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Calmodulin Increases the Sensitivity of Type 3 Inositol-1,4,5-trisphosphate Receptors to Ca^{2+} Inhibition in Human Bronchial Mucosal Cells

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ABSTRACT

Inositol-1,4,5-trisphosphate (IP_3) releases Ca^{2+} from intracellular stores by binding to its receptor (IP_3R), a multigene family of Ca^{2+}-release channels consisting of IP_3R1, IP_3R2, and IP_3R3. IP_3R1 is stimulated by low cytoplasmic Ca^{2+} concentrations and inhibited by high concentrations. Discrepant reports appeared about the effect of cytoplasmic Ca^{2+} on IP_3R3, showing either a bell-shaped dependence or only a stimulatory phase with no negative feedback by high Ca^{2+} concentrations. We investigated how calmodulin interfered with the feedback of cytosolic Ca^{2+} on the unidirectional IP_3-induced Ca^{2+} release in permeabilized 16HBE14o- bronchial mucosal cells, where IP_3R3 represents 93% of the receptors at the mRNA level and 81% at the protein level. Calmodulin inhibited the Ca^{2+} release induced by 1.5 μM IP_3 with an IC_{50} value of 9 μM. This inhibition was absolutely dependent on the presence of cytosolic Ca^{2+}. Ca^{2+} inhibited the IP_3R with an IC_{50} value of 0.92 μM Ca^{2+} in the absence of calmodulin and with an IC_{50} value of 0.15 μM Ca^{2+} in its presence. It is concluded that: 1) IP_3R3 can be inhibited by calmodulin, 2) IP_3R3 is inhibited by high Ca^{2+} concentrations, and 3) calmodulin shifts the inhibitory part of the Ca^{2+}-response curve toward lower Ca^{2+} concentrations.

Many hormones, neurotransmitters, and growth factors induce the hydrolysis of phosphatidylinositol-4,5-bisphosphate and thereby produce inositol-1,4,5-trisphosphate (IP_3) as an intracellular messenger (Berridge, 1993). IP_3 releases Ca^{2+} from intracellular stores by binding to the IP_3 receptor (IP_3R), a multigene family of Ca^{2+}-release channels consisting of IP_3R1 (Furuichi et al., 1989), IP_3R2 (Südhof et al., 1991), and IP_3R3 (Blondel et al., 1993). This Ca^{2+} release results in the generation of complex cytoplasmic Ca^{2+} signals, including Ca^{2+} oscillations and propagating Ca^{2+} waves (Lechleiter et al., 1991).

Cytosolic Ca^{2+} has a bell-shaped effect on IP_3R1, with low concentrations stimulating the Ca^{2+} release and high concentrations inhibiting it (Iino, 1990; Bezprozvanny et al., 1991; Finch et al., 1991; Parys et al., 1992). The regulation of IP_3R2 and IP_3R3 by Ca^{2+} is, however, less well understood. IP_3-induced Ca^{2+} release in permeabilized rat basophilic leukemia cells, which predominantly express IP_3R2 (De Smedt et al., 1994), is not inactivated by cytosolic Ca^{2+} (Horne and Meyer, 1995), and the partially purified cardiac IP_3R2 also lacks the inhibition at high Ca^{2+} concentrations in single-channel recordings (Ramos-Franco et al., 1998). In contrast, the IP_3-induced Ca^{2+} release in permeabilized chicken B cells genetically modified to express only IP_3R2 was inhibited by 1 μM Ca^{2+} (Miyakawa et al., 1999). The effects of high Ca^{2+} concentrations on IP_3R3 have been studied using different techniques, and the reports are so far discrepant. The IP_3Rs in RIN-m5F insulinoma cells, which are between 60% (De Smedt et al., 1994) and 96% (Wojcikiewicz, 1995) of type 3, were not inhibited by up to 100 μM Ca^{2+} when incorporated in planar lipid bilayers (Hagar et al., 1998). In contrast, patch-clamp experiments on outer nuclear membranes of *Xenopus* oocytes overexpressing IP_3R3 revealed that micromolar Ca^{2+} did inhibit IP_3-induced channel activity (Mak et al., 1998a). Reports on the effects of high Ca^{2+} on IP_3R3 in permeabilized cells are also discrepant. IP_3-induced Ca^{2+} release in permeabilized 16HBE14o- cells, which predominantly express IP_3R3 (Sienaert et al., 1998), was inhibited by micromolar Ca^{2+} (Missiaen et al., 1998; Sienaert et al., 1998). In contrast, the release in permeabilized chicken B cells expressing only IP_3R3 was not inhibited by 1 μM Ca^{2+}, but higher concentrations were not tested (Miyakawa et al., 1999). One possible explanation for these divergent results is that experimental conditions and/or regulatory mechanisms

