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Calcium-induced degradation of the Inositol (1,4,5)-trisphosphate receptor/Ca^{2+}-channel

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Ca^{2+}-induced degradation of the neuronal inositol (1,4,5)-trisphosphate receptor, a protein which regulates Ca^{2+}-release from intracellular stores, has been examined. The IP_3-receptor, immunopurified from rat cerebellum, appeared to be an excellent substrate for purified Ca^{2+}-activated neutral protease (calpain). Incubation of membranes or immunopurified IP_3-receptor with Ca^{2+} and cerebellar cytosol also resulted in degradation of the receptor. Two main fragments with approximate molecular masses of 130 and 95 kDa were generated, both of which appeared to derive from the carboxyterminal Ca^{2+}-channel-containing part of the protein. These data suggest that activation of the IP_3-receptor, by causing increases in intracellular $[Ca^{2+}]$, might result in degradation of the N-terminal, IP_3-binding part of the receptor.

Calpain; Ca^{2+}-channel; IP_3-receptor; Signal transduction; Cerebellum

1. INTRODUCTION

Changes in intracellular $[Ca^{2+}]$, which regulate a wide variety of cellular functions, are partly regulated by changes in the levels of inositol 1,4,5-trisphosphate (IP_3) [1]. This compound, an intracellular messenger which mediates the effects of a number of Ca^{2+}-mobilizing neurotransmitters, hormones and growth factors, achieves its effects by binding to an intracellular IP_3-receptor protein (IP_3-R) [2,3].

The isoforms of the IP_3-receptor, all of which have approximate molecular masses of 260 kilodalton (kDa) when analysed by SDS-PAGE [4,5], contain both a cytoplasmic IP_3-binding domain and a transmembrane Ca^{2+}-channel domain [6,7]. Binding of IP_3 to the cytoplasmic receptor domain induces Ca^{2+} flux through the channel domain and thereby leads to increases in cytoplasmic $[Ca^{2+}]$ [8,9].

The IP_3-induced increases in $[Ca^{2+}]$ may modulate the sensitivity of the IP_3-R [10,11]. Such modulation might be caused by Ca^{2+}-regulated phosphorylation/dephosphorylation, or binding of other Ca^{2+}-regulated proteins [12,13]. In the present study, we have examined whether the neuronal isoform [5,14] of the IP_3-receptor, particularly enriched in the endoplasmic reticulum of cerebellar Purkinje cells [15,16], is subjected to Ca^{2+}-regulated degradation.

2. MATERIALS AND METHODS

2.1. *Materials*

Leupeptin, chymostatin, trypsin inhibitor, pepstatin and phenylmethane sulfonyl fluoride (PMSF) were purchased from Boehringer Mannheim, Na^+-deoxycholate (DOC) and Ca^{2+}-activated neutral proteases (80 kDa) were purchased from Sigma, and protein A-Sepharose was purchased from Pharmacia Fine Chemicals. Calpeptin was a generous gift from T. Tsujinaka, University of Osaka and dr. N.O. Solum, Rikshospitalet, Oslo. The catalytic subunit of cAMP-dependent protein kinase was a gift from dr. S.O. Døskeland, University of Bergen. BCA Protein Assay Reagents were purchased from Pierce.

2.2. *Preparation of antibodies*

A synthetic 18-amino acid peptide corresponding to the C-terminal end of the IP_3-receptor [6] was synthetized in the Biotechnology Center, University of Oslo. The peptide was coupled to hemocyanin by glutaraldehyde prior to injection, and antibodies were raised in rabbits by monthly subcutaneous injection of the conjugated peptide mixed with Freund's adjuvant.

