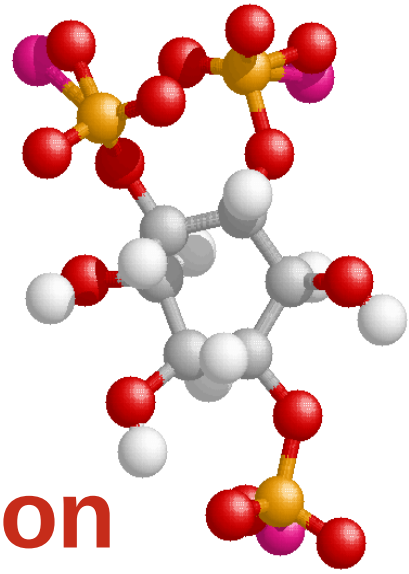
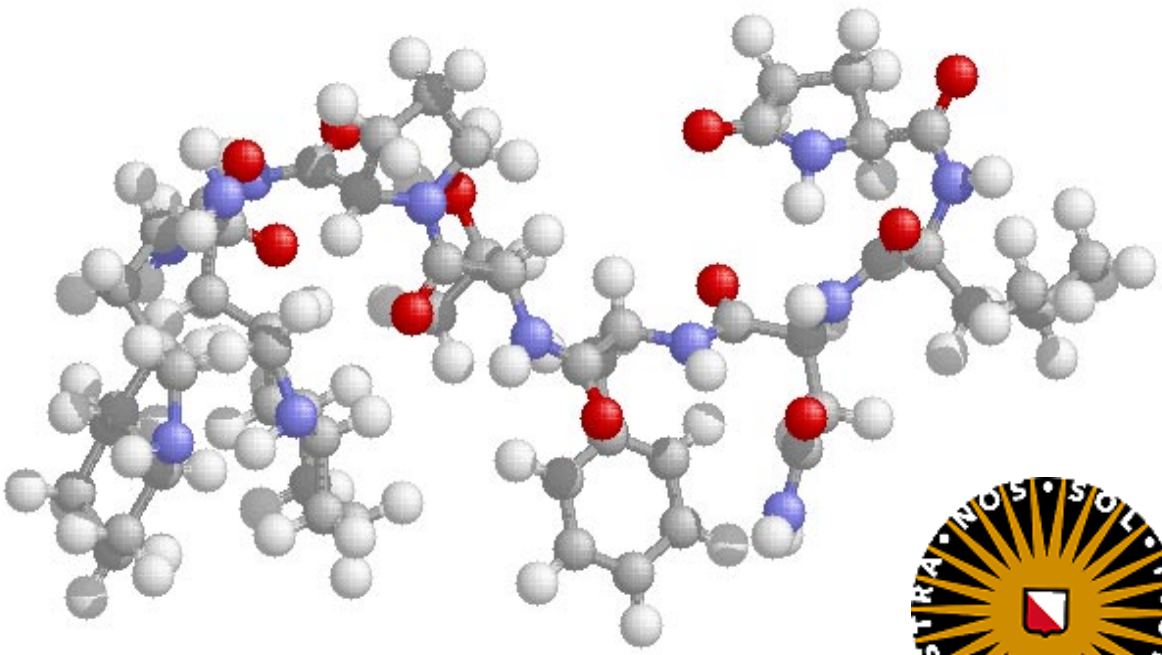


The role of inositol phosphates in the signal transduction of AKH-I, -II and -III in the locust fat body



Jeroen de Meijer
Jun-1995/Aug-1996

Illustrations on front page:
Top Right: Inositol-(1,4,5)-trisphosphate.
Bottom: *Locusta migratoria* Adipokinetic Hormone III (Lom-AKH-III).

The role of inositol phosphates in the signal transduction of AKH-I, -II and -III in the locust fat body

The role of inositol phosphates in the signal transduction of AKH-I, -II and -III in the locust fat body

J. de Meijer 1996

A report of a 15 month research period at the Biochemical Physiology Research Group, Department of Experimental Zoology, University of Utrecht, under convoy of ir. S.F. Vroemen.

Abstract

Inositol phosphates play an important role in the locust adipokinetic hormone (AKH) signal transduction. They induce the release of calcium from intracellular stores such as the endoplasmatic reticulum. This release results in a netto influx of extracellular calcium. Calcium plays an important role in the regulation of the glycogen phosphorylase activity.

Inositol phosphates are formed by hydrolysis of phosphatidyl inositol phosphate into inositol(1,4,5)-trisphosphate ($Ins(1,4,5)P_3$) and diacylglycerol by phospholipase C (PLC). $Ins(1,4,5)P_3$ can then be transformed into a range of other InsPs, with 1 to 6 phosphates.

Goals of this study were to elucidate the role of the different InsPs, and also of PLC, in the signal transduction of the three locust AKHs.

This was done in various ways:

-Determination of total and individual InsP formation after addition of AKH. This was done using radioactive labelled inositol and seperation of InsPs on an ion exchange column.

-Determination of the $Ins(1,4,5)P_3$ concentration after addition of AKH using a radio-immuno assay.

-Involvement of PLC was investigated with the use of U73122, a PLC inhibitor, and evaluating its effect on InsP formation and glycogen phosphorylase activition.

The results show that AKH is able to induce a rise in InsP levels already after 15 seconds, and maximally after 1 minute, although initial rises induced by AKH-II are smaller than those generated by AKH-I and -III. AKH-I is the only AKH for which the InsP levels return to basal after 2.5 minutes. After approximately 5 minutes, a second, probably non-phisiological, increase occurs.

Both AKH-I and -III show a high level of $InsP_3$ at 1 minute that slowly decreases in time. With AKH-III however, after 15 minutes mostly $InsP_1$ and $InsP_2$ were left, while AKH-I-induced fat bodies end up with mostly increased $InsP_4$ and $InsP_{5\text{-}6}$ levels, as do AKH-II-stimulated ones. At 15 minutes $InsP_3$ levels are still high only for AKH-III.

Very suprisingly, AKH-II shows only rises in $InsP_{5\text{-}6}$ after 1 minute, and only a minor part is $InsP_3$ at 2.5 minutes. This is supported by the radio-immuno assay, which shows almost no rise of $Ins(1,4,5)P_3$ concentrations for 40 nM AKH-II at 1 minute. This could suggest a role in signal

transduction for the high InsPs. At 4 nM though, AKH-II was equally potent as AKH-I and -III. The radio-immuno assay also showed that the effect of AKH-I and -III on Ins(1,4,5)P_3 levels is dose-dependent.

The U73122 assay shows that inhibition of PLC lowers the glycogen phosphorylase activity as well as the InsP levels. This suggests that AKH signal transduction towards glycogen phosphorylase involves PLC and InsPs.

Contents

Abstract 5

Introduction 9

1.1 The fat body 9
1.2 The adipokinetic hormones 9
1.3 Glycogen phosphorylase 10
1.4 The role of cyclic AMP 10
1.5 The role of intra- and extracellular calcium 11
1.6 The role of inositol phosphates 13

Materials and Methods 15

2.1 Animals 15
2.2 Chemicals 15
2.3 Preparation of fat body 16
2.4 Total InsP measurement in the fat body 16
2.5 Determination of salt concentrations needed for inositol phosphates elution 17
2.6 Measurement of individual InsP in the fat body 17
2.7 Measurement of radioactivity 18
2.8 Data interpretation InsP measurements 18
2.9 Measurement of Ins(1,4,5)P_3 levels in the fat body ... 18
2.10 Protein determination 19
2.11 Involvement of phospholipase C in glycogen phosphorylase activation 20
2.12 Glycogen phosphorylase assay 20
2.13 Involvement of phospholipase C in InsP formation 20

Results 22

3.1 The effect of AKH on the formation of total InsPs ... 22
3.2 Determination of elutionbuffer concentrations of individual InsPs 24
3.3 The effect of AKH on the formation of the total InsPs 26
3.4 Ins(1,4,5)P_3 determination using a radio-immuno assay 28
3.5 Role of PLC in AKH-induced InsP formation and glycogen phosphorylase activition 29

Discussion and Conclusions 32

References 35
Supplement 38
Acknowledgements 39

1 Introduction

1.1 The fat body

The migratory locust (see ***Fig. 1***) is an insect capable of flying very long distances (about 400 kilometers) without resting. To accomplish this they are equiped with a very efficient energy metabolism. This is one of the reasons that it is such a good animal to do metabolic research on.

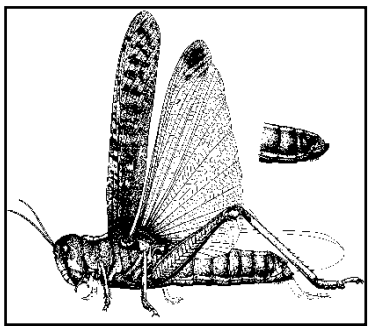


Figure 1;
The migratory locust, Locusta migratory migratorioides.

The locust uses both lipids and carbohydrates as fuels for flight. Both lipid and carbohydrate are stored in the fat body, an organ that occupies the major part of the abdomen and thorax. The carbohydrates are stored as glycogen and the lipids as triacylglycerol and both are mobilised upon flight activity. The fat body plays a major role in the energy metabolism of the locust as it incorporates the functions of vertebrate adipose tissue and liver.

1.2 The adipokinetic hormones

When the insect starts to fly, the cerebral ganglion in the brain stimulates the corpus cardiacum glandular lobe, a neuroendocrine gland, via the nervi corporis cardiaci-I and -II, to release adipokinetic hormones (AKHs) into the hemolymph. These hormones induce the mobilisation of trehalose and diacylglycerol from the fat body by activating triacylglycerol lipase, which hydrolyses the stored triacylglycerol to diacylglycerol, and glycogen phosphorylase, which catalyses the breakdown of stored glycogen to trehalose, resulting in an increased level of available energy in the hemolymph.

There are three adipokinetic hormones, -I, -II and -III, which are all released into the hemolymph upon flight activity. AKH-I is a decapeptide and AKH-II and -III are octapeptides. All AKHs are C- and N-blocked (see ***Fig. 2***). They are all able to mobilise trehalose and diacylglycerol.

Figure 2;
The amino acid structure of the three AKHs.

Lom-AKH-I	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2
Lom-AKH-II	pGlu-Leu-Asn-Phe-Ser-Ala-Gly-Trp-NH_2
Lom-AKH-III	pGlu-Leu-Asn-Phe-Thr-Pro-Trp-Trp-NH_2

Up to now, no reason for the need for three AKHs has been found, although the concomitant release of structurally related hormones is found in more animals like the lamprey [Sower 1993] and the sea bream [Powell

1994]. This study might shed a light on the reason for the presence of three AKHs in locust.

1.3 Glycogen phosphorylase

The activity of glycogen phosphorylase is easy to determine and serves well as a measure for AKH activity. This in contradiction to triacylglycerol lipase, for whose activity no reliable assay is available yet. Glycogen phosphorylase is the main, rate limiting, enzyme in the conversion of glycogen to trehalose, and occurs in three forms; an A, B and AB form (see ***Fig. 3***). The A form has two phosphates, the AB form one and the B form none. The activity depends on the amount of phosphorylation, the A form being the most active. The different forms can be converted into one another by phosphorylase kinase and phosphatase. The activity also depends on the amount of AMP, wich can activate the B and AB form allosterically, but not the A form.