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ABBREVIATIONS: IP_3, inositol-1,4,5-trisphosphate; IP_3R, inositol-1,4,5-trisphosphate receptor; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

can interfere with the bell-shaped Ca^{2+} dependence of the IP_3-induced Ca^{2+} release [e.g., the effects of cytosolic Ca^{2+} on the IP_3R depend on the free Mg^{2+} concentration, pH, and the IP_3 and ATP concentrations (Tsukioka et al., 1994; Bootman et al., 1995; Mak et al., 1998b, 1999)]. In the present study, we focus on the effect of the Ca^{2+}-binding protein calmodulin.

Calmodulin binds to IP_3R1 (Maeda et al., 1991; Yamada et al., 1995; Patel et al., 1997; Cardy and Taylor, 1998), and this interaction results in a decreased binding of IP_3 to IP_3R1 (Patel et al., 1997; Cardy and Taylor, 1998; Sipma et al., 1999). Exogenous calmodulin inhibits IP_3-induced Ca^{2+} release in permeabilized A7r5 cells (Missiaen et al., 1999), which express for 75% IP_3R1 and for 25% IP_3R3 (De Smedt et al., 1994). Calmodulin also inhibits the purified cerebellar IP_3R1 incorporated in planar lipid bilayers (Michikawa et al., 1999).

The aim of this work was to investigate the effects of calmodulin on IP_3-induced Ca^{2+} release in permeabilized 16HBE14o- human bronchial mucosal cells, which express for 93% IP_3R3, as judged from the relative levels of steady-state mRNA, and for 81% IP_3R3 as judged from experiments using isoform-specific antibodies (Sienaert et al., 1998).

We now report that calmodulin inhibited the IP_3-induced Ca^{2+} release if the free cytosolic Ca^{2+} concentration was 0.1 μM or higher. This inhibition occurred with an IC_{50} value of 9 μM calmodulin. Calmodulin shifted the inhibitory part of the Ca^{2+}-response curve of the IP_3-induced Ca^{2+} release toward lower Ca^{2+} concentrations. We conclude that IP_3R3 is inhibited by calmodulin and that the Ca^{2+} concentrations needed to inactivate IP_3R3 are decreased by the presence of calmodulin.

Materials and Methods

$^{45}Ca^{2+}$ fluxes were performed on saponin-permeabilized 16HBE14o- cells derived from human bronchial surface epithelium (Cozens et al., 1994) at 25°C as described previously (Missiaen et al., 1998). The nonmitochondrial Ca^{2+} stores were loaded for 45 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 NaN_3 and 150 nM free Ca^{2+} (23 μCi/ml) and then washed once in 1 ml of efflux medium containing 120 mM KCl, 30 mM imidazole-HCl (pH 6.8), 1 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), and 4 μM thapsigargin. Thapsigargin was added to block the endoplasmic-reticulum Ca^{2+} pumps during subsequent additions of Ca^{2+}. The efflux medium was replaced every 2 min for 20 min. The additions of IP_3, Ca^{2+}, and calmodulin are indicated in the figures. The free Ca^{2+} concentration 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 (Martell and Smith, 1982). The association constants for BAPTA were H-BAPTA, 6.36; H-HBAPTA, 5.47; and Ca-BAPTA, 6.97 (Tsien, 1980). 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%; Calbiochem, San Diego, CA) was made Ca^{2+}-free by batch treatment with 50 mg/ml Chelex 100 (Bio-Rad Laboratories, Hercules, CA) for 1 h at 10°C. Calmodulin was dissolved as a 1 mM stock in 30 mM imidazole-HCl (pH 6.8). Control samples were treated with the same buffer.

