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Activation of fat body in *Periplaneta americana* (Blattoptera: Blattidae) by hypertrehalosemic hormones (HTH): New insights into the mechanism of cell signalling*

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Abstract. In cockroaches and certain other insects the concentration of trehalose in the hemolymph is increased by hypertrehalosemic hormone (HTH), a neuropeptide originating in the corpus cardiacum. A vital step in the action of HTH to promote conversion of glycogen stored in the fat body to trehalose is the activation of phosphorylase. The means by which HTH activates phosphorylase, with particular emphasis on its role in the regulation of intracellular calcium, is discussed. Additional information supporting the view that HTH stimulated synthesis of trehalose, and possibly its release from the trophocyte, is regulated by fatty acids and eicosanoids is presented.

A characteristic feature of insect hemolymph is the presence of the non-reducing disaccharide trehalose. This sugar comprises two glucosyl residues that are readily available to the insect and can be utilized for various purposes. The concentration of trehalose in hemolymph is, as a rule, much higher than that of the monosaccharide glucose which generally is also present. In the cockroach, *Periplaneta americana*, for example, the average concentration of trehalose is approximately 40 mM whereas glucose usually does not exceed 1–2 mM. Before trehalose can be utilized by the insect it must it be hydrolysed to yield its constituent glucose molecules. This is accomplished either by an intracellular trehalase after trehalose has passed into the cell, or by taking up glucose released from hemolymph trehalose by trehalase in the hemolymph. Hemolymph trehalose is important, both directly and indirectly, in supporting the function of many different tissues in the insect. For example, trehalose is the major source of energy used to fuel flight muscle in many species of insects. In the ovary, trehalose is the source of glucosyl residues used for the synthesis of glycogen laid down in the follicular cells to serve as a source of energy and for other purposes during development of the embryonic insect. Of special significance to insects is the use of trehalose to provide glucosyl residues that the epidermal cells incorporate into chitin during the formation of the cuticle at each moult. The work described here is an overview of recent studies in the author's laboratory which were undertaken to better understand the signalling mechanism that transmits the message carried by the hypertrehalosemic hormone (HTH) in the cockroach (*Periplaneta americana*). More general reviews of HTH function are those by Keeley et al. (1991) and Steele (1985).

The organ primarily responsible for the synthesis of trehalose is the fat body. Because the biochemical pathway for the synthesis of trehalose in fat body is well known it need not be described here. Dietary carbohydrate is an important source of precursors for the synthesis of trehalose, much of which is returned to the hemolymph if the concentration of the sugar has fallen below the level normally required. At other times trehalose is derived from glycogen stored in the fat body. It will be readily appreciated that the demand for trehalose (i.e. glucosyl residues) is likely to vary, depending on the intensity of physiological activity, with the result that high demand could prove limiting in the absence of a mechanism to maintain or elevate the level of trehalose, or to sustain any increase in the rate of turnover. To this end it now appears that some species employ hormones to increase the rate of trehalose synthesis. This mechanism has been studied in greatest detail in several species of cockroach. The corpus cardiacum in *Periplaneta americana* contains two neuropeptides, each of which causes the level of hemolymph trehalose to increase more than two-fold. These hypertrehalosemic hormones (HTH-I and HTH-II) have the following structures:

HTH-I: pGlu-**Val**-**Asn**-Phe-**Ser**-Pro-Asn-Trp-NH_2

HTH-II: pGlu-**Leu**-**Thr**-Phe-**Thr**-Pro-Asn-Trp-NH_2

As shown, these hormones are octapeptides having five amino acids in common (Scarborough et al., 1984). The hormones are stored in the corpus cardiacum prior to their release into the hemolymph where they have access to the fat body. The purpose in having two hormones, both appearing to have similar functions, is not known.

There are sound reasons for the belief that glycogen phosphorylase plays a key role in determining the rate of trehalose synthesis, a view supported by the marked acti-