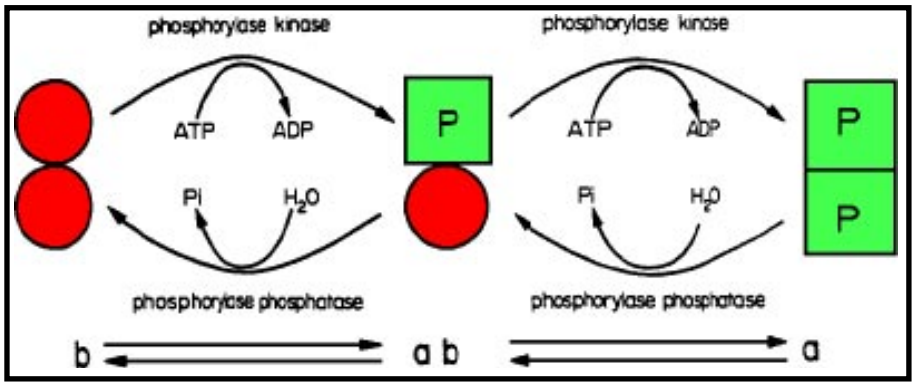


Figure 3;
The different forms of phosphorylase can be converted into each other reversibly.

The activation of glycogen phosphorylase is quite complex and involves G-proteins, calcium, cyclic AMP as well as inositol phosphates (InsPs). Previous studies, though, have elucidated a lot of its secrets.

1.4 The role of cyclic AMP

Upon binding, AKH is able to activate adenylyl cyclase, which results in a rise in the intracellular cyclic AMP level [Vroemen 1995b]. This cyclic AMP is then able to activate glycogen phosphorylase. At a physiological dose of 4 nM there is a difference in potency between the AKHs (AKH-III > AKH-II > AKH-I) in elevating cAMP levels, and accordingly in glycogen phosphorylase activition, although no difference was found at 40 nM [Vroemen 1995b].

The enzyme responsible for the formation of cAMP is adenylyl cyclase (AC). This enzyme can be activated by the alpha subunit of a G_s-protein,

and inhibited by the alpha subunit of a G_i-protein [Gilman 1995]. A G-protein is a trimeric protein with an alpha, beta and gamma subunit. Upon activation the alpha subunit dissociates from the beta-gamma subunit. The alpha subunit is then able to activate other proteins. The beta-gamma subunit is thought to play a role in the signal transduction as well, but not much is known about it yet. Experiments with cholera toxin (CTX), which irreversible activates G_s, and pertussis toxin (PTX), which irreversible inactivates G_i (thus keeping adenylyl cyclase active), have shown that CTX is able to cause a significant rise in intracellular cAMP levels whereas PTX has no effect [Vroemen 1995b]. This suggests that the signal transduction of AKH to AC is mediated through G_s and not through G_i. Moreover the effects of CTX and AKH on cAMP levels were not additive, suggesting that AKH and G_s act via the same pathway.

1.5 The role of intra- and extracellular calcium

Calcium plays an important role in the signal transduction towards glycogen phosphorylase. Without extracellular calcium, AKH isn't able to activate glycogen phosphorylase and to raise cAMP levels [Vroemen 1995a]. Extracellular calcium itself though, in the absence of AKH, is not able to alter the activity of glycogen phosphorylase.

Vroemen et al. [1995a] and van Marrewijk et al. [1993] have shown that AKH is able to realise a rise in the influx as well as the efflux of calcium in the fat body, although the influx is larger then the efflux. Van Marrewijk [1993] also showed that AKH not only requires the influx of extracellular calcium to activate glycogen phosphorylase, but the release of calcium from intracellular stores as well. Several studies have proposed that the depletion of the intracellular calcium pools in some way leads to an influx of extracellular calcium. This mechanism, called capacitative calcium entry, has been shown to occur in vertebrate cells [Putney 1986, Putney 1990, Berridge 1995]. The depletion itself triggers the entry of exracellular calcium. The problem with this theory is: how do the calcium pools 'talk' to the calcium channels in the membrane? Irvine suggested a model in which there is a direct link between two receptors, one being an inositol-trisphosphate ($InsP_3$) receptor in the membrane of the calcium pools which is modulated by both calcium and $InsP_3$ (see ***Fig. 4***)[Irvine 1990, Irvine 1991, Irvine 1992]. This receptor is bound to a protein in the plasma membrane that is controlling calcium entry. The

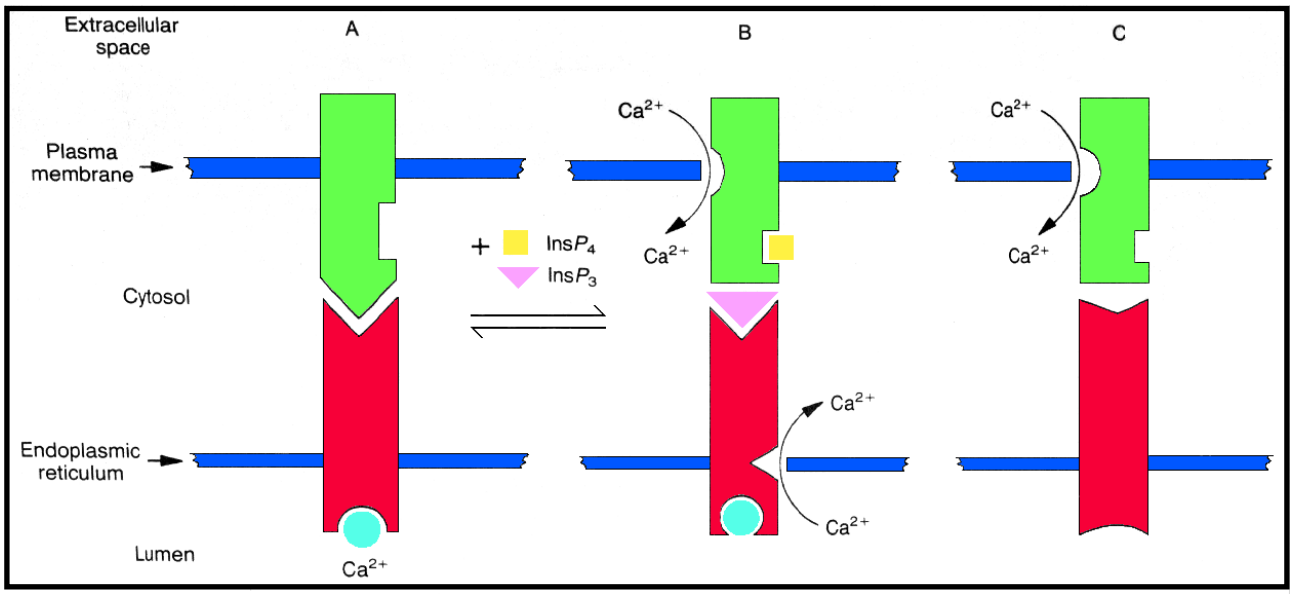


Figure 4;
The model of Irvine. Two bound proteins (A) are disconnected when both $InsP_3$ and $InsP_4$ bind (B), upon which emtying of the pools and influx of extracellular calcium occurs. When $InsP_3$ dissociates calcium influx only occurs when luminal calcium levels are low (C).

receptors dissociate if calcium levels in the pool are low or $InsP_3$ is high, resulting in calcium entry. It has been shown that inositol-tetrakisphosphate ($InsP_4$) is able to modulate calcium entry in lacrimal acinar cells [Changya 1989a, Changya 1989b]. $InsP_4$ could modulate calcium entry by interacting directly (or indirectly) with the membrane-bound protein.

The influx of extracellular calcium is probably needed for the activation of a calcium dependent AC, resulting in the production of cAMP. cAMP itself, however, is able to activate glycogen phosphorylase in the absence of extracellular calcium [Van Marrewijk 1993].

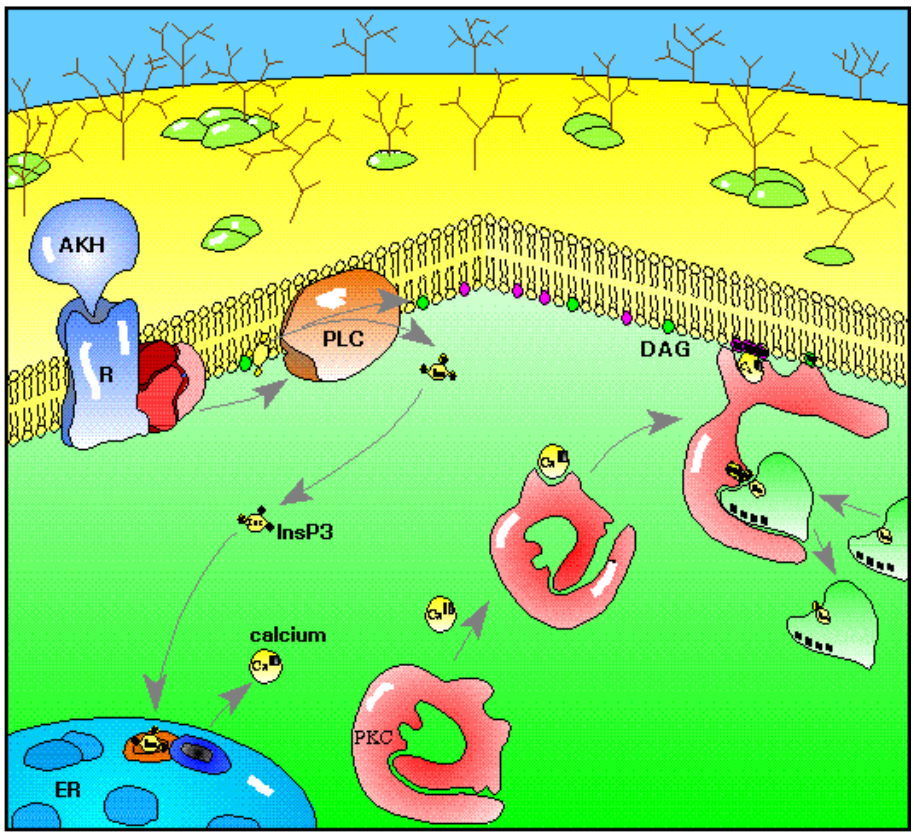


Figure 5;
The role of DAG (green dots in the membrane) and Ins(1,4,5)P_3 (yellow) on PKC (red).PLC, phospholipase C; R, AKH receptor; PKC, protein kinase C; ER, endoplasmatic reticulum.

1.6 The role of inositol phosphates

AKH also activates phospholipase C (PLC). The activation of PLC by AKH is thought to be mediated by a G-protein, but this has not yet been demonstrated in the locust. PLC is able to hydrolyse phosphatidylinositol-4,5-bisphosphate (PtdIns) into Ins(1,4,5)P_3 and diacylglycerol (DAG). DAG plays an important role in the activation of protein kinase C (PKC), which can activate a broad range of proteins. PKC also needs calcium for its activation (see ***Fig. 5***).

InsPs are continuously reused in an inositol-cycle, from inositol through PtdIns and numerous forms of InsPs, back to inositol (see ***Fig. 6***)[Batty 1989]. This reconversion of InsPs to inositol can by blocked by LiCl.

Ins(1,4,5)P_3 is able to empty intracellular calcium stores, like the endoplasmatic reticulum [Berridge 1989]. So, Ins(1,4,5)P_3 could be the trigger for the influx of extracellular calcium, as explained before. This would mean that Ins(1,4,5)P_3 (or other InsPs) plays a crucial role in the regulation of glycogen phosphorylase.

