubscript{3}R at higher free Sr\textsuperscript{2+} concentrations (10 and 30 \mu M), which by themselves were not inhibitory. Because Sr\textsuperscript{2+} binds to calmodulin (24) but does not inhibit the IP\textsubscript{3}R in the absence of calmodulin, we conclude that the dependence of the calmodulin inhibition on the presence of Sr\textsuperscript{2+} or Ca\textsuperscript{2+} represents the formation of
**Effects of Calmodulin on the IP₃ Receptor**

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Fig. 4. The effect of calmodulin on the IP₃R in permeabilized A7r5 cells at various Sr²⁺ concentrations. The IP₃-induced Ca²⁺ release in efflux medium containing 1 mM BAPTA was calculated as the difference between the Ca²⁺ release in the presence and that in the absence of IP₃ (3). The free [Sr²⁺] during the IP₃ application is indicated on the abscissa. The closed bars represent the Ca²⁺ release induced by 1 μM IP₃ in the absence of calmodulin. o and oo, significantly different from the Ca²⁺ release induced by 1 μM IP₃ and [Sr²⁺] in the absence of calmodulin (p < 0.02) as determined using Student’s t test for paired samples. Means ± S.E. are shown for seven experiments.

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**REFERENCES**

1. Berridge, M. J. (1993) Nature 361, 315–325
2. Iino, M. (1990) J. Gen. Physiol. 95, 1103–1122
3. Finch, E. A., Turner, T. J., and Goldin, S. M. (1991) Science 252, 443–446
4. Bezprozvanny, I., Wostrats, J., and Ehrlich, B. E. (1991) Nature 351, 751–754
5. Parysz, J. B., Sernett, S. W., DeLisle, S., Snyder, P. M., Welsh, M. J., and Campbell, K. P. (1992) J. Biol. Chem. 267, 18776–18782
6. Kakiuchi, S., Yasuda, S., Yamazaki, R., Teshima, Y., Kanda, K., Kakiuchi, R., and Suhre, K. (1992) J. Biochem. (Tokyo) 92, 1041–1048
7. Maeda, N., Kawasaki, T., Nakade, S., Yokota, N., Taguchi, T., Kasai, M., and Mikoshiba, K. (1991) J. Biol. Chem. 266, 1109–1116
8. Yamada, M., Miyawaki, A., Saito, K., Nakajima, T., Yamamoto-Hino, M., Ryo, Y., Furuiuchi, T., and Mikoshiba, K. (1995) Biochem. J. 308, 83–88
9. Patel, S., Morris, S. A., Adams, C. E., O’Beirne, G., and Taylor, C. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11627–11632
10. Cardy, T. J. A., and Taylor, C. W. (1998) Biochem. J. 334, 447–455
11. Sipina, H., De Smet, P., Sienka, I., Vanlingen, S., Missiaen, L., Parysz, J. B., and De Smedt, H. (1999) J. Biol. Chem. 274, in press
12. Zhang, B. X., Zhao, H., and Muallem, S. (1993) J. Biol. Chem. 268, 10997–11001
13. Missiaen, L., De Smedt, H., Droogmans, G., and Casteels, R. (1992) Nature 357, 599–602
14. Martell, A. E., and Smith, R. M. (1989) Critical Stability Constants, Plenum Press, New York
15. Tsien, R. Y. (1980) Biochemistry 19, 2396–2404
16. Missiaen, L., De Smedt, H., Droogmans, G., and Casteels, R. (1992) J. Biol. Chem. 267, 22961–22966
17. Iino, M., and Endo, M. (1992) J. Biol. Chem. 267, 76–78
18. Horne, J. H., and Meyer, T. (1995) Biochemistry 34, 12738–12746
19. Richardson, A., and Taylor, C. W. (1993) J. Biol. Chem. 268, 11528–11533
20. Combettes, L., Hamact-Merah, Z., Coquil, J.-F., Rousseau, C., Clarè, M., Swilens, S., and Champoll, P. (1994) J. Biol. Chem. 269, 17561–17571
21. Bootman, M. D., Missiaen, L., Parysz, J. B., De Smedt, H., and Casteels, R. (1995) Biochem. J. 306, 445–451
22. Marshall, I. C. B., and Taylor, C. W. (1994) Biochem. J. 301, 591–598
23. Sienka, I., Missiaen, L., De Smedt, H., Parysz, J. B., Sipina, H., and Casteels, R. (1997) J. Biol. Chem. 272, 25899–25906
24. Cox, J. A., Malno, A., and Stein, E. A. (1981) J. Biol. Chem. 256, 3218–3228
25. Parysz, J. B., De Smedt, H., Missiaen, L., Bootman, M. D., Sienka, I., and Casteels, R. (1995) Cell Calcium 17, 239–249