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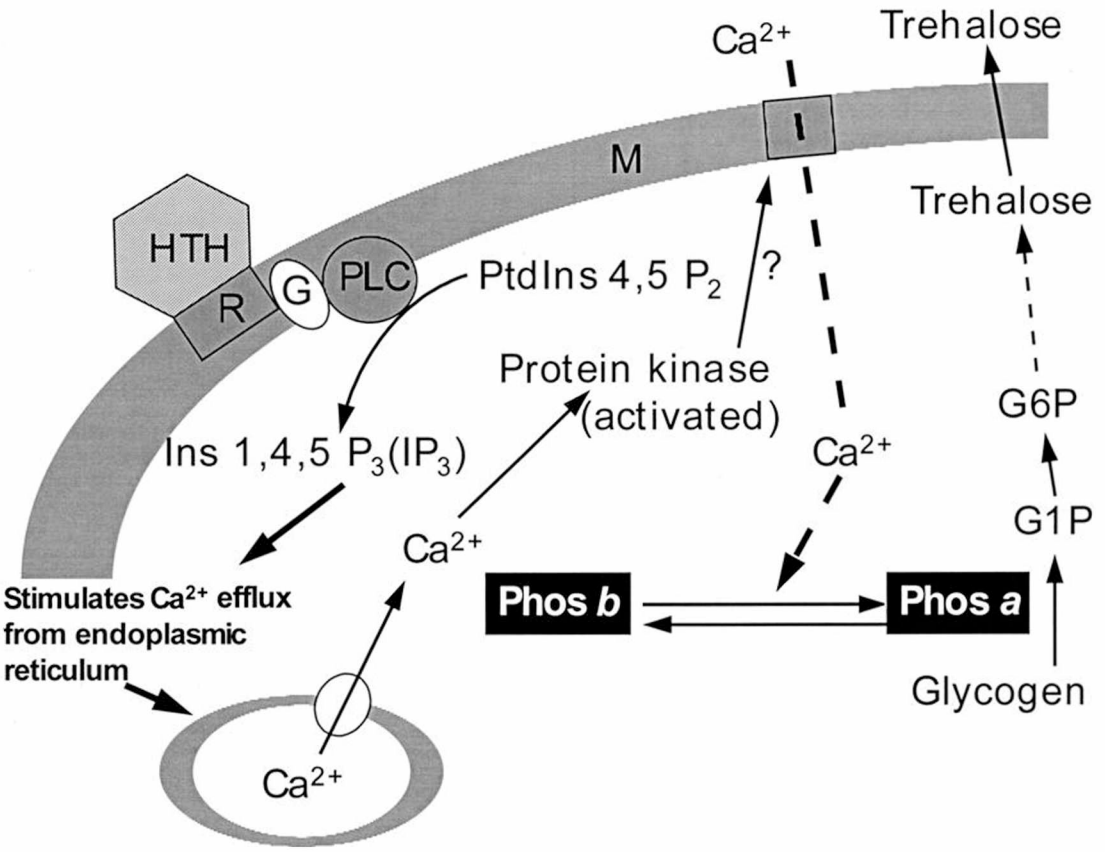

Fig. 1. The recruitment of extracellular Ca^{2+} from the extracellular fluid by fat body trophocytes following a challenge by hypertrehalosemic hormone. The increase in the cytoplasmic concentration of calcium is essential for the activation of glycogen phosphorylase. The abbreviations used are: G – G protein; G1P – glucose 1-phosphate; G6P – glucose 6-phosphate; HTH – hypertrehalosemic hormone; IP_3 – inositol 1,4,5-trisphosphate; M – membrane; PtdIns $4,5P_2$ – phosphatidylinositol 4, 5-bisphosphate; PLC – phospholipase C; Phos *a* – phosphorylase *a*; Phos *b* – phosphorylase *b*; R – receptor.

vation of the enzyme by both HTHs. Nevertheless, it is unlikely that phosphorylase is the sole regulatory site in the trehalose biosynthetic pathway for the following reason. Adenosine 3',5'-monophosphate (cyclic AMP), methyl xanthines and the calcium ionophore A23187 are all potent activators of phosphorylase (McClure & Steele, 1981) yet none of these agents, in contrast to the HTHs, has a significant effect on the efflux of trehalose from the fat body (McClure & Steele, 1981; Steele et al., 1988). Although it is reasonably certain that the reaction catalyzed by phosphorylase is the flux generating reaction our findings suggest that HTH initiates production of regulatory factors that act at other sites downstream from phosphorylase to control the efflux of trehalose from the fat body. These sites possibly include, but need not be restricted to, the reaction catalysed by trehalose 6-phosphate phosphatase and the transport of free trehalose across the plasma membrane into the hemolymph.

A key element in the activation of cockroach fat body by HTH is the requirement for extracellular Ca^{2+}. If Ca^{2+} is omitted from the medium bathing the fat body in vitro, HTH is unable to increase trehalose efflux from the tissue. Under these circumstances phosphorylase in dispersed fat body trophocytes was not activated by 20 nM synthetic HTH-I. When 1 mM Ca^{2+} was added to the medium the activity of the enzyme increased by approximately 100%, an effect comparable to that obtained with corpus cardiacum extract containing the native hormone (McClure & Steele, 1981). Ca^{2+} is required for the activation of phosphorylase kinase which, in turn, converts phosphorylase *b* to the more active phosphorylase *a* with the result that the release of glucosyl residues from glycogen is increased. Like HTH, cyclic AMP stimulates conversion of phosphorylase *b* to phosphorylase *a,* an effect also mediated by activation of phosphorylase kinase, indicating that the cyclic nucleotide has the potential to serve as a second messenger for the hormone.

