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Differential induction of inositol phosphate metabolism by three adipokinetic hormones

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Abstract

Many (in)vertebrates simultaneously release several structurally and functionally related hormones; however, the relevance of this phenomenon is poorly understood. In the locust e.g. each of three adipokinetic hormones (AKHs) is capable of controlling mobilization of carbohydrate and lipid from fat body stores, but it is unclear why three AKHs coexist. We now demonstrate disparities in the signal transduction of these hormones. Massive doses of the AKHs stimulated total inositol phosphate ($InsP_n$) production in the fat body biphasicly, but time courses were different. Inhibition of phospholipase C (PLC) resulted in attenuation of both $InsP_n$ synthesis and glycogen phosphorylase activation. The AKHs evoked differential formation of individual [^{3}H]$InsP_n$ isomers ($InsP_{1-6}$), the effect being most pronounced for $InsP_3$. 40 nM of AKH-I and -III induced a substantial rise in total $InsP_n$ and [^{3}H]$InsP_3$ at short incubations, whereas the AKH-II effect was negligible. At a more physiological dose of 4 nM, the AKHs equally enhanced Ins(1,4,5)P_3 levels. The $InsP_3$ effect was most prolonged for AKH-III. These subtle differences in $InsP_n$ metabolism, together with earlier findings on differences between the AKHs, support the hypothesis that each AKH exerts specific biological functions in the overall syndrome of energy mobilization during flight. © 1997 Elsevier Science Ireland Ltd.

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1. Introduction

Concomitant release of distinct structurally related hormones is a general, however poorly understood phenomenon in animal physiology. In many invertebrates and lower vertebrates, nearly identical hormones with apparently similar functions are released at the same time. For example, three gonadotropin-releasing hormones are synthesized by sea breams (Powell et al., 1994) and lampreys (Sower et al., 1993) and two or more adipokinetic hormones (AKHs) in several insect species. Three AKHs occur in the migratory locust; one deca- and two octapeptides. They are encoded by separate genes (Bogerd et al., 1995) and only differ in one (AKH-I vs. -III) or three (-I vs. -II, -II vs -III) amino acids (Oudejans et al., 1991). In the present study, we use the locust as a model system to explore the rationale for this intriguing phenomenon, focusing on the induction of inositol phosphate ($InsP_n$) metabolism by the AKHs.

Long distance flight of insects is one of the most energy demanding processes in nature. *Locusta migratoria* AKHs, which show many actions similar to the catabolic actions of mammalian glucagon (Goldsworthy, 1994), control fuel supply to the flight muscles. Their target organ is the fat body, which contains both carbohydrate and lipid reserves. The fat body combines many of the properties of vertebrate liver and adipose tissue (Dean et al., 1985). Upon flight activity, AKH

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activates both glycogen phosphorylase and triacylglycerol lipase, leading to an increased release of fat body trehalose and diacylglycerol (Beenakkers et al., 1985). The structural features of the fat body, which is capable of functioning in vitro, renders this tissue a suitable model for studies on signal transduction mechanisms in an intact animal system (Van Marrewijk et al., 1991). The three AKHs express interesting differences in potency: AKH-I is most potent in lipid mobilization, followed by AKH-II and -III (Oudejans et al., 1992). However, in many other respects AKH-III is most potent: in stimulating cyclic AMP (cAMP) production, glycogen phosphorylase activation (Vroemen et al., 1995a), and Ca^{2+} efflux (Vroemen et al., 1995b); in inhibition of acetate uptake, fatty acid synthesis (Lee and Goldsworthy, 1995a), and RNA synthesis (Kodrík and Goldsworthy, 1995). In the assays for acetate uptake and fatty acid synthesis, AKH-II is the least potent; in the other actions AKH-I is the least potent. We recently discovered that the inactivation rates of the three AKHs in hemolymph differ dramatically, decreasing in the direction AKH-III $\gg$ -II > -I (Oudejans et al., 1996); the same pattern was found for their susceptibility to cleavage by chymotrypsin (Lee and Goldsworthy, 1995b). So far, it is not known whether the AKHs use the same or different receptors on the fat body.

Over the last few years, a considerable amount of data on the signal transduction of AKHs has been obtained. In the presence of extracellular Ca^{2+}, AKH enhances fat body cAMP levels, a Ca^{2+}-dependent event, and glycogen phosphorylase activity via the stimulatory G protein G_s (Vroemen et al., 1995a). The final step in this process, the activation of phosphorylase via cAMP, is independent of extracellular Ca^{2+} (Van Marrewijk et al., 1993). AKH-induced mobilization of fat body lipid reserves appeared to be dependent on extracellular Ca^{2+} as well (Lum and Chino, 1990). The importance of Ca^{2+} ions was further illustrated by the AKH-evoked increases in Ca^{2+} fluxes into and from the fat body, likely resulting in enhanced intracellular Ca^{2+} levels (Vroemen et al., 1995b). The activation of phosphorylase not only needs extracellular Ca^{2+}, but the release of Ca^{2+} from intracellular stores is required as well (Van Marrewijk et al., 1993). We have proposed that hormone-induced Ca^{2+} influx into fat body is mediated through depletion of intracellular Ca^{2+} stores, likely in a way similar to capacitative calcium entry in vertebrate cells (Berridge, 1995).