We constructed a model for the signal transduction of AKH in the locust fat body (see ***Fig. 7***). Briefly, AKH binds to its receptor. The receptor activates a stimulatory G-protein. This G-protein is able to activate adenylyl cyclase which will result in a rise in cAMP levels in the cytoplasm. The receptor also activates, probably also via a G-protein, PLC. PLC will hydrolyse PtdIns into DAG and Ins(1,4,5)P_3 and Ins(1,4,5)P_3 will empty the intracellular calcium stores, which will result in a net influx of extracellular calcium. This calcium in combination with the rise in cAMP levels is able to activate glycogen phosphorylase, which indirectly results in the release of trehalose into the hemolymph. The aim

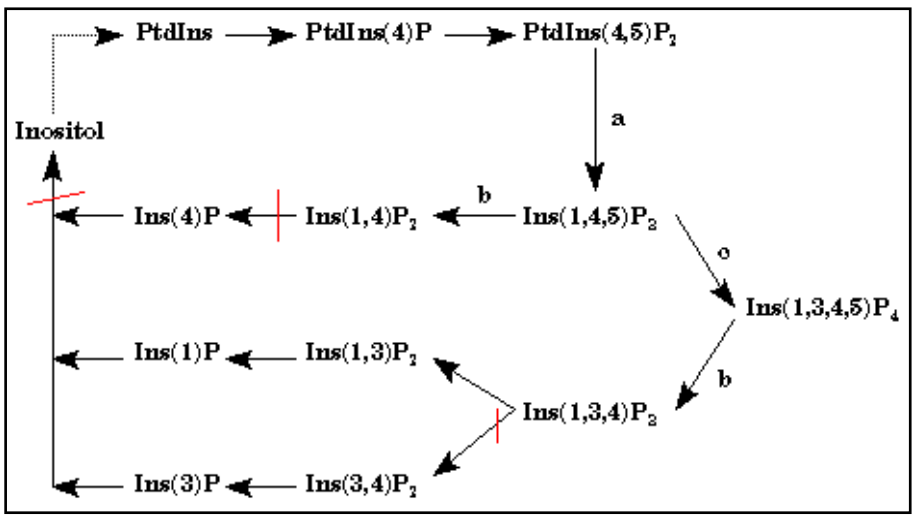


Figure 6;
Inositol-cycle: reconversion of InsPs to inositol via different pathways. a=PLC; b=5-fosfatase; c=3-kinase. Red lines indicate the point of action of Li^+.

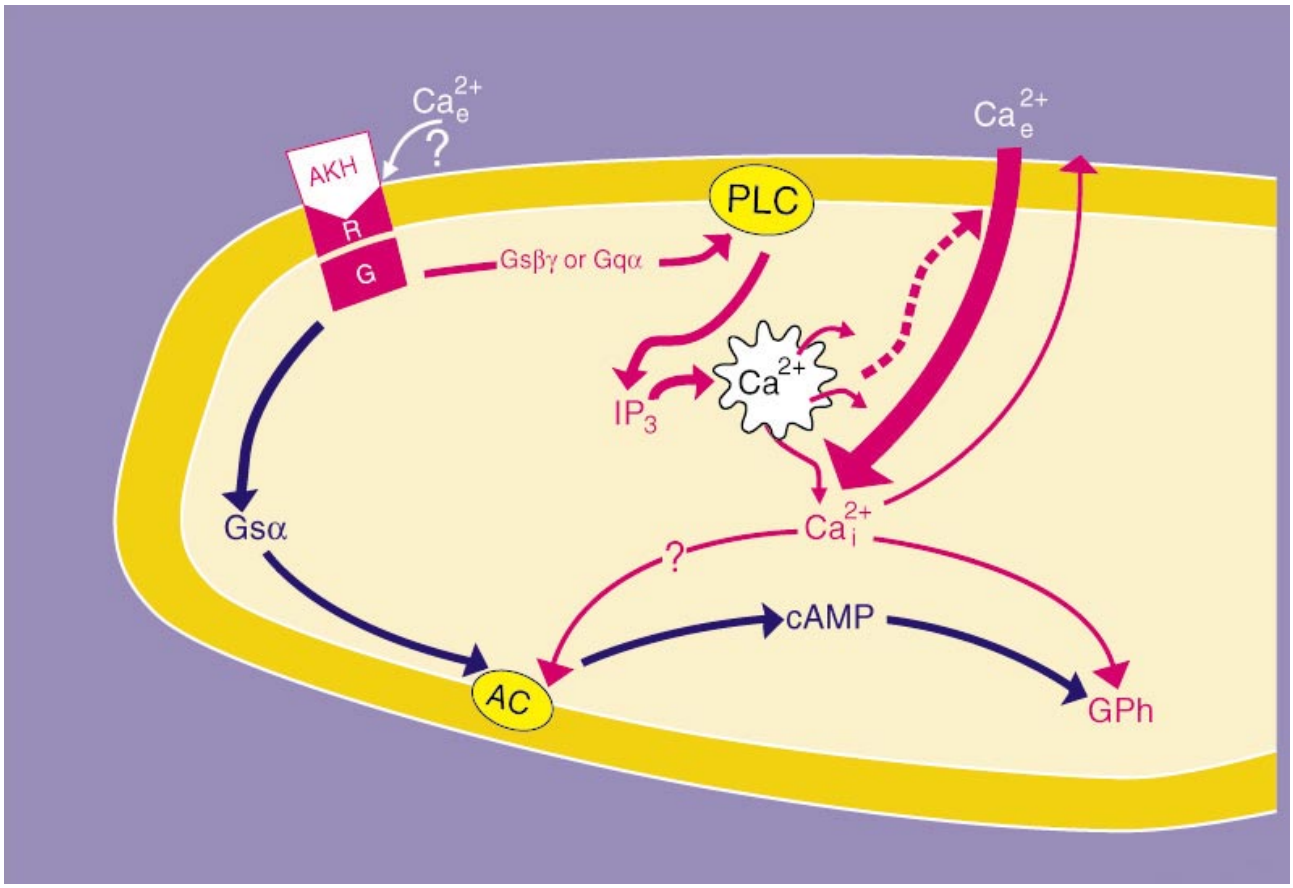


***Figure 7**;*
Our proposed model for the AKH signal transduction in the locust fat body. AC, adenylyl cyclase; GPh, glycogen phosphorylase; IP_3, Ins(1,4,5)P_3; G, G-protein; R, AKH-receptor.

of this study is to look specificly at the role that InsPs play in the signal transdcution of all three AKHs in the migratory locust.

2 Materials and Methods

2.1 Animals

The adult males of the migratory locust, *Locusta migratoria migratorioides* were used, 15 to 16 days after imaginal ecdysis. The animals were raised at a temperature of 30 °C and a relative humidity of 40% in crowded conditions with 12 hours of light a day. The animals were fed daily with reed grass and oatmeal [Van Marrewijk 1983].

2.2 Chemicals

AKH-I and -II (synthetic) were purchased from Peninsula Laboratories (San Carlos, CA, U.S.A.). AKH-III was produced as described by Oudejans [1991]. Adenosine phosphates came from Boehringer Mannheim B.V. (Almere, The Netherlands) like most biochemicals and enzymes. Triethylammonium hydrogen carbonate buffer (TEAB) came from Fluka (Buchs, Switserland). The radioactive InsP standards, Ins(1)P_1 (13 Ci/mmol), Ins(1,4)P_2 (9,9 Ci/mmol), Ins(1,4,5)P_3 (21 Ci/mmol) and Ins(1,3,4,5)P_4 (21 Ci/mmol), were from Du Pont de Nemours ('s-Hertogenbosch, The Netherlands); myo[2-^{3}H] inositol (18 Ci/mmol) and the Ins(1,4,5)P_3-RIA-kit from Amersham ('s Hertogenbosch, The Netherlands) and the ion exchange columns (Waters Sep-Pak Accell Plus QMA) from Millipore Corporation (Milford, MA, U.S.A.). Emulsifier-Safe scintillation liquid came from Packard (Groningen, The Netherlands), penicillin/streptomycin (Pen/Strep)from Gibco BRL (Breda, The Netherlands) and U73122 was obtained from Calbiochem (Breda, The Neherlands). All other chemicals came from Baker (Deventer, The Netherlands) and Merck (Amsterdam, The Netherlands).

For experiments, stock solutions of the AKHs were used with a concentration of 20 pmol/µl. In these stocks AKH-I was dissolved in 10% methanol, AKH-II in 20% methanol and AKH-III in 10% methanol, 1 M NaAc. Adenosine phosphates were added from a stock solution with a concentration of 40 mM. Double glass distilled water (bidest) was used throughout all experiments.

2.3 Preparation of fat body

Legs, wings and the last two segments of the abdomen were removed from the locust. Next, the head was removed by turning it 180 degrees relative to the thorax, and pulling it out together with the intestines. The animal was cut open lengthwise on the ventral side and spread open with pins to paraffine under preparation medium (Medium II, see *Supplement*). The fat body was dissected in this medium and all residues of the respiratory system, Malpighian tubes and other tissues which did not belong to the fat body were removed. Next, the fat body was split into two equal lateral parts of which one served as a control, and the contralateral half was used for experimental treatment.

2.4 Total InsP measurement in the fat body

After preparation the half fat bodies were preincubated for 16 hours in a solution containing 1 µl myo[2-^{3}H] Inositol, 10 µl 100x Pen/Strep stock and 969 µl preincubation medium (Medium XV, see *Supplement*) (total volume 980 µl) in a 30 °C shaking waterbath.

After preincubation, a solution containing 2 µl AKH stock, 8 µl 2.5 M LiCl and 10 µl Medium XV was added to one half. To the other half, the same solution, however containing 2 µl of the solvent of the AKH (10 or 20 % methanol) was added and they were incubated for 15, 30, 60, 150, 300, 900 or 1800 seconds in a 30 °C shaking waterbath. The incubation was stopped by putting the fat body in liquid nitrogen. The frozen fat body was then sonificated during 40 seconds in a solution containing 940 µl chloroform:methanol (1:2), 250 µl bidest, 8 µl 10% TCA and 2 µl fytate hydrolysate.

The pH was set to 7.0 with 1 M KOH and/or 10% TCA. After 10 minutes of extraction with repeated shaking, 310 µl bidest and 310 µl chloroform were added to obtain a seperation between InsP in the waterphase, and PtdIns in the chloroformphase. After centrifugation at 12,000 ***g*** for 5 minutes, half of the upper phase (600 µl) was added to 9.4 ml water to create a 10 ml sample. This sample was run over an ion exchange column (Waters Sep-Pak Accell Plus QMA) which was eluted with 5 ml water, 10 ml 10 mM TEAB and 10 ml 500 mM TEAB respectively, at 0.8 ml/min. The 500 mM fraction, containing total InsP,was collected and 400 µl was counted in a scintillation counter. The columns were regenerated with 5 ml 2 M NaCl, 5 ml 50% methanol and 5 ml 500

mM TEAB respectively.

250 µl of the lower phase was washed in 1 ml of chloroform:water:methanol (3:47:48). After washing, 125 µl was taken off and the chloroform was evaporated by heating at 80 °C during 1 hour. The radioactivity in the residue, containing the PtdIns, was counted in a scintillation counter as described in paragraph 2.7, and was used to correct for the differences in size and/or preloading of the fat body halves.

