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Inositol trisphosphatase and bisphosphatase activities in the retina of crab

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Inositol trisphosphate appears to be an excitatory second messenger in the transduction cascade of invertebrate visual photoreceptors. The high time-resolution of visual transduction demands an efficient system for the removal of the second messenger. It is now demonstrated that soluble extracts of crab retina promote rapid magnesium-dependent release of inorganic phosphate from D-*myo*-1,4,5,-inositol trisphosphate. Experiments in which the inositol trisphosphate had been labelled with ^{32}P in the 4′ and 5′ positions indicated that both inositol trisphosphatase and bisphosphatase activities are present. The breakdown involves loss of at least one of the pair of vicinal phosphates, which is sufficient to inactivate the compound.

Inositol trisphosphate; Inositol trisphosphatase; Retina, Invertebrate; Visual transduction; *(Leptograpsus variegatus)*

1. INTRODUCTION

When *myo*-inositol 1,4,5-trisphosphate ($InsP_3$) is pressure injected into the ventral photoreceptors of *Limulus* it excites them in a manner similar to light [1,2]. Devary et al. [3] have shown that a light- and G-protein-activated phospholipase C is present in fly photoreceptor membranes. However, the precise physiological role of $InsP_3$ in visual transduction by rhabdomeral photoreceptors remains controversial [4,5]. If $InsP_3$ is part of this pathway then the synthetic and degradative pathways of the phosphoinositide cycle should be especially well-developed in rhabdomeric photoreceptors. In particular, good temporal resolution by these visual systems requires that the effective concentration of any diffusible messenger can be reset to the resting level very rapidly after a transient stimulus. In other systems $InsP_3$ degradation usually proceeds by initial removal of the 5′-phosphate [6–9] or by phosphorylation to inositol tetrakisphosphate [10] followed by specific dephosphorylation [11]. The role of inositol trisphosphatase activity was investigated because, in the case of the *Limulus* ventral photoreceptor, it is known that inositol 1,4-bisphosphate is physiologically inactive [1]. Retinal $InsP_3$ase activity has previously been observed in crab [12] and fly [3]; here data are presented on the specificity and sub-cellular distribution of inositol trisphosphatase activity in the invertebrate retina.

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2. MATERIALS AND METHODS

2.1. *D*-myo-*inositol 1,4,5-trisphosphate*

$InsP_3$ and [^{32}P]$InsP_3$, prepared from human erythrocytes [6,13], were the kind gift of Dr R.F. Irvine. $InsP_3$ was also obtained from Sigma.

2.2. *Preparation of retinal extracts*

Retinae of the crab *Leptograpsus variegatus* were dissected and homogenised by standard procedures described previously [12,14]. Crude S12 fractions were prepared by centrifugation of the homogenate at approx. 12000 × *g* for 2 min. S120 refers to the supernatant after centrifugation for 90 min at 120000 × *g*: the P120 fraction was prepared by resuspending the pellet from the high-speed spin in the original volume of dissection buffer.

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Red screening lipid was removed from the S120 fraction by repeated filtration through Whatman GF/C glass microfibre filters to yield the 'GF/C' fraction.

2.3. *Assay of phosphate release from inositol trisphosphate*
For details of the assays see [14].

2.4. *Analysis of breakdown products of $InsP_3$*
Duplicate assays were performed in a final volume of 250 μl using a Tris-sucrose buffer, pH 7.5 [14]. The substrate was 100 μM [^{32}P]$InsP_3$ containing approx. 8000 dpm per assay tube, predominantly in the 5'-phosphate with none in the 1'-phosphate [6,13]. The assay was started by the addition of 20 μl aliquots of an S12 fraction of crab retina and allowed to proceed for 15 or 30 min. At the end of this time the assay was stopped by the addition of 250 μl of 12% (w/v) trichloroacetic acid. Samples were incubated on ice for 30 min before the precipitated protein was pelleted at approx. 12000 × *g*. The samples were diluted to 2.5 ml with distilled water and neutralized with a small volume of 0.1 M NaOH. Each sample was loaded onto a 0.5 cm Dowex-formate column in a pasteur pipette. The columns were washed with 1 ml distilled water and eluted with 7.5 ml fractions according to the regime of Downes and Michell [13] in order to separate inositol trisphosphate from the bisphosphates and monophosphates. The ammonium formate/formic acid eluates were collected in scintillation vials and the distribution of radioactivity amongst the fractions was determined by Cerenkov counting.