In view of their proposed Ca^{2+} mobilizing actions, it is important to gain an understanding of the role of $InsP_n$ in transducing the AKH signal. The work to date on the role of $InsP_n$ in AKH signaling has been limited to the influence of AKH-I, we report in this paper on the influence of all three AKHs on $InsP_n$ metabolism, which may provide insight into the biological significance of the simultaneous occurrence of closely related hormones. Although this addition helps to understand the broader issue, there remains an important lacuna in our information. So far, all studies on the role of $InsP_n$ in insect signal transduction mechanisms report agonist-induced increases in $InsP_n$ levels (Berridge et al., 1983), however none of them unequivocally demonstrates that inhibiting phospholipase C (PLC), the enzyme responsible for $InsP_n$ formation, results in a consequent inhibition of its presumed effector. In this paper, we provide conclusive evidence for a role of PLC in AKH signaling. Furthermore, a model is presented for the concerted action of the three AKHs, based on both these new data and previous findings.

2. Materials and methods

2.1. Animals

Adult male locusts, *Locusta migratoria*, were reared under crowded conditions as described previously (Van Marrewijk et al., 1983). For experiments, insects were used 14–16 days after the imaginal ecdysis.

2.2. Chemicals

Synthetic AKH-I, -II and -III were from Peninsula Laboratories Europe (St. Helens, UK) and were used from 20 pmol/μl stocks in 10% methanol (AKH-I) or 10% methanol, 1 M sodium acetate (AKH-II and -III). Emulsifier-Safe scintillator was purchased from Packard (Groningen, The Netherlands), triethyl ammonium hydrogen carbonate buffer (TEAB) from Fluka Chemie (Buchs, Switzerland), myo-[2-^{3}H]inositol (18 Ci/mmol) from Amersham (Den Bosch, The Netherlands) and ^{3}H-labeled standards of 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) from Du Pont de Nemours (Den Bosch, The Netherlands). U73122 was obtained from Calbiochem (Breda, The Netherlands) and dissolved (10 mM) in DMSO, and penicillin/streptomycin from Gibco BRL (Breda, The Netherlands). All other chemicals and auxiliary enzymes were purchased from local distributors.

2.3. Isolation and incubation of fat body

Fat body preparation and incubation as well as prelabeling of phosphatidyl inositols ($PtdInsP_n$) and $InsP_n$ to equilibrium (during 16 h) were carried out as described previously (Van Marrewijk et al., 1996). For comparisons between the effects of the AKHs, control experiments were routinely included, applying the three hormones (and their solvent as a control) to quarter fat bodies of the same animal. Likewise, a fair comparison between different time points was obtained by perform-

ing control experiments in which quarter fat bodies prepared from the same animal were incubated for different times in the presence of the same effector. Unless otherwise stated, hormonal effects were studied at a massive dose of 40 nM in order to reduce the number of experiments required to obtain significant effects. If essential, however, also lower doses were applied.

2.4. Analysis of [^{3}H]$InsP_n$ formation in fat body

Stimulation of $InsP_n$ metabolism by AKH in the presence of 20 mM LiCl (to inhibit reconversion of $InsP_n$ to inositol), extraction and separation of $InsP_{1-6}$ were done as described previously (Van Marrewijk et al., 1996), with the following modifications: individual radiolabeled $InsP_{1-6}$ were eluted with 10 ml each of, successively, 110 mM TEAB ($InsP_1$), 210 mM TEAB ($InsP_2$), 310 mM TEAB ($InsP_3$), 410 mM TEAB ($InsP_4$) and 500 mM TEAB ($InsP_{5,6}$). These TEAB concentrations were determined by continuous gradient elution of radiolabeled $InsP_{1-4}$ standards, and their validity for a stepwise elution was confirmed in control experiments using these standards. If no separation of $InsP_{1-6}$ was desired, total $InsP_n$ was eluted from the anion exchange column with 10 ml 500 mM TEAB. To adjust for differences in the uptake of [^{3}H]inositol per half fat body, the amount of $InsP_n$ per sample was expressed as a ratio of dpm [$InsP_n$]/dpm [$PtdInsP_n$ plus $InsP_n$], the latter representing total radioactivity in the fat body.

2.5. Measurement of Ins(1,4,5)P_3

Extraction of Ins(1,4,5)P_3 from fat body for determination of its intracellular concentration was carried out as described before (omitting phytate hydrolysate) (Van Marrewijk et al., 1996), however the aqueous phase resulting after centrifugation of the homogenate was directly used in the RIA kit (Amersham, Den Bosch, The Netherlands). Ins(1,4,5)P_3 levels were expressed as pmol per mg fat body protein. Protein was determined by the method of Schacterle and Pollack, 1973.