2.5 Determination of salt concentrations needed for inositol phosphates elution

Fat bodies were treated as described in paragraph 2.4 until chromatography. The chloroformphase was discarded. To the sample (600 µl of the upper (water) phase with 9.4 ml water) 1 µl of each InsP standard was added (Ins(1)P_1, Ins(1,4)P_2, Ins(1,4,5)P_3, Ins(1,3,4,5)P_4). This sample was run over the ion exchange column. Next, a linear gradient of TEAB, stretching from 0 to 500 mM, in a volume of 60 ml, was run over the column at 0.8 ml/min using a gradient mixer. 2 ml fractions were collected and 400 µl of each fraction was counted in a scintillation counter. As a control, several samples containing different combinations of InsP standards were eluted stepwise with several combinations of TEAB concentrations.

2.6 Measurement of individual InsP in the fat body

Treatment of the fat bodies was the same as described in paragraph 2.4, with the following differences: the column was eluted with 5 ml water, 10 ml sample, 5 ml water, 10 ml 10 mM TEAB, 10 ml 110 mM TEAB, 10 ml 210 mM TEAB, 10 ml 310 mM TEAB, 10 ml 410 mM TEAB and 10 ml 500 mM TEAB, respectively (the concentrations as determinded in the experiment described in paragraph 2.5). The 110, 210, 310, 410 and 500 mM fractions ($InsP_1$, $InsP_2$, $InsP_3$ and $InsP_4$, respectively) were collected and 400 µl of each fraction was counted in a scintillation counter.

2.7 Measurement of radioactivity

To 400 µl of sample, 4 ml of Emulsifier-Safe was added. Samples from the total InsP measurement were counted 2 times for 2.5 minutes in a Packard 4550 liquid scintillation counter. Samples from the individual InsP measurement were counted 2 times for 5 minutes.

2.8 Data interpretation InsP measurements

The rises in InsP levels were calculated as followed: the amount of radioactive PtdIns is considered as a measure for the size of the fat body. In order to eliminate differences in the sizes of the fat body halves, the dpm of the InsP fraction is divided through the dpm of the corresponding PtdIns fraction. And because only about 1 % of the radioactive PtdIns is converted into InsP, no correction for conversion was made.

Then, to calculate the relative raise in InsP in relation to the control, InsP/PtdIns from the AKH-stimulated fraction was divided through InsP/PtdIns from the control fraction ("relative raise"). In order to calculate the absolute raise in InsP, instead of the relative, the $(InsP/PtdIns)_{Control}$ is substracted from $(InsP/PtdIns)_{AKH}$ ("absolute raise").

For statistical data analysis the 'Dean and Dixon' test for outliers was used [Dean 1951] on the $(InsP/PtdIns)_{AKH}$ and $(InsP/PtdIns)_{Control}$ values and on the AKH/Control values. Results are expressed as the mean ± S.E.M.. Significance of differences was tested using the Students *t*-test for paired or unpaired comparisons [Sokal 1969], depending on the experimental setup.

2.9 Measurement of Ins(1,4,5)P_3 levels in the fat body

Fat body was treated as described in paragraph 2.4 till after the seperation by chloroform:methanol. The protein layer (infranatant) was collected and washed in 1 ml chloroform, and then dried for 10 min at 50 °C. Next, 1 ml 0.5 M KOH was added. The solution was sonificated briefly (4 seconds), heated (56 °C) for 1 hour, and put at room temperature overnight to enhance resuspension of the proteins. 50 µl of this solution was mixed with 450 µl of water and used in the protein determination assay of Schacterle and Pollack [1973].

600 µl of supernatant was put under a constant flow of nitrogen gas at 40 °C, to evaporate the methanol and the chloroform remains, until about 150 µl was left. The precise volume was determined using an automatic pipet. 7 µl extract was mixed with 43 µl water and used as sample for the Amersham Ins(1,4,5)P_3 radio-immuno assay. All Ins(1,4,5)P_3 measurements were carried out in silanized tubes.

volumes in µl	Ins(1,4,5)P3 standard stock	water	sample	diluted standard	Assay buffer	3H-Ins(1,4,5)P3 tracer	Binding Protein
NSB	50				50	50	50
Bo		50			50	50	50
standard curve				50	50	50	50
sample		43	7		50	50	50

Table 1;
Mixing table for Ins(1,4,5)P_3 assay.

The radio-imuno assay was carried out as follows; As blanc, a B_0 sample was made containing 50 µl water instead of 43 µl water and 7 µl extract. Nonspecific binding (NSB) was measured by adding 50 µl unlabelled Ins(1,4,5)P_3 standard stock instead of 43 µl water and 7 µl of fraction. To all samples 50 µl assay buffer, 50 µl radioactive Ins(1,4,5)P_3 tracer and 50 µl binding protein were added. For the standard curve, 4 dilutions of the Ins(1,4,5)P_3 standard stock were used; 25 pmol, 6.25 pmol, 1.56 pmol and 0.39 pmol per tube (see ***Table 1***). All samples were thoroughly shaken, put on ice for 15 minutes and centrifuged at 12,000 ***g*** for 10 minutes. Next, the pellet was dried and dissolved in 400 µl 0.15 M NaOH. Samples were counted in a scintillation counter.

2.10 Protein determination

For protein determination a modified assay of Schacterle and Pollack [1973] was used. 500 µl diluted sample was mixed with 500 µl alkaline copper reagent, and left at room temperature for 10 minutes. Then 2 ml Folin reagens was added and incubated for 5 minutes at 55 °C. Next, the sample was allowed to cool down to room temperature and its extinction was measured at 750 nm. Water was used as blanc and standard calibration solutions (BSA) were used to make a standard curve.

2.11 Involvement of phospholipase C in glycogen phosphorylase activation

Fat bodies were divided into eight pieces and pooled to get identical half fat bodies (each consisting of 4 pieces). Fat bodies were incubated in preincubation medium (Medium III, see *Supplement*) for 2 hours to obtain resting levels of glycogen phosphorylase and then washed in washing medium (Medium XI, see *Supplement*). Subsequently, fat bodies were incubated for 30 minutes in incubation medium (Medium IV, see *Supplement*) in the presence of 10 µl 10 mM U73122, an inhibitor of phospholipase C, or 10 µl of its solvent DMSO (dimetyl sulfoxide). Then incubation with or without AKH -I, -II or -III for 15 minutes followed. Incubation was terminated by homogenizing the tissue, using a chilled glass Teflon homogenizer, in 1 ml homogenisation medium (Medium I, see *Supplement*). The homogenates were centrifuged for 15 minutes at 12,000 ***g*** at 0 °C. The infranatant was used for spectrophotometric determination of the phosphorylase activity.

2.12 Glycogen phosphorylase assay

This assay was done as previously described by van Marrewijk [1980]. Briefly, the infranatant (10 to 130 µl) was put into a cuvet with 345 µl of a mixture containing 200 µl Buffer I (see *Supplement)*, 50 µl Buffer II (see *Supplement)*, 25 µl 100 mM MgAc, 35 µl 12 mM NADP, 25 µl 56 mg/ml glycogen, 5 µl 0.4 mM glucose-1,6-bisphosphate, 5 µl enzymes (1 µl phosphoglucomutase (1.9 I.U.) and 1.2 µl glucose-6-P dehydrogenase (0.38 I.U.) centrifuged and dissolved in 5 µl Buffer II). Water was added to get a final volume of 475 µl. Active forms (A and AB) of glycogen phosphorylase were measured by measuring the reduction of $NADP^+$ at 340 nm. Next, 25 µl 40 mM 5'-AMP was added to measure both active and inactive forms of phosphorylase, representing the size of the fat body piece. The glycogen phosphorylase activity ratio was calculated by dividing the the activity without AMP through the activity with AMP.

2.13 Involvement of phospholipase C in InsP formation

To determine whether the effect of phospholipase C on glycogen phos-

phorylase (if any) is mediated through InsPs, the effect of U73122 on InsP formation by AKH was measured.

This experiment was done as described in paragraph 2.4, except for the fact that fat bodies were cut in 3 parts (control, AKH and AKH+U73122) and 30 min before AKH addition, 10 µl 10 mM U73122 was added (or 10 µl DMSO for the control and AKH treatments).

3 Results

3.1 The effect of AKH on the formation of total InsPs

It is generally believed that InsPs play an important role in signal transduction in general, and also in the signal transduction of AKH. As the effect of a hormone is expected to occur in the first few minutes, we expected a rise in the formation of InsPs about one minute after addition of AKH, especially since previous studies in this lab have shown that the three AKHs show a significant increase in calcium influx after 1 minute. We didn't expect to find differences in the InsP formation induced by the three AKHs, as previous studies didn't show differences in calcium influx caused by the AKHs.

All three AKHs show a rapid increase in InsP levels as soon as 15 seconds after AKH addition, although the AKH-II-induced rise is clearly smaller than the ones brought about by the other hormones (see ***Fig. 8***). For AKH-III the rise is already significant ($P<0.01$) at this time. For AKH-I and -II the increase is significant after 1 minute ($P<0.01$). These findings confirm the idea that InsPs play a role in AKH signal transduction.

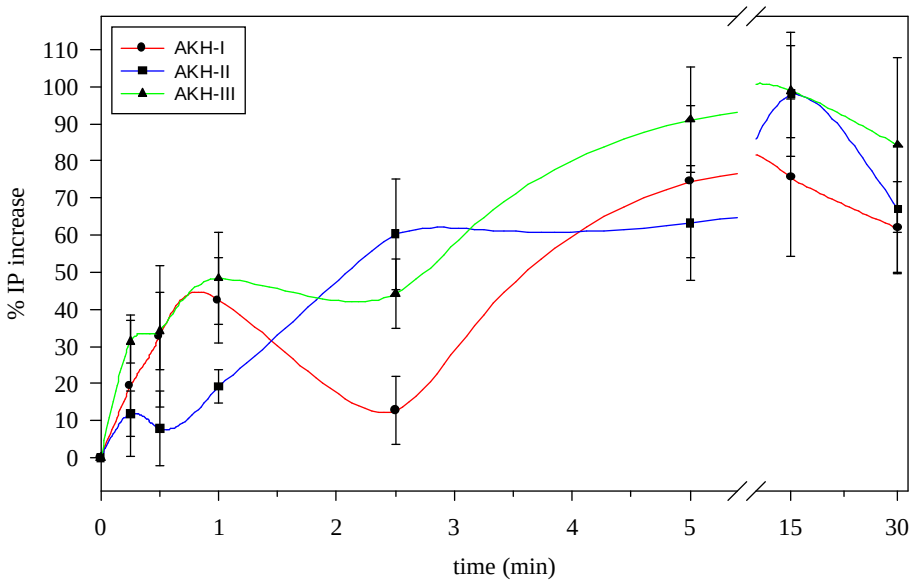


Figure 8;
The effect of AKH on the total InsP levels (in % of control).

The curve is biphasic for all AKHs. We expect the first part to be the phase important for second messenger functions, and the second phase to be non physiological, due to high concentrations of AKH and lithium in the incubations. To examine this, a characterisation of the individual

InsP content of the peaks was done, which will be discussed later. Biphasic in this case implies that there are two seperate peaks with a plateau in between. Only AKH-I shows a dip at 2.5 minutes that is also significant compaired to the level at 1 minute ($P<0.05$), as is the rise after 2.5 minutes ($P<0.05$). There is no significant difference between the level at 2.5 minutes and the level at t=0, which suggests that InsP levels are back to control level. Such a decrease can't be found for AKH-II and -III, where the InsP levels reach a plateau level at about 2.5 minutes.