2.5. *Determination of total endogenous phosphate*
Duplicate samples of up to 40 μl were pipetted into acid-washed test-tubes and dried under vacuum. The samples were digested with 5 M sulphuric acid and 60% perchloric acid [15]. 125 μl of distilled water was added to the digested samples followed by 650 μl of ammonium molybdate-Malachite Green reagent [16] modified according to [14]. After 10 min the absorbances of the samples were read at 660 nm. Phosphate concentration was calculated by reference to inorganic phosphate standards, similarly processed.

2.6. *Time courses of $InsP_3$ hydrolysis by retinal fractions*
The substrate was 45 μM inositol 1,4,5-trisphosphate, assay buffer conditions were as stated previously. Assays were stopped at 5 min intervals with an equal volume of 12% (w/v) trichloroacetic acid, centrifuged and the amount of phosphate released into the supernatant was measured [16].

3. RESULTS

A homogenate of crab retina was centrifuged at 12000 × *g* for 2 min and the supernatant was assayed for phosphate release in the presence of 100 μM $InsP_3$ and 10 mM magnesium chloride. In a typical experiment, 36 μg of protein released 39 nmol of inorganic phosphate from 25 nmol of $InsP_3$ in a volume of 250 μl over 30 min at 25°C. The total phosphate of tissue origin present in the assay was estimated as 4.5 nmol by total acid hydrolysis. $InsP_3$ was completely stable for up to 1 h if no extract was added to the assay. These data suggest that enzymes in the retina are capable of degrading $InsP_3$ to inositol monophosphate. One cannot exclude the possibility that the inositol tris/tetrakisphosphate [10] pathway operates in crab retinae but the relatively low content of endogenous phosphate precludes the possibility that such a pathway is significant under the assay conditions used.

S12-mediated hydrolysis of ^{32}P-labelled $InsP_3$ was investigated in order to confirm the source of the inorganic phosphate release and to determine the extent and specificity of the $InsP_3$ breakdown. The breakdown products were separated by Dowex-formate column chromatography into three fractions which comprised: inositol trisphosphate, inositol bisphosphates and a fraction including inositol monophosphates and inorganic phosphates [7,13]. As shown in fig.1, the S12 fraction of crab retina promoted $InsP_3$ hydrolysis in a time- and magnesium-dependent manner. The combined action of inositol trisphosphatase and inositol bisphosphatase activities removes at least one of the vicinal phosphates, which would be expected to terminate biological activity of the molecule [1,6]. The data showing that radioactivity is found in the $InsP_2$ fraction after 15 min in the presence of magnesium indicates that initial phosphate removal is not confined to the 5'-position because the ratio of 5'- to

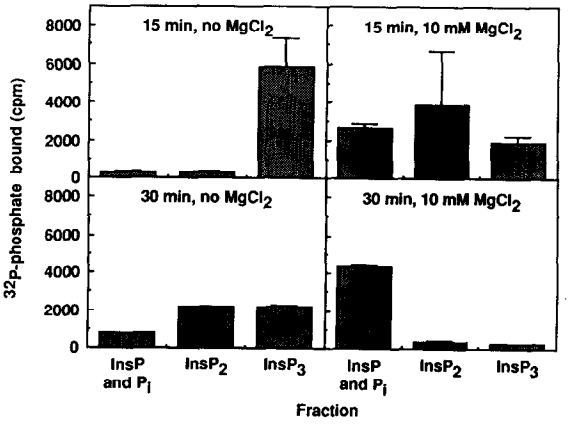

Fig.1. Fractionation of the products of inositol 1-[^{32}P]-4,5-trisphosphate after incubation, under the stated conditions, with a crude S12 supernatant of crab retina. Error bars represent the standard deviations of duplicate assays.

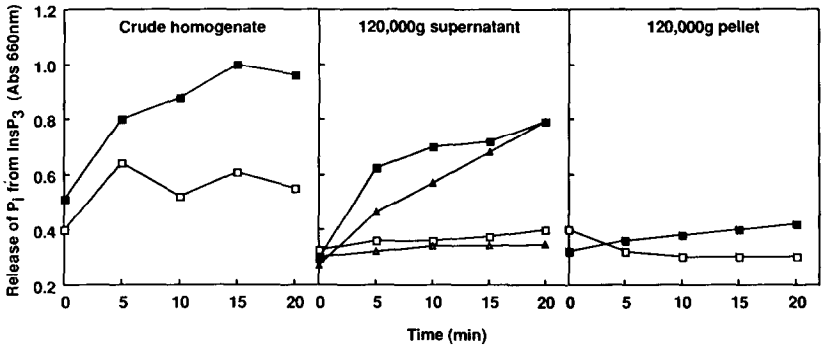

Fig.2. Release of inorganic phosphate from inositol 1,4,5-trisphosphate by fractions derived from a homogenate of crab retina. Assays were performed in the presence (●) or absence (○) of 10 mM $MgCl_2$. (▲, △) Filtered samples.