2.6. Determination of phosphorylase activity

Fat body tissue was homogenized as described previously (Cunha-Melo et al., 1988) and glycogen phosphorylase activity was measured using a protocol of Van Marrewijk et al., 1980. The extent of activation of the enzyme was expressed as the 'activity ratio' between active phosphorylase, including phosphorylase *a* and a minor part of *ab* (Van Marrewijk et al., 1988), and total phosphorylase, including the *a*, *ab* and *b* forms.

2.7. Statistical analysis

Results are expressed as the mean ± S.E.M. for n independent experiments. Statistical analysis of the data was made using a t-test for paired comparisons (Sokal and Rohlf, 1969). Statistical outliers were identified using Dixon's test (Dean and Dixon, 1951).

3. Results

3.1. Effect of AKH on the synthesis of total $InsP_n$

When fat body tissue that had been prelabeled with [^{3}H]inositol was treated with 40 nM AKH-I, -II or -III, the formation of radiolabeled $InsP_n$ was stimulated by each of the AKHs (Fig. 1). At each incubation time used, the amount of ^{3}H-labeled total $InsP_n$ was higher in AKH-treated samples than in control samples. For all three AKHs the effect appeared to be biphasic, with an initial rise in total $InsP_n$ levels as early as 15 s after the addition of AKH (although almost absent for AKH-II), and reaching a maximum within 1 min. Surprisingly, a second (and stronger) increase in $InsP_n$ levels ($P < 0.001$ compared with the control) occurred after 5 min and persisted until at least 30 min following hormonal stimulation. The time courses for total $InsP_n$ formation induced by the three hormones were dissimilar. For AKH-I and -III, the rise in total $InsP_n$ was significant ($P < 0.01$) at 15 s and reached a maximum (ca. +45%) at 1 min. The AKH-II-induced $InsP_n$ formation was significantly lower than the AKH-I and -III effects during the first min ($P < 0.05$) and became significantly different from the control ($P < 0.01$) after 1 min (ca. +20%). In contrast to the other AKHs, for which total $InsP_n$ levels strongly increased or remained constant at 2.5 min, the curve for AKH-I displays a dip in $InsP_n$ formation ($P < 0.001$) at this time point, resulting in a level not significantly different from the control. After 5 min, each of the hormones strongly induced $InsP_n$ synthesis, which reached maxima (ca. +90%) after 15 min of incubation.

3.2. Effect of U73122 on $InsP_n$ formation and glycogen phosphorylase activation

To ascertain that PLC is involved in the intracellular transduction of the AKH signal towards glycogen phosphorylase, the highly specific PLC inhibitor U73122 (100 μM) was applied to fat body tissue 45 min prior to exposure to 40 nM AKH-I, -II or -III. First the effect of the inhibitor on AKH-induced $InsP_n$ formation was studied to verify the ability of the compound to inhibit PLC in the fat body. For AKH-I and -III, an incubation period of 1 min was chosen, whereas for AKH-II we chose 2.5 min because the rise in $InsP_n$

levels caused by this hormone was still low at 1 min (Fig. 1). U73122 completely inhibited AKH-I, -II and -III-induced $InsP_n$ synthesis (Fig. 2A), precluding that a possible partial inhibition of phosphorylase activation would be due to incomplete inhibition of PLC by