The problem with depicting the % InsP increase relative to the control (as done in ***Fig. 8***) is that differences in control levels are eliminated. The

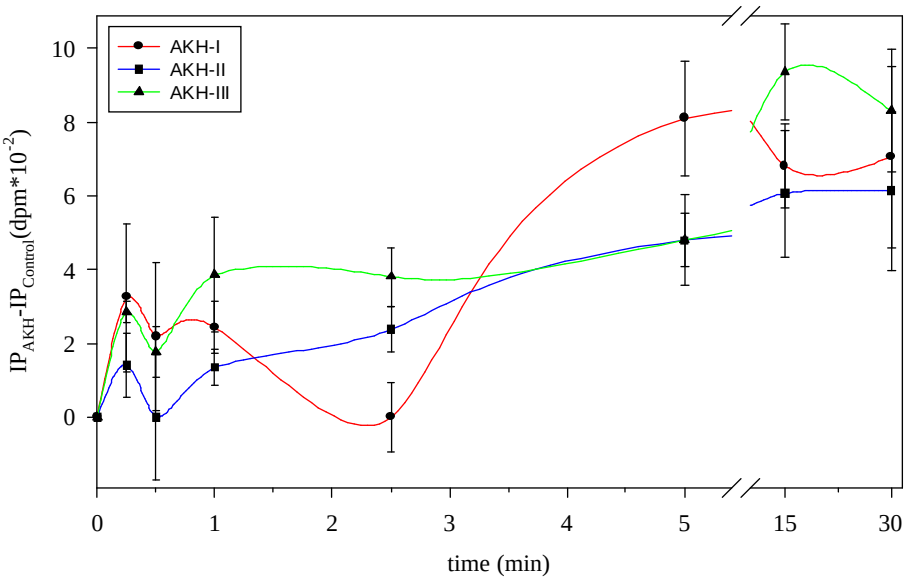


Figure 9;
The effect of AKH on the InsP levels absolutely to the control.

InsP levels of the control, however, will also increase due to the LiCl present in the solution. If, for instance, the control level is 6 and the AKH-induced level 9, the "relative" increase of 50% and the "absolute" rise is 3. If however the control level is 3 and the AKH-induced level is 6, one will get a 100% "relative" increase, but still an "absolute" rise of 3.

To meet this problem, also the "absolute" rises ($InsP_{AKH}$-$InsP_{control}$) were calculated (see ***Fig. 9***). This graph shows no big differences with the "relative" graph, which supports the quality of the results. In the figure it is clearly shown that AKH-I-induced InsP levels are back to control level at 2.5 minutes. The figures also show clearly that there is a quick rise in InsP levels already after 15 seconds.

It should be stressed that for AKH-I at 2.5 minutes, the "absolute" total amount of InsP does not decrease (this is impossible because of the presence of LiCl!) but only the difference between AKH-stimulated and control levels decreases.

	mM TEAB	counts (dpm)
InsP1	20	29
	30	629
	120	779
	130	0
InsP2	120	26
	130	667
	220	795
	230	0
InsP3	220	33
	230	384
	320	616
	330	2
InsP4	320	39
	330	539
	420	680
	430	27

Table 2;
Column test with stepwise elution.

	mM TEAB	counts (dpm)
InsP1	110	696
	120	0
InsP2	210	787
	220	0
InsP3	310	562
	320	21
InsP4	410	812
	420	6

Table 3;
Column test with stepwise elution.

3.2 Determination of elutionbuffer concentrations of individual InsPs

Fig. 10 clearly shows seperate peaks for the four InsP standards added, from left to right Ins(1)P_1, Ins(1,4)P_2, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. These data suggest 120, 220, 320 and 420mM TEAB to be the concentrations at which the individual InsPs elute from the SepPak columns. As there could be differences in these concentrations between a gradient elution and a stepwise elution, several tests were done to determine the validity of the determined concentrations. A sample containing one InsP standard was eluted with respectively the determined concentration (X) minus 30mM, followed by X minus 20, then X minus 10, X and X plus 10 mM (i.e. 90, 100, 110, 120 and 130 mM for $InsP_1$). When the determined concentrations are correct the X plus 10 mM samples should give zero counts. The results showed no significant counts for the X plus 10 fraction, so this suggests that the determined concentrations are correct (data not shown). To be sure that raising the concentration in small steps has no influence on the complete elution of an InsP isomer, another test was done. In this test we used one InsP standard per sample, and the column was eluted with respectively 120 and 130 mM TEAB for $InsP_1$, 220 and 230 mM for $InsP_2$, 320 and 330 mM for $InsP_3$ and 420 and 430 mM for $InsP_4$. If the determined concentrations are correct there should be no radioactivity in the 130, 230, 330 and 430 mM fractions. The same test

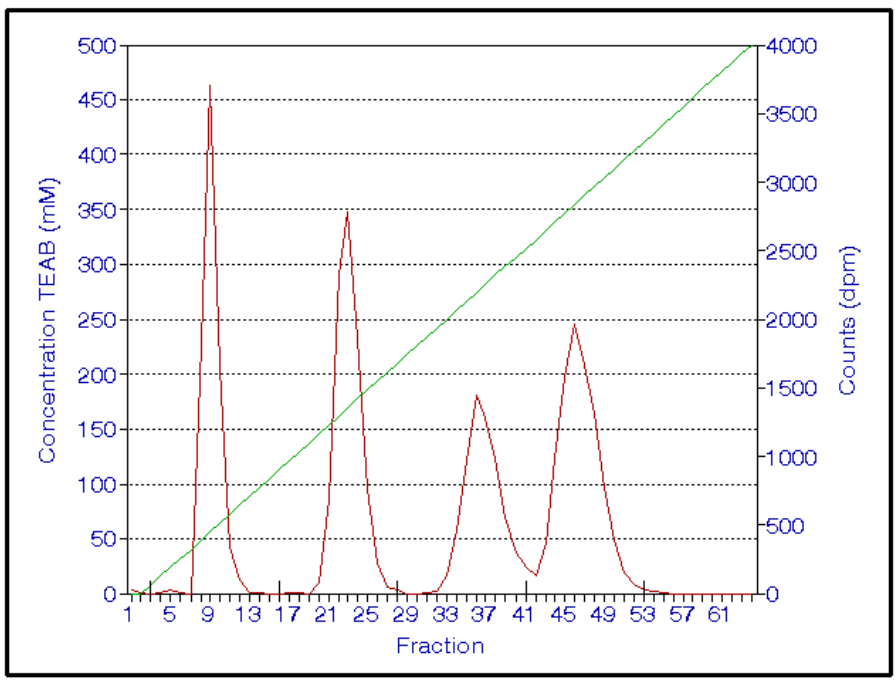


Figure 10;
Determination of the elution concentrations of $InsP_1$ through $InsP_4$. Green line, [TEAB]; red line, radioactivity in fraction.

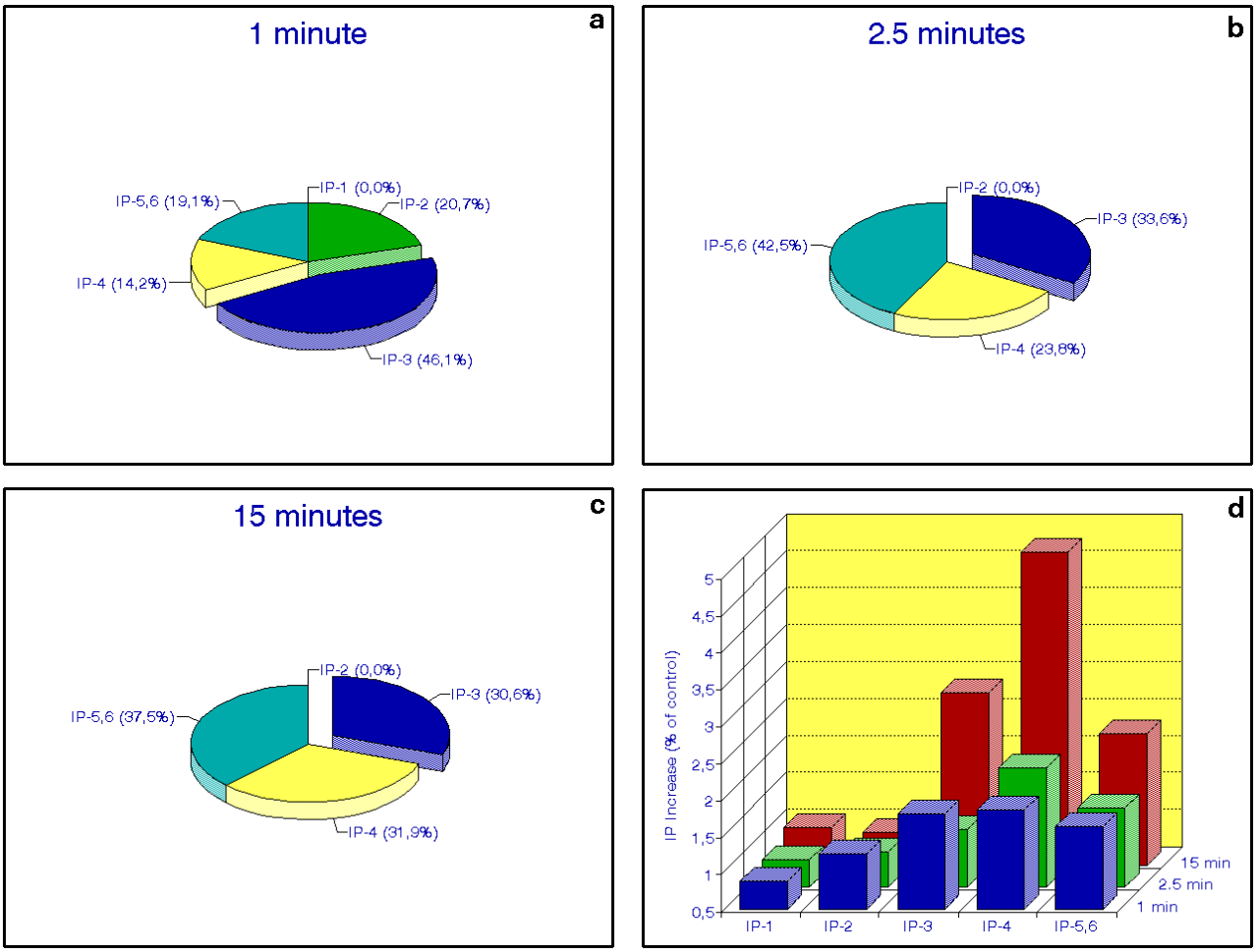


***Figure 11**;*
Determination of the effect of AKH-I on individual InsP (IP) levels in the fat body. A, b and c depict the 'absolute' values, d depicts the 'relative' values (see text).

was also done by eluting at 20 and 30 mM ($InsP_1$), 120 and 130 mM ($InsP_2$), 220 and 230 mM ($InsP_3$) and 320 and 330 mM ($InsP_4$) TEAB, respectively. These fractions should all give no counts if the determined concentrations are correct, as they are the concentrations at which the InsPs lower than the applied standard elute (or 10 mM higher than these).