Results

IP_3-Induced Ca^{2+} Release in Permeabilized 16HBE14o- Cells. The nonmitochondrial Ca^{2+} stores of permeabilized 16HBE14o- cells were first loaded to equilibrium with $^{45}Ca^{2+}$ and then incubated in efflux medium containing 1 mM BAPTA and no added Ca^{2+}. Thapsigargin (4 μM) was added to the efflux medium to allow a unidirectional Ca^{2+} efflux. Figure 1A (filled circles) illustrates that a 2-min exposure to 1.5 μM IP_3 and 0.3 μM free Ca^{2+} accelerated the rate of Ca^{2+} loss. The traces were corrected for the passive Ca^{2+} efflux in an identical medium in the absence of IP_3. This concentration of IP_3 released 45 ± 4% of the Ca^{2+} released by a saturating dose of 100 μM IP_3 in the presence of 0.3 μM free Ca^{2+} (n = 3).

Effect of Calmodulin on IP_3-Induced Ca^{2+} Release. Figure 1 also illustrates the effect of 20 μM calmodulin (open symbols), added at the time of IP_3 addition, on the Ca^{2+} release induced by 1.5 μM IP_3 in the presence of 0.3 μM free Ca^{2+} (Fig. 1A) and in the absence of added Ca^{2+} (Fig. 1B). Exogenously added calmodulin inhibited the IP_3-induced Ca^{2+} release in the presence of 0.3 μM Ca^{2+} but was unable to inhibit the release in the absence of added Ca^{2+}.

The inhibition by calmodulin was not caused by contaminating Ca^{2+} in the calmodulin sample for two reasons. First, calmodulin was made Ca^{2+}-free by pretreatment with Chelex 100 (see *Materials and Methods*). Second, the inhibition still occurred when the free Ca^{2+} concentration was set at 0.3 μM using 6 mM BAPTA instead of the routinely used 1 mM BAPTA (data not shown).

Inhibition of IP_3R by Calmodulin Is Dose-Dependent. The Ca^{2+} release induced by 1.5 μM IP_3 and a whole range of calmodulin concentrations in a medium containing 0.3 μM free Ca^{2+} (filled symbols) and in a medium with 1 mM

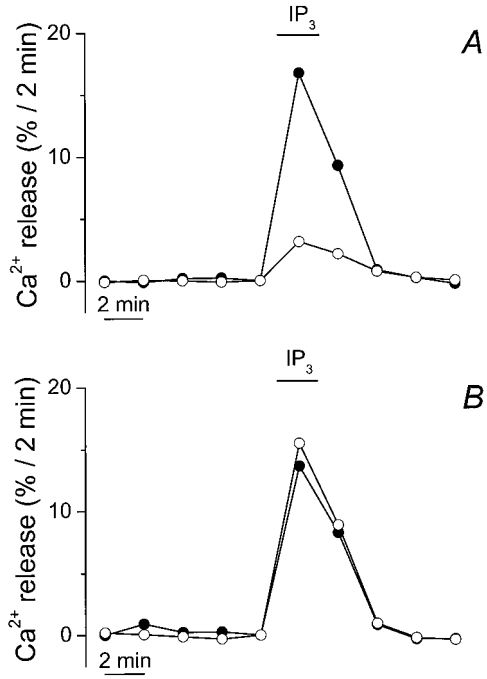

Fig. 1. Effect of calmodulin on the IP_3-induced Ca^{2+} release in permeabilized 16HBE14o- cells. The nonmitochondrial Ca^{2+} stores were loaded to steady state with $^{45}Ca^{2+}$ and then incubated in efflux medium containing 1 mM BAPTA and no added Ca^{2+}. During the time period indicated by the horizontal bar, 1.5 μM IP_3 and 0.3 μM free Ca^{2+} (A) or 1.5 μM IP_3 alone (B) were added for 2 min in the absence (●) or presence (○) of 20 μM calmodulin. The traces were corrected for the passive Ca^{2+} efflux in an identical efflux medium in the absence of IP_3. Ca^{2+} release is plotted as fractional loss (i.e., the amount of Ca^{2+} released in 2 min divided by the total store Ca^{2+} content at that time). Values are mean of four experiments. The S.E. was always less than 5%.

BAPTA and no added Ca^{2+} (open symbols) is shown in Fig. 2. Calmodulin inhibited the IP_3R with an IC_{50} value of 9 μM in the presence of 0.3 μM free Ca^{2+}. No inhibition was observed in the absence of added Ca^{2+}.