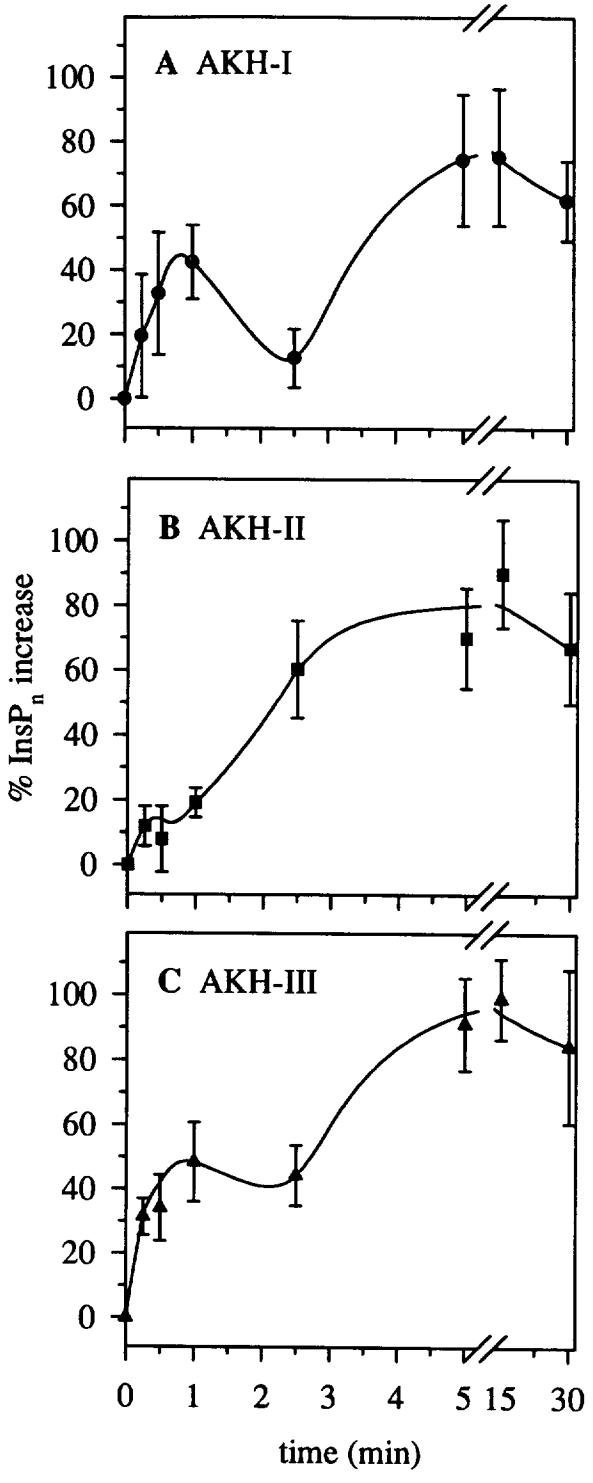

Fig. 1. Time course of total [^{3}H]$InsP_n$ synthesis in fat body induced by 40 nM AKH-I (A), -II (B) and -III (C). Radioactivity of total $InsP_n$ is expressed as the percentage increase compared to the control at each time point ($n = 12$). See text for significance of differences.

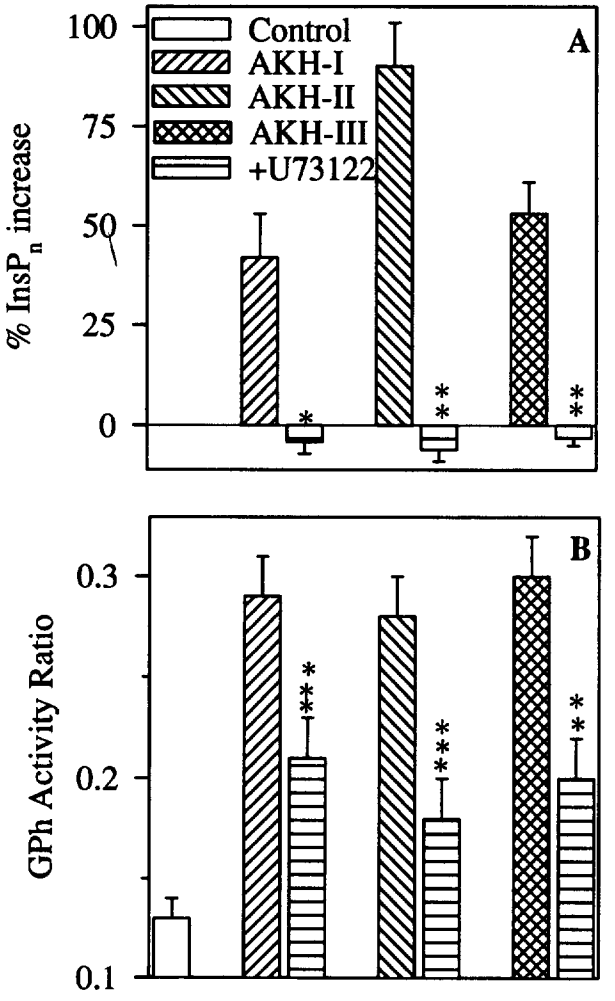

Fig. 2. Effect of U73122 (100 μM) on total $InsP_n$ formation (A) and glycogen phosphorylase activation (B) induced by 40 nM AKH-I, -II and -III. Radioactivity of the extracted $InsP_n$ is expressed as the percentage increase compared to the control for each AKH ($n = 8$), and phosphorylase activity as the activity ratio between active and total phosphorylase ($n = 13$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from AKH treatments without U73122.

U73122. Subsequently, we examined the effect of the inhibitor on AKH-I, -II and -III-induced glycogen phosphorylase activation. Fat body quarters were incubated with each hormone for 10 min. Stimulation of phosphorylase activity by all three AKHs was attenuated in the presence of U73122, implying that PLC is involved in AKH signaling in the direction of phosphorylase (Fig. 2B).