The results of these two test are shown in ***Table 2***. These results show that the InsP standards elute from the column at 120, 220, 320 and 420 mM respectively. This suggests that the determined elution concentrations are correct. However, at a 10 mM higher concentration then each determined concentration, the next InsP already starts to elute from the column. In order to avoid a possible contamination of an InsP fraction with the next InsP, also elution tests were done with slightly lower TEAB concentrations of 110, 120, 210, 220, 310, 320, 410 and 420 mM TEAB, respectively. The results (see ***Table 3***) show that also with 110, 210, 310 and 410 mM the InsP are eluted completely seperately from the column. So the final elution concentrations were set at 110 mM TEAB for $InsP_1$, 210 mM for $InsP_2$, 310 mM for $InsP_3$ and 410 mM for $InsP_4$. Although we could not determine the elution concentration for $InsP_{5\text{-}6}$, we chose 500 mM TEAB since at this concentration all InsPs elute from the col-

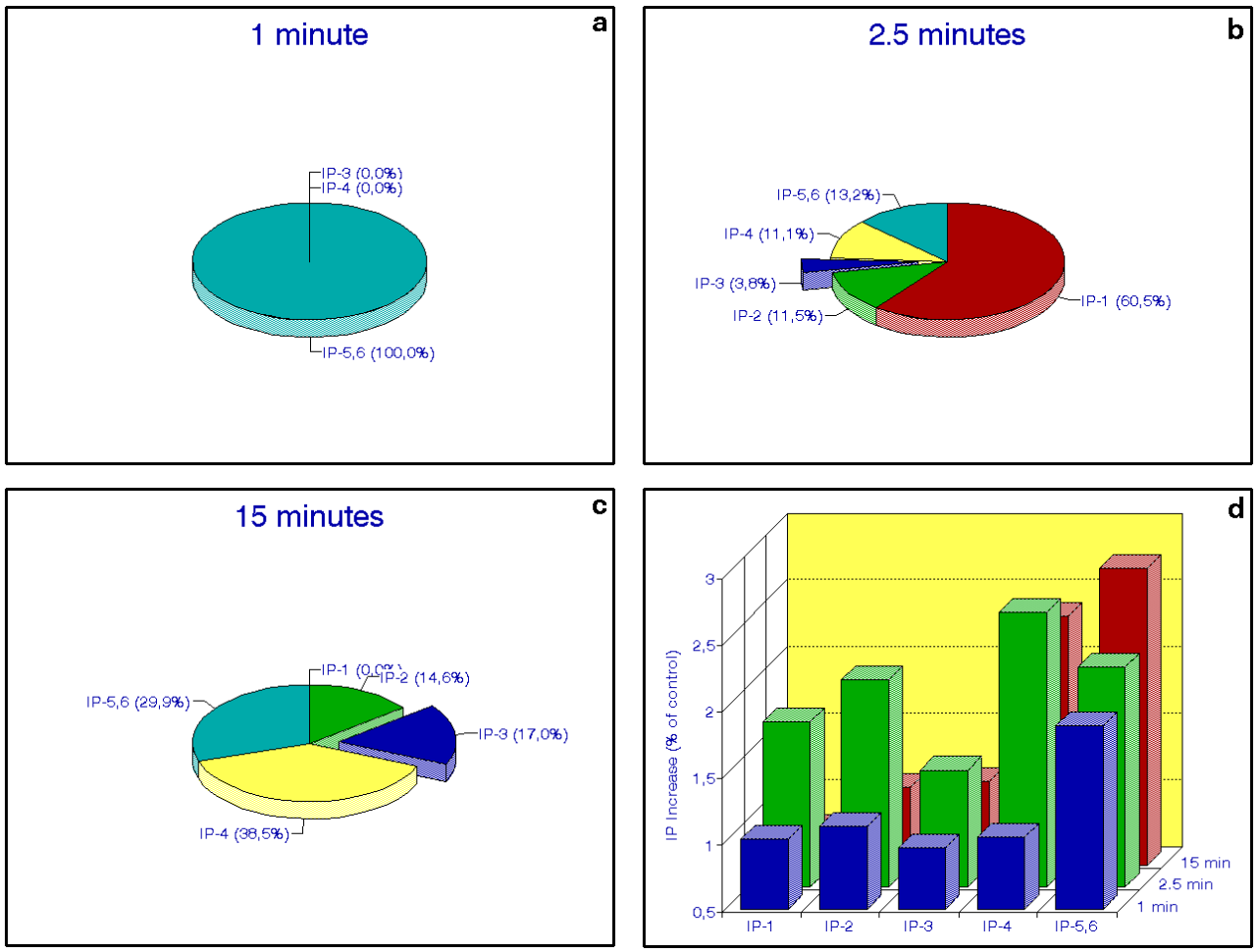


***Figure 12**;*
Determination of the effect of AKH-II on individual InsP (IP) levels in the fat body. A, b and c depict the 'absolute' values, d depicts the 'relative' values (see text).

umn. Morover, since biological samples all contain glycerophosphoinositol, these compounds should first be eluted with a TEAB concentration of 10 mM.

3.3 The effect of AKH on the formation of individual InsPs

Considering the proposed model in chapter 1, one would expect $InsP_3$ levels to be raised significantly by AKH after 1 minute, since $Ins(1,4,5)P_3$ is thought to be the second messenger inducing calcium fluxes and, as shown by the total InsP determination in paragraph 3.1, at 1 minute the first InsP peak occurs (see ***Fig. 8***). One would expect $InsP_3$ levels to return to basal levels after about 2.5 minutes as the activation shouldn't be too long and according to the total InsP determination.

Therefore we measured the formation of individual InsPs induced by the AKHs after 1 and 2.5 minutes. To investigate the nature of the accumulation shown in paragraph 3.1 at later time points, the experiment was also done at 15 minutes of AKH incubation. At this time point one would

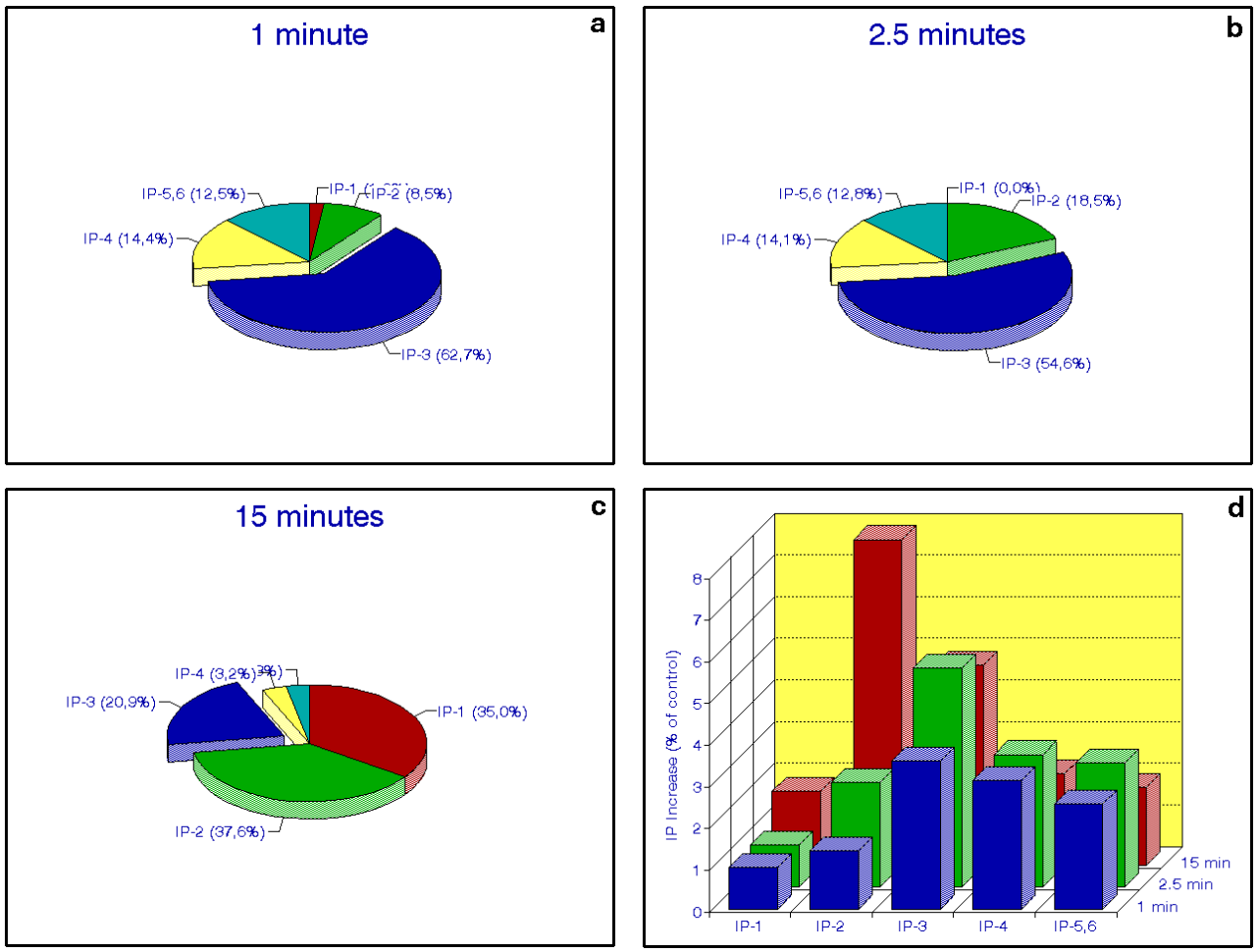


***Figure 13**;*
Determination of the effect of AKH-III on individual InsP (IP) levels in the fat body. A, b and c depict the 'absolute' values, d depicts the 'relative' values (see text).

expect, because of the presence of LiCl, an accumulation of $InsP_2$ and/or $InsP_1$.

The same problem with depicting a relative InsP increase compaired to the control, as described in paragraph 3.1, occurs here. So, also here, the absolute rises above control levels were calculated. These values are depicted in circle diagrams (see ***Fig. 11, 12*** and ***13***). Each slice represents the contribution (in %) of an individual InsP to the total InsP rise (100%) at the given time point.

Figure 11a, b and c show the results of the experiments with AKH-I. They demonstrate that after 1 minute a considerable part of the total raise in InsP levels is $InsP_3$ (46 %). This part significantly ($P<0.05$) decreases at 2.5 minutes and even further after 15 minutes. One should remember that, as the total rise in InsP levels at 2.5 minutes is very small (as shown in ***Fig. 8***), the absolute amount of InsP3 is almost zero at 2.5 minutes. So far these results support the discussed model. Strangely, already a great part of the total rise in InsP consists of $InsP_4$ and $InsP_{5-6}$ at 1 minute, and this percentage even increases at 15 minutes. So, instead of an accumulation of $InsP_2$ and $InsP_1$, we see an accumulation of the

highly phosphorylated InsPs. This is also seen with AKH-II (see ***Fig. 12a, b, c***), although this hormone induces a large accumulation of the less phosphorylated InsPs at 2.5 minutes which disappear again after 15 minutes, in benefit of the highly phosphorylated InsPs. Suprisingly, at 1 minute, AKH-II only appears to rise the level of $InsP_{5\text{-}6}$, while $InsP_3$ starts to show only in a small amount at 2.5 minutes, and in a larger amount at 15 minutes. It should be noted that the absolute levels of radioactivity were by far the highest in the $InsP_1$ and $InsP_2$ fractions (both in controls as in AKH-treated fat bodies), and that the foregoing remarks solely concern the rises in the levels of individual InsPs. The questions that rise from these results are: doesn't AKH-II use $InsP_3$ as a second messenger for its signal transduction? Does it operate via $InsP_4$ (which is also thought to have second messenger capabilities) or even $InsP_5$ or $InsP_6$?

AKH-III (see ***Fig. 13a, b, c***) is the only AKH that meets our expectations completely. The $InsP_3$ concentration diminishes nicely in time and at 15 minutes the largest part of the InsP accumulation consists of $InsP_2$ and $InsP_1$. The highly phosphorylated InsPs have almost completely vanished at 15 minutes.