Effect of Calmodulin on Ca^{2+} Concentration Dependence of IP_3-Induced Ca^{2+} Release. Figure 3 illustrates how 20 μM calmodulin interfered with the activation of the IP_3R by Ca^{2+} in the presence of a constant IP_3 concentration (1.5 μM). The filled symbols illustrate the effects of Ca^{2+} on the IP_3-induced Ca^{2+} release in the absence of calmodulin. Low Ca^{2+} concentrations slightly activated the release, and high Ca^{2+} concentrations inhibited it. The open circles illustrate that a similar pattern also occurred in the presence of 20 μM calmodulin. Ca^{2+} inhibited the IP_3R with an IC_{50} value of 0.92 μM Ca^{2+} in the absence of calmodulin and with an IC_{50} value of 0.15 μM Ca^{2+} in its presence. The inactivation by Ca^{2+} therefore occurred at lower Ca^{2+} concentrations in the presence of calmodulin.

Discussion

16HBE14o- cells express for 81 to 93% IP_3R3, as judged from experiments using isoform-specific antibodies and from the relative levels of steady-state mRNA as determined by quantitative ratio reverse transcription-polymerase chain reaction (Sienaert et al., 1998). Although a small fraction of the IP_3Rs are IP_3R1 and IP_3R2 isoforms, the properties of the IP_3-induced Ca^{2+} release in 16HBE14o- cells were very similar to those in genetically engineered B cells that exclusively express IP_3R3 (Miyakawa et al., 1999); that is, the release was less sensitive to IP_3 and much less affected by ATP than in cell types expressing predominantly IP_3R1 (Missiaen et al., 1998). The properties of the IP_3-induced Ca^{2+} release in 16HBE14o- cells can therefore be considered as representative of the characteristics of IP_3R3.

We observed that calmodulin inhibited the IP_3-induced Ca^{2+} release in 16HBE14o- cells in the presence of Ca^{2+} and that calmodulin shifted the inhibitory part of the Ca^{2+}-response curve toward lower Ca^{2+} concentrations. IP_3-induced Ca^{2+} release in permeabilized RIN-m5F cells, which express between 60% (De Smedt et al., 1994) and 96% (Wojcikiewicz, 1995) of type 3 IP_3R, was also inhibited by calmodulin (Adkins et al., 2000). Binding studies have provided evidence for both Ca^{2+}-dependent and -independent interactions between calmodulin and IP_3R1 (Maeda et al., 1991; Yamada et al., 1995; Patel et al., 1997; Cardy and Taylor, 1998; Adkins et al., 2000). Calmodulin interacts with at least two different binding sites, of which the functional significance has not yet been unequivocally demonstrated (Yamada et al., 1995; Sipma et al., 1999; Adkins et al., 2000). A Ca^{2+}-dependent binding site is localized in the regulatory domain of IP_3R1 (Yamada et al., 1995) and could be involved in the Ca^{2+}-dependent inhibition of IP_3R1 by calmodulin (Michikawa et al., 1999; Missiaen et al., 1999). This site was also identified in IP_3R2 but not in IP_3R3 (Yamada et al., 1995), possibly because its affinity is too low to be detected by affinity chromatography (Adkins et al., 2000).

The significance of the Ca^{2+}-independent interaction of IP_3R1 with calmodulin is much less clear, but a role in the inhibition of IP_3-induced Ca^{2+} release was also proposed (Patel et al., 1997). Moreover, calmodulin was found to inhibit in a Ca^{2+}-independent way IP_3 binding to the bacterially expressed ligand-binding domain of IP_3R1 (Sipma et al., 1999), and similar observations were made for the ligand-binding domains of IP_3R2 and IP_3R3 (Vanlingen et al., 2000). These effects may be mediated by a conserved low-affinity calmodulin-binding site identified in the N-terminal region of IP_3R1 (Adkins et al., 2000).

The inhibition of IP_3-induced Ca^{2+} release by calmodulin in cell types expressing predominantly IP_3R3, such as RIN-m5F insulinoma cells (Adkins et al., 2000) or 16HBE14o- bronchial epithelial cells (present work), could therefore indicate the interaction of calmodulin to IP_3R3 at a low-affinity binding site that could have been missed by calmodulin affinity chromatography. Alternatively, the effect of calmodulin may be indirect and mediated by a protein associated with IP_3R3 and in fact can even be the IP_3R1 or IP_3R2 subunits present with the predominant IP_3R3 as heterotetramers.