The permissive and stimulatory effect of calcium and cyclic AMP respectively in the activation of phosphorylase prompts questions about the nature of the second messenger employed by HTH to initiate trehalose efflux from the fat body. Cyclic AMP is unlikely to be a second messenger for HTH for two reasons. Firstly, the concentration of cyclic AMP in the fat body does not increase in response to HTH (Orr et al., 1985) and, secondly, unlike the hormone, it fails to stimulate the release of trehalose (Steele et al., 1988). The absolute requirement for Ca^{2+} by HTH to bring about an increase in trehalose efflux from fat body or dispersed trophocytes suggests that the hormone prompts a response by the trophocyte that leads to an increase in the intracellular concentration of Ca^{2+}. The manner in which this is accomplished has not been described in any detail and many important questions remain to be answered. Nevertheless, numerous studies on

vertebrate organisms (Berridge, 1987), as well as insects (Keeley et al., 1996; Pancholi et al., 1991; Vroemen et al., 1997) give evidence that the second messenger associated with the action of HTH in elevating the level of intracellular Ca^{2+} is inositol 1, 4, 5-trisphosphate (IP_3). A study in our laboratory has shown that both HTH-I and HTH-II increase the concentration of IP_3 in dispersed fat body trophocytes from the cockroach, *Periplaneta americana* (unpublished data). The increase is of the order of 100% and was detected as early as 15 s following the addition of HTH. This increase in IP_3 preceeds the time at which the increase in the efflux of trehalose from the trophocytes can be detected, a prerequisite that must be fulfilled in order that IP_3 qualify as the second messenger for HTH. A model purporting to show the manner in which HTH, acting via the second messenger IP_3, brings about the activation of phosphorylase is shown in Fig. 1. Some evidence has been obtained showing that IP_3 acts to release Ca^{2+} from the endoplasmic reticulum (Park & Keeley, 1996) as it does in mammalian tissue. Nevertheless, this Ca^{2+} pulse from the endoplasmic reticulum appears to be insufficient for full activation of phosphorylase in the absence of Ca^{2+} in the extracellular medium. The model postulates that the Ca^{2+} primarily responsible for the activation of phosphorylase is recruited from the extracellular fluid. Because of the normally steep Ca^{2+} concentration gradient that exists between the extracellular fluid and the cytoplasm the increase in cytoplasmic Ca^{2+} to the level required for activation of phosphorylase could be achieved by opening Ca^{2+} channels in the cell membrane. This might be accomplished by a conformational change in the proteins that comprise the Ca^{2+} channel as a result of phosphorylation by a protein kinase. The model also allows for the suggestion that such a protein kinase could be activated by the pulse of Ca^{2+} released from the endoplasmic reticulum by the increased level of IP_3.

The model to explain the activation of phosphorylase by HTH is based on the premise that there is a rapid influx of Ca^{2+} from the extracellular fluid which is sufficient to increase the intracellular concentration of the ion to the level required for activation of phosphorylase. Using dispersed trophocytes to investigate this problem we have shown that HTH causes the influx of $^{45}Ca^{2+}$ to be maximal during the first 30 s after addition of the hormone to the trophocytes, following which it returns to near normal levels 2 min later (unpublished data). It may be helpful to note that an efflux of Ca^{2+} from the trophocytes begins at this time and continues for about 4 min. This efflux of Ca^{2+} is most intense during the first minute. The Ca^{2+} efflux is an important part of the mechanism since it enables the normal Ca^{2+} level of the cell to be re-established.

Treatment of cockroach fat body in vitro with corpus cardiacum extract containing HTH causes a doubling of the tissue concentration of each glycolytic intermediate between glucose 1-phosphate and fructose 1,6-bisphosphate inclusively with the exception of glucose 6-phosphate (Sevala & Steele, 1991). The concentration of glucose 6-phosphate increases four-fold. For theoretical reasons, because the reaction catalyzed by phosphoglucoisomerase is considered to be near-equilibrium, the greater increase in glucose 6-phosphate is enigmatic. Regardles of the reason, the large increase in the concentration of glucose 6-phosphate, because of mass action, appears to be responsible for the increase in trehalose synthesis that occurs when the fat body is challenged with HTH. The increase in the concentration of the glycolytic intermediates is attributable solely to the activation of phosphorylase which increases the pool of glucose 1-phosphate by phosphorolytic cleavage of the glycogen. Thus it might be anticipated that any agent that activates phosphorylase would lead to an increase in the rate of production of trehalose. This interpretation, however, is incorrect for the reason that agents such as cyclic AMP, methyl xanthines and the calcium ionophore A23187 activate phosphorylase to levels exceeding that obtained with HTH (McClure & Steele, 1981), but with little or no increase in the production of trehalose by the fat body (Steele et al., 1988). This can lead to only one conclusion, namely that HTH causes the fat body to produce additional regulatory agents to control the flux of metabolites through the trehalose biosynthetic pathway. In this context fatty acids and their derivatives appear to have great potential to act as signalling or regulatory agents in the cockroach fat body. Among vertebrates, arachidonic acid is the precursor for a large number of prostaglandins and leukotrienes, many of which have numerous signalling and regulatory functions. In recent years increasing attention has been paid to the possibility that these compounds may also be important as regulators in insect tissues (Stanley-Samuelson, 1994).