Also the relative rise compaired to the control was calculated, and these are shown in ***Fig. 11d***, ***12d*** and ***13d***. In ***Fig. 11d*** you can clearly see the accumulation of the highly phosphorylated InsPs at 15 minutes for AKH-I, the role of $InsP_3$ at 1 minute, and more importantly the decrease at 2.5 minutes wich is also seen in ***Fig. 9***. In ***Fig. 12d*** the accumulation of $InsP_4$ and $InsP_{5\text{-}6}$ at 15 minutes for AKH-II is clearly shown, as well as the large amount of $InsP_{5\text{-}6}$ at 1 minute and the considerable amount of $InsP_1$ at 2.5 minutes and its (strange) disappearence at 15 minutes.

Briefly, AKH-III seems to support our model (of $InsP_3$ being the second messenger that acts within a short period, and the second rise in total InsP being of less physiological importance), AKH-I partly and AKH-II not. An important disadvantage of the above experiments is that (for instance) the $InsP_3$ fraction contains both possible forms of $InsP_3$, and not only the second messenger, $Ins(1,4,5)P_3$. To eliminate this inaccuracy a radio-immuno assay specific for $Ins(1,4,5)P_3$ was introduced.

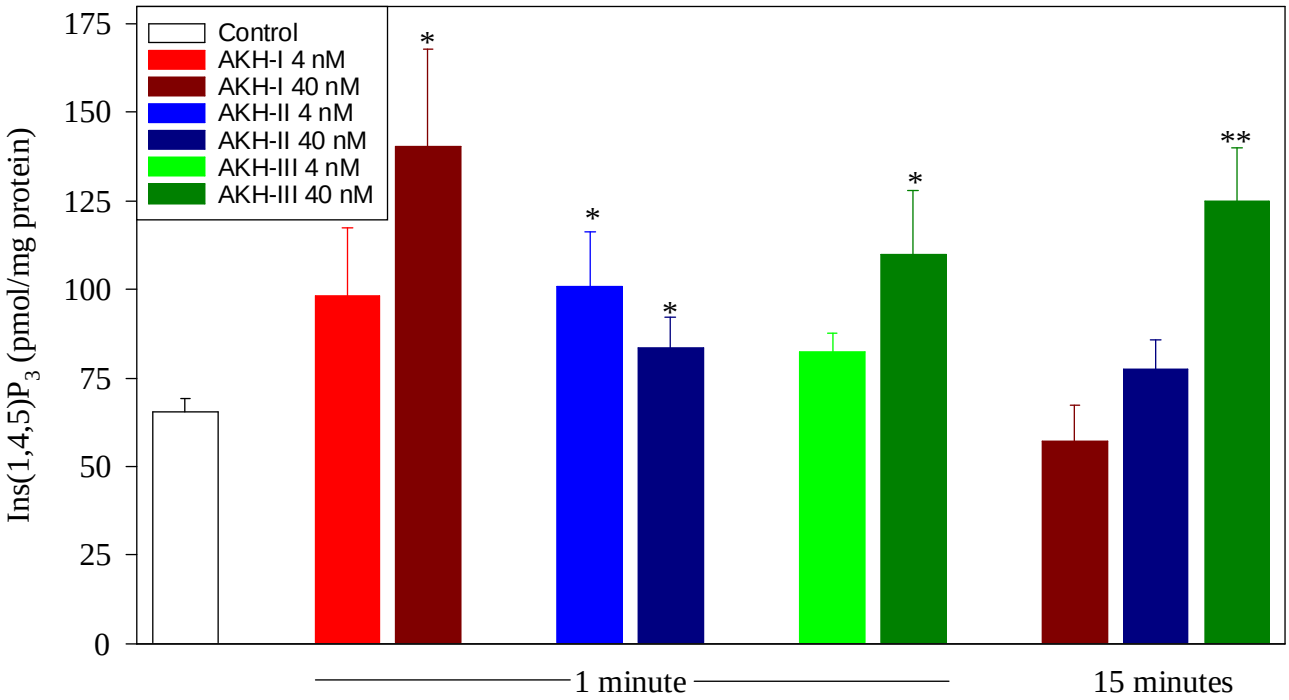
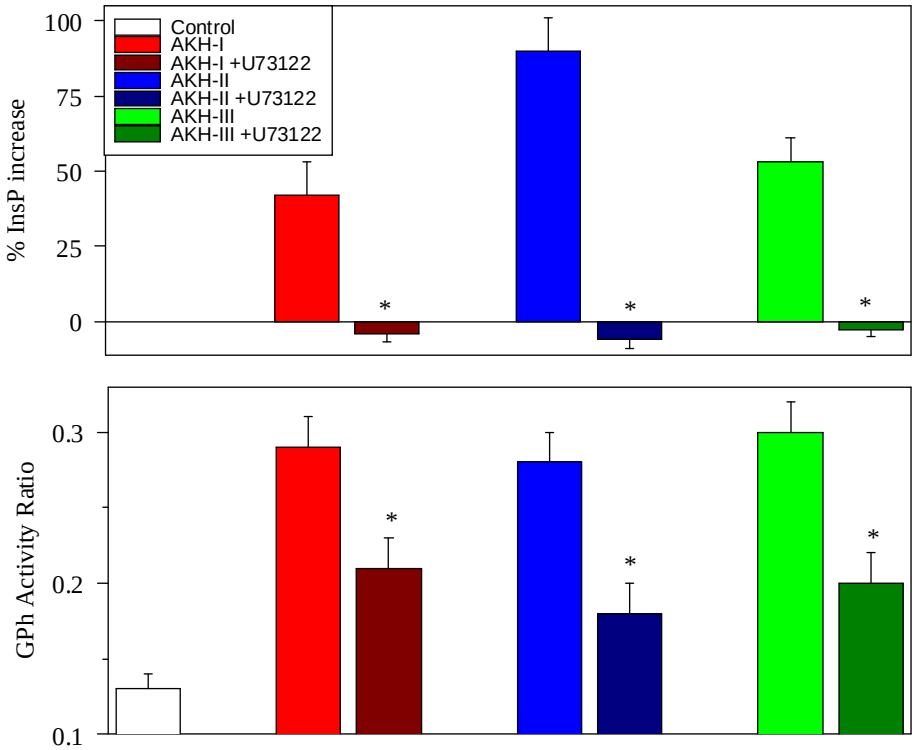


Figure 14;
The effects of AKH-I, -II and -III on InsP(1,4,5)P_3 levels (in % of control).

3.4 Ins(1,4,5)P_3 determination using an radio immuno assay

The amount of Ins(1,4,5)P_3 in AKH-treated fat bodies was determined using a 40 nM concentration of AKH as well as a physiological concentration of 4 nM. Previous research by Vroemen [1995a] has shown that there is a dose-response relationship for cAMP formation induced by AKH-I, -II and -III. We expected to find a similar dose-response relationship for hormone induced InsP(1,4,5)P_3 formation. We also expected to see a significant rise in InsP(1,4,5)P_3 compaired to the control, as our experiments with labelled InsP showed rises in InsP_3. ***Fig. 14*** shows the results of the radio-immuno assay. All three AKHs are able to induce a significant rise in Ins(1,4,5)P_3 within 1 minute ($P < 0.01$ for AKH-I and -III and $P<0.05$ for AKH-II) although at 40 nM, rises in Ins(1,4,5)P_3 levels induced by AKH-II are much smaller then those induced by AKH-I and -III, as was found in our experiments with radiolabelled InsPs. At a physiological dose of 4 nM however the three AKHs are equally potent. AKH-I and -III show a clear dose-response relationship, but suprisingly AKH-II does not. After a long time of incubation there is virtually no Ins(1,4,5)P_3 left in AKH-I-treated fat bodies, wich supports the idea that the accumulation is of less physiological meaning. Fat bodies treated with AKH-III, and to a much lesser extend AKH-II, still contain quite high levels of Ins(1,4,5)P_3.

Figure 15;
The effect of U73122 on the InsP levels and glycogen phosphorylase activity.



3.5 Role of PLC in AKH induced InsP formation and glycogen phosphorylase activition

The model in chapter 1 suggests that the signal transduction from AKH to glycogen phosphorylase is mediated via PLC and $InsP_3$. To provide some further clues whether this pathway is really involved, the effect of inhibition of PLC on InsP formation and glycogen phosphorylase activation was studied.

Fig. 15 shows that U73122, a specific inhibitor of PLC, is able to significantly ($P<0.05$ for AKH-I and $P<0.01$ for AKH-II and -III) block the effect of AKH on the increase in InsP levels, and to significantly attenuate the effect of AKH on glycogen phosphorylase ($P<0.001$ for AKH-I and -II and $P<0.05$ for AKH-III). However, the inhibitor is not able to inhibit glycogen phosphorylase activation completely. This is, most probably, due to the fact that, as shown in the model, glycogen phosphorylase is activated by two more or less seperate pathways. It is likely that in the presence of U73122, glycogen phosphorylase is still activated via the adenylyl cyclase/cAMP pathway. A second possibility would be that U73122 isn't able to inhibit PLC completely. This possibility, however, is ruled out by our observation that U73122 is able to block the effect of PLC on InsP levels completely.

This experiment proves that PLC is somehow involved in the activation of glycogen phosphorylase. Whether this involvement includes $InsP_3$ or not can not be said for sure, but it is likely because of the AKH-induced calcium fluxes. It is possible that the pathway is mediated by DAG, the other messenger that results from hydrolysis of PtdIns by PLC, which is known to have a role in the activation and regulation of PKC.

4 Discussion and Conclusions

The research on InsPs is relatively young, though developing very fast. Already a lot of research has been done and a lot of the mechanisms it is involved in have been unravelled. Still, a lot of questions arise when doing research on this fascinating second messenger system. In this study we tried to answer some of these questions for the the signal transduction of AKH in the locust fat body, the most compelling being why there are three AKHs and if there are differences between the AKHs. Because AKH needs the release of calcium from intracellular stores [Van Marrewijk 1993] and InsPs are known to empty intracellular calcium pools [Berridge 1993], we took a look at the total InsP formation induced by the three AKHs.

In our expectations, InsP levels would rise somewhere between 0 and 2.5 minutes as a result of AKH stimulation, and return to resting levels after that. Because of the presence of LiCl and a massive dose of AKH, an accumulation of InsPs could be expected and was indeed seen. The total InsP determination showed that all three AKHs are able to induce a significant rise in InsP levels 1 minute after AKH administration which suggests an early role for InsPs in the signal transduction pathway. The level of InsP formation induced by AKH-II though, is considerably lower then those induced by AKH-I and -III. This is remarkable because no differences between the AKHs have been found in the induction of calcium infux, which is coupled to the emptying of the intracellular calcium stores by $InsP_3$ [Van Marrewijk 1993]. Further, only AKH-I shows InsP levels that return to the control level at 2.5 minutes suggesting a desensitisation process that is not induced by AKH-II or -III. A strange phenomenon is the second phase in the total InsP time course. The accumulation is also seen by others [Van Marrewijk 1996] and is generally believed to be nonphysiological and a result of prolonged incubations with a massive dose of AKH and LiCl (therefore containing mostly $InsP_1$ and $InsP_2$)[Berridge 1983]. To get a better insight in the results of the total InsP determination we took a look at the distinct InsPs formed upon AKH stimulation.