2.3. *Tissue preparation*

Rat cerebella were homogenized by sonication on ice in 4 vols. of a buffer containing 0.25 M sucrose, 20 mM Tris-HCl (pH 7.4) and 2 mM EDTA. The protease inhibitors PMSF (0.1 mM), leupeptin, pepstatin, chymostatin and trypsin inhibitor (all in 10 μg/ml concentration) were added as indicated. The homogenate was fractionated by centrifugation at $1,000 \times g$ for 10 min to remove the nuclei, followed by centrifugation at $100,000 \times g$ for 60 min to pellet membranes and microsomes from the cytosol [17]. The membrane pellet was resuspended in PBS or Tris-HCl (pH 7.4), at a protein concentration of about 7 mg/ml. The cytosol used as enzyme source was prepared as described, without any protease-inhibiting substances and without EDTA, but with 1 mM DTT. Protein content was analysed by the bicinchoninic acid method [18].

2.4. *Immunoprecipitation of the IP_3-receptor*

Membrane preparations were extracted with 1% (w/v) deoxycholate (DOC) on ice for 30 min, which solubilized 95% of the IP_3-R. The mixture was centrifuged for 30 min at $27,000 \times g$ to remove denatured membrane fragments. The resulting supernatant (approximately 5 mg/ml protein) is referred to as the DOC-extract.

Protein A-Sepharose beads were washed in TBS-Tween 20 (0.05%) and then incubated with anti-IP_3-receptor antiserum (> 5 h). Following washing in TBS-Tween 20, the beads were incubated with the

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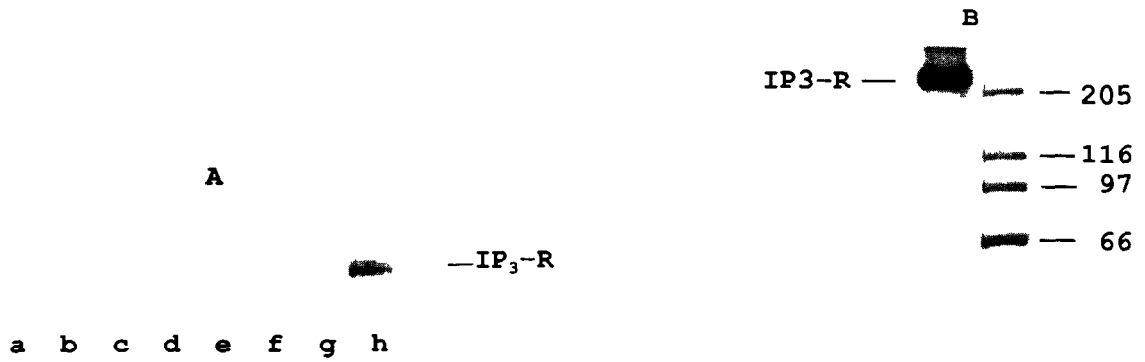

Fig. 1. Characterization of anti-IP_3-receptor antibodies. (A) Immunoblots of solubilized membrane proteins from rat tissues. Membrane proteins from rat cells or tissues were separated by SDS-PAGE, transferred to nitrocellulose, probed with crude rabbit anti-IP_3-receptor antiserum diluted 1.500 and visualized by horseradish peroxidase and diaminobenzidine as described. (a) Astrocytes, (b) GH_3 cells, (c) Vas deferens, (d) Round spermatids, (e) Pachytene spermatocytes, (f) Sertoli cells, (g) Peritubular cells from testis and (h) Cerebellum. (B) Autoradiogram of immunoprecipitated IP_3-receptor from DOC-solubilized cerebellum membranes phosphorylated with cAMP- dependent protein kinase. Protein A-Sepharose-bound IP_3-receptor was incubated with the catalytic subunit of the kinase and [γ-^{32}P]ATP for 5 min, and the phosphorylation was terminated by addition of EDTA (final [EDTA] = 50 mM). SDS-PAGE and autoradiography were performed as described in the text.

DOC-extract diluted 1:3 with TBS-Tween (final [DOC] = 0.33%, w/v) at 4°C overnight. The immunocomplexes were isolated by centrifugation, and analysed by SDS-PAGE.