3.3. Effect of AKH on the synthesis of distinct $InsP_{1-6}$

We assessed the effect of AKH-I, -II and -III on the synthesis of distinct $InsP_{1-6}$ after incubation periods of 1, 2.5 and 15 min. These time periods were chosen to obtain more insight into the nature of the first peak, the dip (or plateau/rise) and the second peak in the total $InsP_n$ curves (Fig. 1). Although the absolute levels of

radioactivity were by far the highest in the $InsP_1$ fractions (Fig. 3), AKH treatment stimulated synthesis of almost all forms $InsP_n$ from prelabeled phosphoinositides at each time point. AKH-I hardly induced a rise in any $InsP_n$ form after 2.5 min, which corresponds with the dip in the total $InsP_n$ curve at this time point. At short incubation times AKH-induced elevations of the high polyphosphates contributed to the total rise in $InsP_n$ levels, whereas prolonged incubation resulted in an accumulation of mainly the lower $InsP_{1-2}$, as expected from the presence of the monophosphatase inhibitor LiCl. Control levels did not change significantly with time. A 1 min incubation with AKH-I and -III, but not -II, led to a substantially enhanced radioactivity in the $InsP_3$ fraction, which is supposed to contain the second messenger Ins(1,4,5)P_3 together with Ins(1,3,4)P_3. For AKH-III, this elevation persisted during prolonged incubation, whereas for AKH-I it returned to the basal level within 2.5 min. Remarkably, AKH-II yielded a small increase of $InsP_3$ only after 2.5 min. With respect to the higher polyphosphates, AKH raised both $InsP_4$ levels (particularly after longer incubations) and $InsP_{5,6}$ levels as well (although no standards were available to analyze the exact nature of the latter fraction).

3.4. Effect of AKH on the concentration of Ins(1,4,5)P_3

Because Ins(1,4,5)P_3 has a pivotal role in intracellular Ca^{2+} signaling in many cell types (Berridge, 1993), we sought to determine its intracellular concentration, as well as the influence of AKH on its level using a radioreceptor assay. This assay provides more detailed information than the one using radiolabeled $InsP_3$ fractions, as the latter does not separate Ins(1,3,4)P_3 from Ins(1,4,5)P_3. To produce results comparable with those from the experiments described above, fat bodies were preincubated for 16 h before application of AKH-I, -II or -III for 1 and 15 min. A substantial amount of Ins(1,4,5)P_3 (66 pmol/mg protein), which did not change with time, was already present in the controls. Upon treatment with 40 nM AKH-I, -II or -III for 1 min, Ins(1,4,5)P_3 levels were significantly elevated, although the AKH-II-induced level was, as expected from our radiolabeling experiments, much lower ($P < 0.05$) than those induced by AKH-I and -III (141, 83, 110 pmol/mg protein for AKH-I, -II, -III respectively; Fig. 4). In contrast to the AKH-II effect, the effects of AKH-I and -III appeared to be dose-dependent ($P < 0.05$, 4 vs. 40 nM effects). At a more physiological dose of 4 nM (Cheeseman and Goldsworthy, 1979), responses to each of the hormones were similar. Finally, in accordance with the results of our radiolabeling assays, only the AKH-III-induced Ins(1,4,5)P_3 level remained significantly elevated after 15 min.