Both AKH-I and -III show high levels of $InsP_3$ at 1 minute (46% and 63% respectively) which is consistant with our model, being that

stimulation by AKH firstly leads to synthesis of Ins(1,4,5)P_3 by PLC. This synthesis is possibly followed by phosphorylation to $InsP_4$ or higher, and subsequently by dephosphorylation to $InsP_2$ and $InsP_1$, as described by Batty [1989] and in ***Fig. 5***. Suprisingly, but consistent with the low rise in InsP levels induced by AKH-II, no significant rise in $InsP_3$ levels was seen for AKH-II at 1 minute. This is even more suprising because AKH-II is equally potent in activating calcium influx into the fat body as AKH-I and -III [Vroemen 1995b]. Because the effect of AKH-II on calcium influx is within 1 minute, the small rise in $InsP_3$ levels at 2.5 minutes (and even more at 15 minutes) can not account for the induction of calcium influx. In accordance with the total InsP determination, AKH-I-induced $InsP_3$ levels return to basal levels at 2.5 minutes. Suprisingly, AKH-I and -II show an accumulation of the highly phosphorylated InsPs at longer incubations, instead of the lowly phosphorylated ones that AKH-III induces (as expected because of the presence of lithium). At 15 minutes $InsP_3$ levels are relatively low, which supports the idea of the second phase being of less physiological importance. Because the assay using radioactive inositol can not discriminate between the different forms of $InsP_3$, a specific Ins(1,4,5)P_3 assay was done.

This radio-immuno assay showed a significant increase in Ins(1,4,5)P_3 levels induced by 40 nM of the three AKHs, although the level induced by AKH-II was again lower then those induced by AKH-I and -III. It could be possible that AKH-II uses another pathway (via other InsPs or even DAG), but this seems unlikely because with a physiological dose of 4 nM hormone the three AKHs induce equal Ins(1,4,5)P_3 levels, so this dose is probably enough to induce calcium influx into the fat body. AKH-I and -III display a dose-response relationship for hormone-induced Ins(1,4,5)P_3 formation, but AKH-II does not. It seems that AKH-II is less efficacious than AKH-I and -III. For both AKH-I and -II, Ins(1,4,5)P_3 levels return to the control level after 15 minutes (40 nM hormone) as could be expected for a second messenger. AKH-III-induced Ins(1,4,5)P_3 levels though remain high after 15 minutes, suggesting some kind of long term effect for AKH-III. No such long term effect can be seen in the induction of calcium influx though.

The fact that, for AKH-II at 1 minute, $InsP_3$ could be found in the radio-immuno assay but not in the individual InsP determination reveals the biggest problems of the latter assay, being the lower sensitivity and the fact that this assay can not discriminate between Ins(1,4,5)P_3 and Ins(1,3,4)P_3. Another problem we encountered was that there was no

radioactive standard for $InsP_5$ and/or $InsP_6$. This prevented us from determining whether or not $InsP_5$ is eluted from the column at 410 nM (thereby possibly contaminating the $InsP_4$ fraction). So the value of 410 nM for the elution-concentration of $InsP_4$ is a little arbitrairy as we could not determine the overlap with $InsP_5$.

A problem encountered in all our tests done with locusts is the high variability between the animals. An attempt to meet this problem was done by Lee [1995] who found a way to create dispersed fat body cells suitable to do assays on. Because our protocols were already running when this technique became available, and because of the lack of time to restart our assays, we did not use this method for this research. Seperate fat body cells, however, do have a great potential for this research and further exploration of this method should certainly be done.

The results of this research provide no direct reason to change our proposed model. The role of InsPs though, could turn out to be more complicated then our model suggests. Irvine suggests roles for other InsPs, such as $InsP_4$, in the regulation of calcium influx induced by emptying of the intracellular calcium stores [Irvine 1990, Irvine 1991, Irvine 1992]. The influx of calcium into the locust fat body could well be regulated by $InsP_4$, especially because we found rises in $InsP_4$ for each AKH at each time point. In our lab, Van Marrewijk [1996] has suggested that $Ins(1,3,4,5)P_4$ may serve as a pool from which $Ins(1,4,5)P_3$ could be rapidly synthesized when needed. Although we know already a lot about the role of InsPs in signal transduction, still a lot of research has to be done.

Lets go for it !!!

References

Batty I.H., Letcher A.J., Nahorski S.R. (1989). Accumulation of inositol polyphosphate isomers in agonist stimulated cerebral-cortex slices. Comparison with metabolic profiles in cell-free preparations. *Biochem. J.,* **258, 23-32**

Berridge M.J. (1995). Capacitative calcium entry. *Biochem. J.*, **312, 1-11**

Berridge M.J., Dawson R.M.C., Downes C.P., Heslop J.P., Irvine R.F. (1983). Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Bioch. J.*, **212, 473-482**

Berridge M.J., Irvine R.F. (1989). Inositol phosphates and cell signalling. *Nature,* **341, 197-205**

Changya L., Gallacher D.V., Irvine R.F., Petersen O.H. (1989a). Inositol 1,3,4,5-tetrakisphosphate nad inositol 1,4,5-trisphosphate act by different mechanisms when controlling Ca^{2+} in mouse lacrimal acinar cells. *FEBS Lett.,* **251, 43-48**

Changya L., Gallacher D.V., Irvine R.F., Potter B.V.L., Petersen O.H. (1989b). inositol 1,3,4,5-tetrakisphosphate is essential for the sustained activation of the Ca^{2+}-dependent K^{+} current in single internally perfused mouse lacrimal acinar cells. *J. Memb. Biol.*, **109, 85-93**

Dean R.B., Dixon W.J. (1951). Simplified statistics for small numbers of observations. *Anal. Chem.*, **23, 636-638**

Gilman A.G. (1995). G proteins and the regulation of adenylyl cyclase. *Bioscience Rep.,* **15, 65-97**

Irvine R.F. (1990). 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates - a possible mechanism. *FEBS Lett.*, **263, 5-9**

Irvine R.F. (1991). Inositol tetrakisphosphate as a second messenger: confusions, contradictions, and a potential resolution. *Bioessays*, **13, 419-428**

Irvine R.F. (1992). Is inositol tetrakisphosphate the second messenger that controls Ca^{2+} entry into cells? In *Inositol Polyphosphates and Calcium Signalling* (Putney J.W. Jr., ed) pp. **161-185**, Raven, New York

Lee M.J., Goldsworthy J. (1995). The preparation of dispersed cells from fat body of the *Locusta migratoria* in a filtration plate asaay for adipokinetic peptides. *Anal. Biochem.*, **288, 155-161**

Oudejans R.C.H.M., Kooiman F.P., Heerma W., Versluis C., Slotboom A.J. en Beenakkers A.M.Th. (1991). Isolation and structure elucidation of a novel adipokenetic hormone (Lom-AKH-III) from the glandular lobes of the corpus cardiacum of the migratory locust, *Locusta migratoria*. *Eur. J. Biochem.*, **195, 351-359**

Powell J.F.F., Zohar Y., Elizur A., Park M., Fischer W.H., Craig A.G., Rivier J.E., Lovejoy D.A., Sherwood N.M. (1994). Three forms of gonadotropin-releasing hormone characterized from the brain of one species. *Proc. Acad. Sci. USA*, **91, 12081-12085**

Putney J.W. Jr. (1986). A model for receptor-regulated calcium entry. *Cell Calcium,* **7, 1-12**

Putney J.W. Jr.. (1990). Capacitative calcium entry revisited. *Cell Calcium,* **11, 611-624**

Schacterle G.R., Pollack R.L. (1973). A simplified method for the quantitative assay of small amounts of protein in biological material. *Anal. Biochem.,* **654-655**

Sokal R.R., Rohlf F.J. (1969). Two way analysis of variance. In: *Biometry* (Emerson R., Kennedy D., Park R.B., Beadle G.W., Withaker D.M., Eds.). **pp. 299-366**. Freeman, San Fransisco

Sower S.A., Chiang Y.-C., Lavas S., Conlon J.M. (1993). Primary structure and biological activity of a third gonadotropin-releasing hormone from the lamprey brain. *Endocrinology,* **132, 1125-1131**

Van Marrewijk W.J.A., Van den Broek A.Th.M., Beenakkers A.M.Th. (1980). Regulation of glycogenolysis in the locust fatbody during flight. *Insect Biochem.,* **10, 675-679**

Van Marrewijk W.J.A., Van den Broek A.Th.M., Beenakkers A.M.Th. (1983). Regulation of glycogen phosphrylase activity in fat body of *Locusta migratoria* and *Periplaneta americana*. *Gen. comp. Endocrinol.*, **50, 266-234**

Van Marrewijk W.J.A., Van den Broek A.Th.M., Gielbert M.-L., Van der Horst D.J. (1996). Insect adipokinetic hormone stimulates inositol phosphate metabolism: roles for both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in signal transduction? *Mol. Cell. Endocrinol.*, **in press**

Van Marrewijk W.J.A., Van den Broek A.Th.M., Van der Horst D.J. (1993). Adipokinetic hormone-induced influx of extracellular calcium into insect fat body cells is mediated through depletion of intracellular calcium stores. *Cell. Signalling*, **5, 753-761**

Vroemen S.F., Van Marrewijk W.J.A., Van der Horst D.J. (1995a). Stimulation of glycogenolysis by three locust adipokinetic hormones involves G_s and cAMP. *Mol. Cell. Endocrinol.,* **107, 165-171**

Vroemen S.F., Van Marrewijk W.J.A., Schepers C.C.J., Van der Horst D.J. (1995b). Signal transduction of adipokinetic hormones involves Ca^{2+} fluxes and depends on extracellular Ca^{2+} to potentiate cAMP-induced activation of glycogen phosphorylase. *Cell Calcium,* **17, 459-467**

Supplement

The table shows the composition of the used media and buffers. DTE and trehalose were added just before use of the medium. Buffer II also contains 0.002 % BSA.

mM	Medium						Buffer	
	I	II	III	IV	XI	XV	I	II
NaCl		149	140	110	110	100		
KCl		15	15	15	15	15		
MgCl2			4	4	4	4		
CaCl2(2 aq)			2	2		2		
NaH2PO4*2H2O						4		
NaHCO3						4		
HEPES	50	10	10	10	10	10		
Trehalose				80		80		
Glucose		20	20					
DTE	2			2			3.5	
NaF	20							
EDTA	5						5	
Imidazol							12.5	5
k-phosphatebuffer							100	20
MgAc								1
pH	7	7.2	7.2	7.2	7.2	7.2	7	7

Acknowledgements

I would like to thank Dick van der Horst for his great positive influence. He was, and is, always interested and positive about everything you (try to) do, not just concerning work but also private.

Next, I would like to thank Wil van Marrewijk for his good advice and our nice teabreaks. To both I would say; check your e-mail regularly!

And of course, Aloys van den Broek, who will always think of just that little experiment you should do. A man with really great scientific knowledge.

I also want to thank Hans Jonker and Wilbert Jansen for there partnership through the years of my study biology.

Not last and certainly not least (how cheap), I would like to thank Simon “topper” Vroemen, for his great guidence throughout the last 15 months he had to cope with me. Thanks for all the work you’ve done for me, and for all those nice drinks and dinners we had and will have in the future.

Thanks to everybody else who made the Biochemical Physiology Research Group such a nice place to be. I’ll miss it, and whenever you guys need an AIO.....

And Dick, I ment it when I said; “When you need somebody to help during the differentation course, you’ll know where to find me!”. Thanks for letting me assist in this course by the way.