The Ca^{2+}-induced inhibition of IP_3R1 in cerebellar microsomes in the absence of added calmodulin was prevented by 400

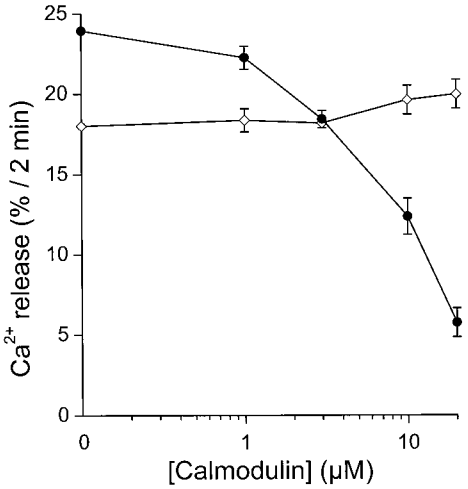

Fig. 2. Inhibition of the IP_3-induced Ca^{2+} release by calmodulin in permeabilized 16HBE14o- cells is dose-dependent. Ca^{2+} release induced by 1.5 μM IP_3 in the absence (◇) or presence (●) of 0.3 μM free Ca^{2+} was measured at the indicated calmodulin concentration. Values are mean ± S.E. for three experiments.

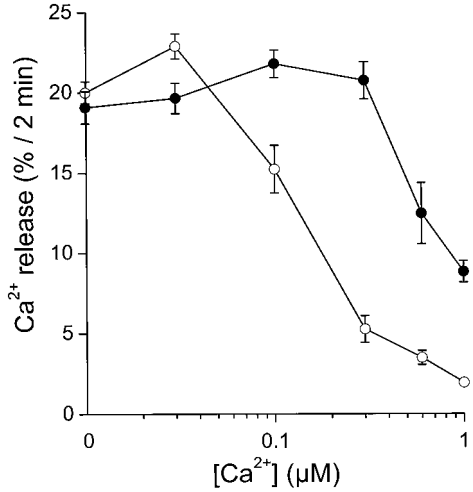

Fig. 3. Effect of calmodulin on the Ca^{2+} concentration dependence of the IP_3-induced Ca^{2+} release in permeabilized 16HBE14o- cells. The stores were challenged for 2 min with 1.5 μM IP_3 and the indicated free Ca^{2+} concentration in the absence (●) or presence (○) of 20 μM calmodulin. Values are mean ± S.E. for four independent experiments.

μM *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), a calmodulin inhibitor (Michikawa et al., 1999). Ca^{2+} also caused a significant inhibition of the IP_3R3 in the absence of added calmodulin in permeabilized 16HBE14o- cells. This could mean either that sufficiently high levels of endogenous calmodulin were still present after permeabilization or that calmodulin was not strictly necessary but only stimulated the Ca^{2+}-induced inhibition of IP_3R3. It was technically impossible to discriminate between these two possibilities, because the calmodulin inhibitor W-7 (50 μM) induced an appreciable release of $^{45}Ca^{2+}$ on its own (data not shown), probably via nonspecific lipophilic interactions.

High levels (>10 μM) of calmodulin were found in brain, testis, and pituitary gland (Kakiuchi et al., 1982). Intermediate levels (5–10 μM) were found in lung, prostate, and adrenal gland, whereas low levels (<5 μM) occurred in liver, kidney, and spleen. In addition, calmodulin is compartmentalized, and its distribution changes during increases in intracellular Ca^{2+} concentration (Luby-Phelps et al., 1995). The concentration range over which calmodulin inhibited IP_3R3 (IC_{50} = 9 μM in the presence of 0.3 μM free Ca^{2+}) is therefore potentially physiologically relevant.

We conclude that IP_3R3 in human bronchial mucosal cells is inhibited by calmodulin and that the Ca^{2+} concentrations needed to inactivate IP_3R3 are decreased by the presence of calmodulin. The present data therefore confirm our previous finding that the type 3 IP_3R can be inhibited by Ca^{2+} (Missiaen et al., 1998). The present work extends these observations by showing that the Ca^{2+} concentration needed to inactivate IP_3R3 is largely dependent on the presence of calmodulin.

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