2.5. *Phosphorylation of the IP_3-receptor*
This was performed as described [16].

2.6. *Analysis of Ca^{2+}-dependent proteolysis*
IP_3-receptor, immunopurified on the protein A-Sepharose beads as described above, was washed twice in TBS-Tween. The beads, or alternatively, a crude membrane fraction, were incubated in buffer (20 mM Tris-HCl, pH 7 4), in the absence or presence of 1 mM Ca^{2+} and cytosol or calpain, for the times and temperatures indicated. The reactions were terminated either by addition of SDS-containing buffer [19] or, in the case of the protein A-Sepharose beads, addition of protease inhibitors followed by washing in ice cold TBS-Tween before resuspension in SDS-buffer and analysis by SDS-PAGE.

2.7. *SDS polyacrylamide electrophoresis*
This was performed in 7.5% polyacrylamide gels in accordance with Laemmli [19].

2.8. *Immunoblotting*
Proteins, separated by SDS-PAGE, were electrotransferred to nitrocellulose [20]. The transferred proteins were incubated with a 1:500 dilution of the rabbit antiserum followed either by a horseradish peroxidase-conjugated secondary antiserum, or by ^{125}I-labelled protein A. The antigen–antibody complexes were visualized either by 0.05% diaminobenzidine, 0.03% $NiCl_2$ and 0.03% H_2O_2 in 50 mM NH_4CO_3, or by autoradiography.

3. RESULTS

The neuronal form of IP_3-receptor, purified from rat cerebellum, is composed of subunits of apparent 260 kDa mass [2]. When rabbit antibodies were raised against a synthetic peptide derived from the extreme C-terminal end of the IP_3-R [6], a single protein with an apparent molecular mass of 260 kDa was recognized in different cell types when examined by SDS-PAGE and Western immunoblots (Fig. 1A). Moreover, the immunoreactive protein was an excellent substrate for cAMP-dependent protein kinase in vitro (Fig. 1B), it was enriched in microsomal fractions (not shown) and it was highly concentrated in the cerebellum (Fig. 1A). These are all properties that have been described for the neuronal isoform of the IP_3-receptor [2,16]. The immunoreactive 260 kDa protein therefore appears to represent this form of the IP_3-R.

When a membrane fraction from rat cerebellum was incubated with [γ-^{32}P]ATP in the presence of the catalytic subunit of cAMP-dependent protein kinase, the 260 kDa IP_3-receptor was strongly labelled (not shown). When this ^{32}P-labelled fraction was further incubated with a crude cytosol fraction from rat cerebellum, addition of 1 mM Ca^{2+} to the reaction mixture led to a rapid disappearance of the 260 kDa phosphoprotein (not

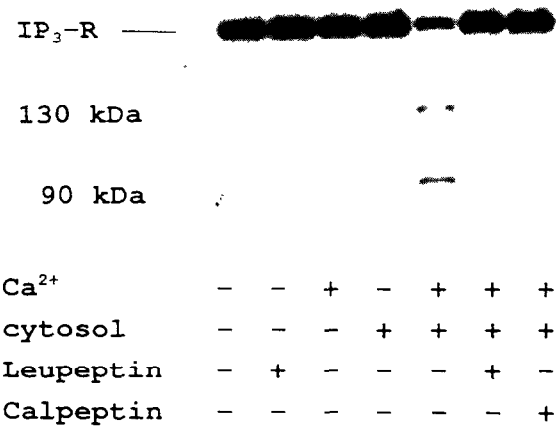

Fig. 2. Immunoblot of membrane preparations incubated with Ca^{2+}, cytosol, leupeptin and calpeptin. 200 μg membrane protein was incubated for 15 min at 30°C as described in the text. 35 μg of the proteins were separated by 7.5% SDS-PAGE and probed with anti-IP_3-receptor antibodies. The immunocomplexes were visualized by ^{125}I-protein A and autoradiography