4′-labelling achieved by the method of Downes and Michell is of the order of 3:1 [6]. Whether the degradation also involves the action of an inositol monophosphatase cannot be determined from these data.

Both membrane bound and cytosolic inositol trisphosphatases have been described and are known to coexist in various mammalian preparations [6,8,17]. The S12 fraction, used as the source of the inositol trisphosphatase in the experiments described so far, contains both membrane and cytosolic components and cannot be used to provide information regarding the distribution of inositol trisphosphatase. Fig.2 shows the time courses of magnesium-independent and magnesium-stimulated $InsP_3$ hydrolysis in authentic membrane and cytosolic fractions prepared from a crude homogenate of crab retina. After high-speed centrifugation the inositol trisphosphatase was recovered largely in the S120 fraction. This is a novel finding in the invertebrate retina and it raises the question of the functional significance of the two types of activity. There was minimal loss of activity following glass microfibre filtration to remove the red screening lipid.

4. DISCUSSION

In order to assign second-messenger status to a particular compound it is helpful to demonstrate the presence of the relevant synthetic and degradative enzymes in the tissue. The data presented on inositol trisphosphatase and bisphosphatase activities are consistent with the hypothesis that inositol trisphosphate is a second-messenger in the invertebrate rhabdomeric retina [1,2]. The observation [3] that there is a membrane-bound inositol trisphosphatase in invertebrate retina is confirmed; however, in the crab, the membrane-bound activity represents only a small proportion of the total. It would be helpful to determine the subcellular distribution of breakdown enzymes for $InsP_3$ in situ, in order to understand the spatial organisation of the transduction machinery. This will require the generation of specific antisera against the isolated phosphatase enzymes.

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REFERENCES

[1] Fein, A., Payne, R., Corson, D.W., Berridge, M.J. and Irvine, R.F. (1984) Nature 311, 157–160.
[2] Brown, J.E., Rubin, L.J., Ghalayini, A.J., Tarver, A.P., Irvine, R.F., Berridge, M.J. and Anderson, R.E. (1984) Nature 311, 160–163.
[3] Devary, O., Heichal, O., Blumenfeld, A., Cassel, D., Suss, E., Barash, S., Rubinstein, C.T., Minke, B. and Selinger, Z. (1987) Proc. Natl. Acad. Sci. USA 84, 6939–6943.
[4] Fein, A. (1986) Trends Neurosci. 9, 110–114.
[5] Johnson, E.C., Robinson, P.R. and Lisman, J.E. (1986) Nature 324, 468–470.
[6] Downes, C.P., Mussat, M.C. and Michell, R.H. (1982) Biochem. J. 203, 169–177.
[7] Berridge, M.J., Dawson, R.M.C., Downes, P., Heslop, J.P. and Irvine, R.F. (1983) Biochem. J. 212, 474–482.

[8] Storey, D.J., Shears, S.B., Kirk, C.J. and Michell, R.H. (1984) Nature 312, 374–376.
[9] Connolly, T.M., Bross, T.E. and Majerus, P.W. (1985) J. Biol. Chem. 260, 7868–7874.
[10] Irvine, R.F., Letcher, A.J., Heslop, J.P. and Berridge, M.J. (1986) Nature 320, 631–634.
[11] Burgess, G.M., Kinney, J.S., Irvine, R.F. and Putney, J.W. (1985) Biochem. J. 232, 237–243.
[12] Trowell, S.C. (1988) Comp. Biochem. Physiol. 89B, 285–297.
[13] Downes, C.P. and Michell, R.H. (1981) Biochem. J. 198, 133–140.
[14] Trowell, S. (1985) Eur. J. Cell Biol. 36, 277–285.
[15] Hess, H.H. and Derr, J.E. (1975) Anal. Biochem. 63, 607–613.
[16] Lanzetta, P.A., Alvarez, L.J., Reinach, P.S. and Candia, O.A. (1979) Anal. Biochem. 100, 95–97.
[17] Joseph, S.K. and Williams, R.J. (1985) FEBS Lett. 180, 150–154.