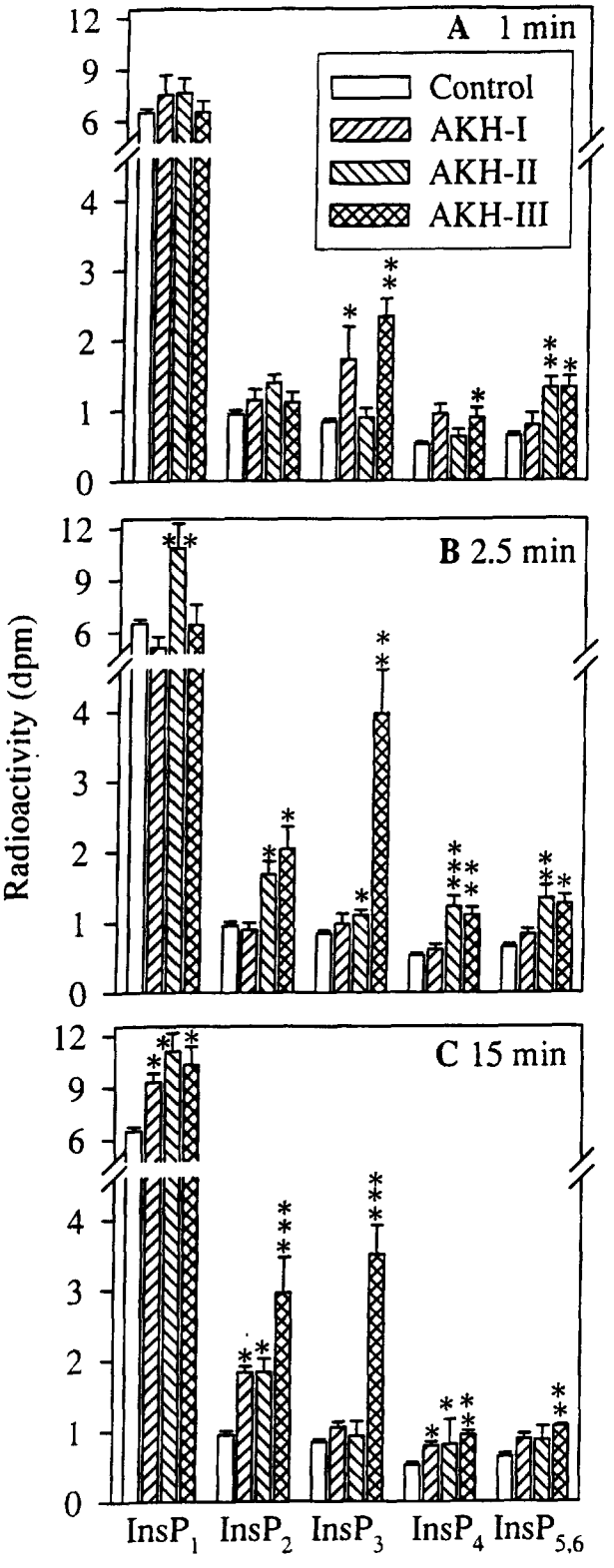

Fig. 3. Effect of 40 nM AKH-I, -II and -III on the radioactivity profiles of distinct $InsP_{1-6}$ after 1 min (A), 2.5 min (B) or 15 min (C) of incubation. Radioactivity of individual $InsP_{1-6}$ is expressed as dpm per 100 dpm prelabeled $PtdInsP_n$ ($n = 11$). The basal $PtdInsP_n$ labeling level amounted approximately to 10^5 ($\pm$ 5000) dpm per fat body half. Control bars represent the average of the individual controls for each of the AKHs (which were not significantly different). * $P < 0.05$, ** $P < 0.01$, *** $P < .001$ significantly different from the control.

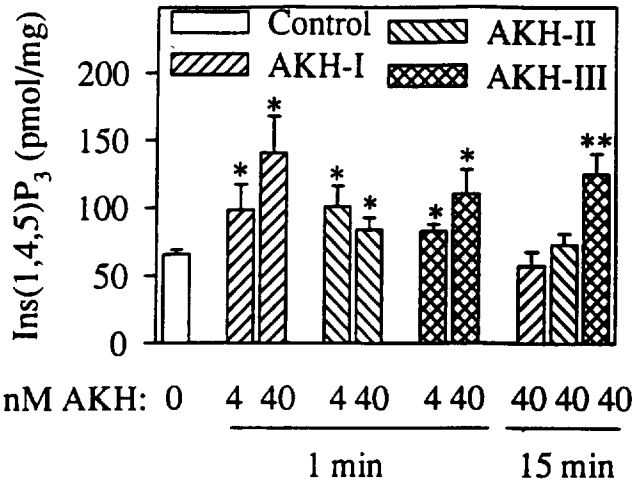

Fig. 4. Intracellular Ins(1,4,5)P_3 concentrations (in pmol/mg protein) after stimulation of fat body with 4 or 40 nM AKH-I, -II or -III for the times shown ($n = 12$). * $P < 0.05$, ** $P < 0.01$, significantly different from the control .

4. Discussion

In an attempt to clarify the rationale for the simultaneous release of structurally and functionally related hormones, we investigated differences in the signaling pathways of the three AKHs in our model system, the locust fat body. Whereas there are interesting differences in the potency of the AKHs to elicit various biological effects, the most striking difference among the AKHs reported so far concerns their rate of inactivation by endopeptidases, which is by far the highest for AKH-III (Oudejans et al., 1996). In this paper we advance understanding of endocrinology by moving from hormone inactivation to signal transduction pathways. As the complex phosphoinositide cycle provides many different forms of $InsP_{1-6}$ with possible second messenger function, we focused on the differential effects of the AKHs on $InsP_n$ metabolism.

As to the effects of the AKHs on total $InsP_n$ formation, the first peak observed after 1 min is consistent with an early role of $InsP_n$ in hormonal signaling. The early rise in $InsP_n$ levels instigated by AKH-II was substantially lower than those generated by the other AKHs. This was unexpected because previous experiments had failed to demonstrate differences in AKH-I, -II and -III-induced Ca^{2+} influx (Vroemen et al., 1995b), a process thought to be linked to $InsP_n$ production via capacitative Ca^{2+} entry (Berridge, 1995). Preliminary experiments on intracellular Ca^{2+} concentrations, however, have shown that AKH-II induces a much shorter rise in the Ca^{2+} levels than the other AKHs (Goldsworthy et al., 1996). Moreover, in contrast to AKH-II and -III-induced $InsP_n$ levels, the $InsP_n$ level brought about by AKH-I returned to the basal value after 2.5 min, suggesting some kind of desensitization mechanism which we cannot explain by our current results. The second, strong elevation of total $InsP_n$ levels after longer incubations, previously found with *Locusta* AKH-I (Van Marrewijk et al., 1996) and *Schistocerca* AKH-I and -II (Stagg and Candy, 1996), may be due to a non-physiological response of the fat body upon prolonged exposure to high doses of AKH (40 nM) and LiCl (20 mM). To get more insight into the validity of this suggestion, as well as the significance of the described discrepancies, we identified the distinct $InsP_{1-6}$ present in the total $InsP_n$ fractions.