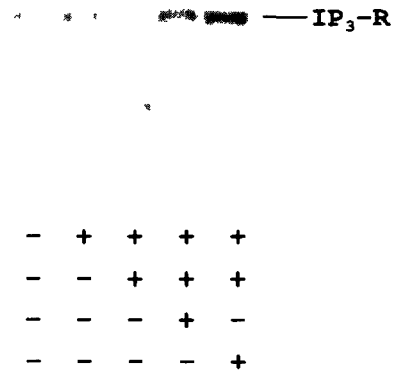

Fig. 3. Immunoblot of immunopurified IP_3-receptor incubated in the presence or absence of cerebellar cytosol, Ca^{2+}, leupeptin and 50 μg/ml calpeptin for 15 min at 37°C as indicated. Following incubation, the proteins were separated by 7.5% SDS-PAGE and probed with anti-IP_3-receptor antibodies and ^{125}I-protein A as described.

shown). Analysis of the same samples by immunoblotting indicated that this disappearance was due to a degradation of the immunoreactive protein, and not to a Ca^{2+}-regulated dephosphorylation of the IP_3-R catalyzed by e.g. calcineurin (Fig. 2). Ca^{2+}-induced degradation was accompanied by a simultaneous increase in two immunoreactive protein bands with apparent molecular masses of 130 and 95 kDa (Fig. 2).

These observations indicated that rat cerebellum contained Ca^{2+}-regulated enzymes capable of degrading the IP_3-receptor. To examine the mechanisms involved, we used antibodies coupled to protein A-Sepharose beads to affinity-purify the IP_3-receptor from rat cerebellar membranes. When such immunocomplexes were incubated with cerebellar cytosol, addition of 1 mM Ca^{2+} to the reaction mixture induced a rapid degradation of the purified IP_3-receptor, with a main 130 kDa protein band being generated as an immunoreactive product (Fig. 3). Addition of 10 μg/ml leupeptin, an inhibitor of thiol-proteases, completely prevented the Ca^{2+}-induced degradation of the IP_3-receptor. In contrast, addition of 0.1 mM PMSF or 10 μg/ml trypsin inhibitor, both of which are inhibitors of serine proteases, as well as 10 μg/ml pepstatin, an inhibitor of lysosomal proteases, did not prevent the Ca^{2+}-induced degradation of the IP_3-receptor (not shown).

These observations indicated that the IP_3-receptor might be a substrate for the Ca^{2+}-activated neutral thiol-dependent protease calpain, [21–23]. This question was further examined by in vitro incubations with purified components. Addition of the purified 80 kDa calpain subunit to the immunopurified IP_3-receptor led to a rapid and efficient degradation of the receptor 260 kDa protein (Fig. 4). Finally, addition of 50 μg/ml calpeptin, a specific inhibitor of calpain [24], completely prevented the Ca^{2+}-induced degradation of the IP_3-receptor catalyzed by cerebellar cytosol (Figs. 2, 3).

4. DISCUSSION

The present study has shown that the IP_3-receptor, an intracellular membrane protein responsible for release of Ca^{2+} from intracellular stores in response to a variety of extracellular signals [1,3], is a target for Ca^{2+}-regulated proteolysis. Moreover, our results suggest that the enzyme responsible for the degradation of the IP_3-receptor belongs to the calpain family. These nonlysosomal proteolytic enzymes are active at physiological pH and require either millimolar or micromolar [Ca^{2+}] for activation [21–23]. It is known that Ca^{2+} can induce autolytic cleavage of calpains, thereby increasing the sensitivities of these enzymes so that they become responsive to physiological [Ca^{2+}] fluctuations [23]. Hence, it is possible that the levels of Ca^{2+} achieved inside cells, either in response to increases in [IP_3] or in response to opening of plasma membrane Ca^{2+}-channels, could induce degradation of the IP_3-sensitive Ca^{2+}-channel itself.

The functional consequences of calpain activation are not fully understood. When compared to the predomi-

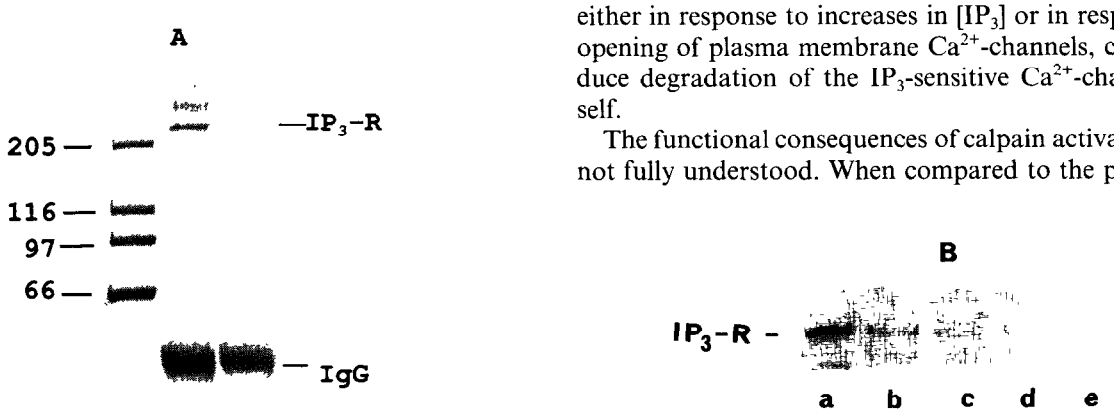

Fig. 4. Effect of purified calpain on IP_3-receptor. (A) Immunopurified IP_3-receptor was treated with 1 U calpain and 1 mM Ca^{2+} for 1 min at 37°C, separated by SDS-PAGE and visualized by Coomassie blue staining. (a) Control, (b) calpain + Ca^{2+}. (B) Immunoblot of immunopurified IP_3-receptor following treatment with 1 U purified calpain at 37°C for the following periods: (a) 0 s, (b) 10 s, (c) 30 s, (d) 1 min, (e) 2 min. The reactions were terminated by SDS-containing buffer. IP_3-receptor was separated by SDS-PAGE, transferred to nitrocellulose, probed with rabbit anti-IP_3- receptor antiserum and visualized by horseradish peroxidase and diaminobenzidine as described.

nating lysosomal proteases present in most cells, the calpains display a relatively narrow substrate specificity [23]. However, a number of proteins related to the metabolism or functions of cellular Ca^{2+} have been reported to be calpain substrates. These include both membrane proteins involved in Ca^{2+}-regulation such as Ca^{2+}-ATPase and the ryanodine receptor [25–28], and enzymes important in Ca^{2+}-regulated signal transduction such as protein kinase C and phospholipase C [23,29,30]. In most cases, calpain-induced degradation of these proteins is believed to terminate their functions. However, calpain-catalyzed limited proteolysis of some of these substrates, e.g. protein kinase C and the Ca^{2+}-ATPase, can generate active forms of these proteins [25,29]. The Ca^{2+}-induced degradation of the IP_3-receptor observed in this study generated two fragments of 130 and 95 kDa. Since these fragments retained immunoreactivity towards a C-terminal-specific antibody, they presumably are derived from that part of the receptor where the Ca^{2+}-channel domain is located.

It has recently been proposed that increases in cytosolic Ca^{2+} may act directly upon the IP_3-receptor to stimulate Ca^{2+}-release, presumably acting as a positive feedback system [31,32]. Whether the Ca^{2+}-regulated cleavage of the cytosolic domain of the IP_3-receptor observed in this study could lead to such increased functional activities of the Ca^{2+}-channel domain of the receptor remains to be investigated.

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