The identity of the $InsP_{1-6}$ detected and the time course of their labeling are consistent with the view that stimulation of PLC first leads to the synthesis of $InsP_3$. The contribution of higher $InsP_{4-6}$ to the total rise in $InsP_n$ was more profound at short incubation times than at longer ones. This is congruent with the possible further phosphorylation to $InsP_4$ and $InsP_{5,6}$, and finally dephosphorylation of these products to $InsP_2$ and $InsP_1$ (Batty et al., 1989). Longer incubation periods mainly resulted in the accumulation of $InsP_1$ and $InsP_2$, as Li^+ prevents recycling of $InsP_1$ to inositol by acting as an uncompetitive inhibitor of primarily inositol monophosphatase (Gee et al., 1988). Berridge et al., 1983 reported that particularly during longer incubation periods, $InsP_1$ accumulates to a much greater extent than other $InsP_{2-6}$, a finding supported by data from proctolin-stimulated locust foregut (Hinton and Osborne, 1995) and AKH-I-treated fat body (Van Marrewijk et al., 1996).

Agonist-induced disproportionate rises in [^{3}H]$InsP_3$ were reported in locust fat body by AKH-I (Van Marrewijk et al., 1996) and -II (Stagg and Candy, 1996), in foregut (Hinton and Osborne, 1995), rectum (Radallah et al., 1995; Nogaro et al., 1995) and metathoracic ganglion (Trimmer and Berridge, 1985). Here we also show substantial elevations in [^{3}H]$InsP_3$ levels upon stimulation for 1 min with AKH-I and -III, consistent with an early role for Ins(1,4,5)P_3 in Ca^{2+} mobilization. In accordance with the weak AKH-II-induced rise in total $InsP_n$, AKH-II did not cause a significant rise in [^{3}H]$InsP_3$ after 1 min, although previous studies had shown AKH-II-induced Ca^{2+} influx within this time span (Vroemen et al., 1995b). However, using a highly specific radioreceptor assay, we observed AKH-II-induced rises in Ins(1,4,5)P_3 which, at a constant level of [^{3}H]$InsP_3$, might be explained by a hormone-induced shift in the relative amounts from Ins(1,3,4)P_3 towards Ins(1,4,5)P_3.

After 15 min, $InsP_3$ levels in AKH-I and -II-treated tissue relaxed to control values, though levels of total $InsP_n$ were greatly enhanced. As at that time point $InsP_1$ and $InsP_2$ constitute the majority of the $InsP_n$, these data indicate that the second rise in total $InsP_n$ is of minor physiological significance. Contrary to the

influence of AKH-I and -II, the $InsP_3$ levels induced by AKH-III remained elevated even after 15 min. We infer that AKH-III may have a more prolonged effect on $InsP_n$ and Ca^{2+} signaling than the other AKHs, in correspondence with preliminary data on increases in fat body Ca^{2+} levels induced by short-term application of the three AKHs. The influence on Ca^{2+} is more prolonged for AKH-III than for AKH-I and -II (Goldsworthy et al., 1996).

In agreement with a possible role of Ins(1,3,4,5)P_4 in Ca^{2+} signaling (Irvine, 1991), [^{3}H]InsP_4 levels were elevated by each AKH at all time points (although our experimental setup did not allow discrimination between the different isoforms of InsP_4). Its concentration was enhanced within 1 min and was still elevated during prolonged incubations, as has also been found in *Schistocerca* fat body (Stagg and Candy, 1996). This is in agreement with the observation that Ins(1,3,4,5)P_4 is essential for sustained Ca^{2+} signaling in mouse lacrimal acinar cells (Morris et al., 1987; Changya et al., 1989). In addition to a direct role in Ca^{2+} signaling, Van Marrewijk et al., 1996 showed the mechanism in locust fat body for the utilization of Ins(1,3,4,5)P_4 as a rapidly mobilizable pool from which Ins(1,4,5)P_3 could be resynthesized, as proposed for a number of vertebrate cells (Cunha-Melo et al., 1988; McIntosh and McIntosh, 1990; Foster et al., 1994). Moreover, synthesis of $InsP_{5,6}$, thought to exert signaling effects in animals and plants (Van der Kaay et al., 1995), is probably enhanced by the AKHs.

We suggest that Ins(1,4,5)P_3 may be a critical second messenger that triggers mobilization of intracellular Ca^{2+} in the fat body of *Locusta migratoria*, as recently suggested for hypertrehalosemic hormone of the cockroach *Periplaneta americana* (Park and Keeley, 1996) and AKH of the desert locust *Schistocerca* (Stagg and Candy, 1996; Pancholi et al., 1991). The observed Ins(1,4,5)P_3 concentrations instigated by 40 nM AKH after 1 min correspond with this view, and are in the same order of those previously reported for AKH-I (Van Marrewijk et al., 1996) and for cockroach hypertrehalosemic hormone (Park and Keeley, 1996). Again, rises induced by AKH-I (+ 114%) and -III (+ 68%) are substantially higher than rises evoked by AKH-II (+ 27%). However, the latter rise might be sufficient for the increase in intracellular Ca^{2+} levels caused by AKH-II, which is substantially shorter than those evoked by AKH-I and -III (Goldsworthy et al., 1996). Unexpectedly, the three AKHs appeared to be equally potent at a physiological dose of 4 nM. Thus, in contrast to its effect on cAMP (Vroemen et al., 1995a), the AKH-II effect on Ins(1,4,5)P_3 peaks at 4 nM (ca. + 43%).

In unstimulated control fat body cells, the amount of Ins(1,4,5)P_3 was quite substantial, the basal level amounting to ca. 65 pmol/mg protein, which corresponds with an estimated cellular concentration of 2.5 μM. Much of this Ins(1,4,5)P_3, however, may well be metabolically inactive in unstimulated cells, as has been discussed previously (Van Marrewijk et al., 1996). We recognize that other mechanisms could be involved as well, e.g. a modulatory role of Ins(1,3,4,5)P_4.

The role of PLC in hormonal signaling in insects had not been unequivocally established. Here we demonstrated that inhibition of PLC resulted in partial reduction of glycogen phosphorylase activation by all three AKHs; this partial effect could not be due to incomplete inhibition of PLC, because U73122 completely inhibited AKH-induced $InsP_n$ formation. More likely, phosphorylase was partially activated via the cAMP pathway, which is not influenced by inhibition of PLC. Our results strongly support a role for $InsP_n$ in AKH signaling towards glycogenolysis. We are currently investigating the possibility that PLC-produced diacylglycerol acts in transduction of AKH signals.

Although the observed differences in $InsP_n$ metabolism are not immense, our data provide us with new clues for understanding the simultaneous occurrence of three different AKHs in locust. Each AKH is encoded by a separate gene (Bogerd et al., 1995), suggesting that each hormone may play its own biological role in the overall syndrome of insect flight. AKH-III is rapidly synthesized in the adipokinetic cells, but undergoes a high turnover and/or a fast release from these cells, suggesting that the hormone is released constitutively and provides the locust with energy when it is not flying (Oudejans et al., 1991). The high potency of AKH-III in many assays is consistent with this function. At the onset of flight, the mRNA levels encoding for all three AKHs are elevated (Bogerd et al., 1995), indicating a role for all three AKHs during flight activity. However, the level of AKH-III in the adipokinetic cells is quite low (Oudejans et al., 1991), its half-life in the hemolymph is very short (Oudejans et al., 1996) and its release during flight has not been established. On the other hand, release of AKH-I and -II during flight has been established (Orchard and Lange, 1984) and relative to AKH-III their half-lives are much longer (Oudejans et al., 1996). On the basis of data available so far, one can speculate on different physiological functions for the AKHs. During the early stages of locust flight the main energy substrate is carbohydrate; there is a subsequent marked shift to lipid oxidation during sustained flight activity (Van der Horst et al., 1978). AKH-I is more potent than the other AKHs in mobilizing lipids, while AKH-II is associated with carbohydrate mobilization (Oudejans et al., 1992; Orchard and Lange, 1983). Therefore, AKH-I would be the major lipid-mobilizing hormone, and AKH-II the major trigger for carbohydrate mobilization, while AKH-III would constitute a modulatory entity during rest. Several points support this view.

First, AKH-II typically occurs at lower titer than AKH-I during flight (Diederen et al., 1987). Second, both hormones are inactivated at the same rate during flight (Oudejans et al., 1996). Therefore, AKH-I may even be considerably more predominant at sustained flight (when lipid is the main fuel) than at the beginning of flight. Moreover, activation of hormone-sensitive lipases in adipocytes involves translocation of the lipases to the lipid storage droplets (Egan et al., 1992), a process highly dependent on Ca^{2+} ions (Clark et al., 1991). The observation that AKH-I induces less Ca^{2+} efflux (Vroemen et al., 1995b), somewhat higher $InsP_n$ levels (this study) and a more prolonged elevation in intracellular Ca^{2+} concentrations (Goldsworthy et al., 1996) than AKH-II, also supports our view that the first AKH is responsible for lipid mobilization. Insect flight demands very dynamic changes in energy mobilization and metabolism. There is an additional complexity in long distance fliers, such as locusts, because these insects shift energy substrate from carbohydrates to lipids during flight. The success of migratory insects can be, in part, understood in light of the coordinated biological actions of three similar, yet unique hormone entities.

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