The dominant species of free fatty acids in dispersed cockroach fat body trophocytes are palmitic, stearic, oleic and linoleic acids; the concentration of palmitic, stearic and linoleic acids being generally in the range of 2–4 nmol/10^5 cells while that of oleic acid is 8–10 nmol/10^5 cells. Addition of HTH-I (100 pmol/ml) increased the concentration of each fatty acid by 30–40% (Ali & Steele, 1997a). A comparable effect was also obtained with HTH-II. Unlike the trophocytes, the concentration of free fatty acids in the remaining cells of the fat body does not increase following treatment with HTH, showing that the effect of the hormones is specific to the trophocytes.

To determine whether the HTH-mediated increase in trophocyte free fatty acids is causally related to the release of trehalose by the hormone, the problem was investigated from a pharmacological perspective. On the assumption that the fatty acids are likely to be derived from position-2 of phospholipid, inhibitors of phospholipase A_2 (PLA_2) were used to block the release of fatty acids from phospholipid. The effect of these inhibitors, which were expected to impede the release of fatty acids from phospholipid, were also examined for possible effects on trehalose efflux from the trophocytes. Bromophenacyl bromide (BPB), a potent inhibitor of PLA_2 in vertebrate tissue, significantly decreased the concentration of linoleic acid in trophocytes at concentrations as low as 10^{-5} M (Ali & Steele, 1997b). The same concentra-

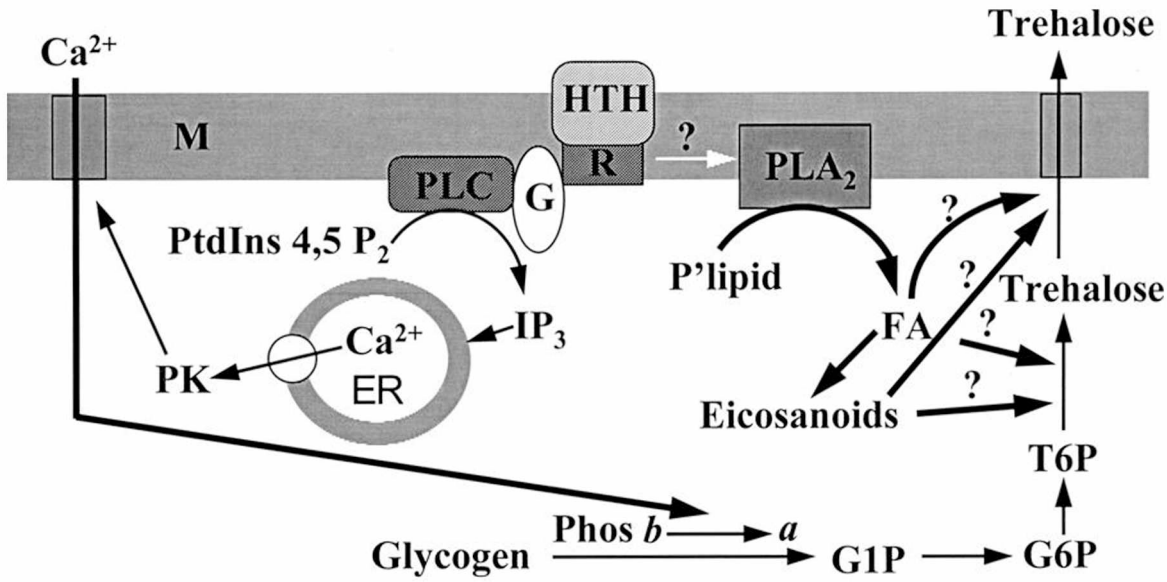

Fig. 2. A scheme showing how hypertrehalosemic hormone, through the dual activation of phospholipase C and phospholipase A_2, may increase the synthesis of trehalose and facilitate its release from the cell. The abbreviations are: ER – endoplasmic reticulum; FA – fatty acid; G – G protein; G1P – glucose 1-phosphate; G6P – glucose 6-phosphate; HTH – hypertrehalosemic hormone; IP_3 – inositol 1, 4, 5-trisphosphate; M – membrane; PtdIns $4,5P_2$ – phosphatidylinositol 4,5-bisphosphate; PLA_2 – phospholipase A_2; PLC – phospholipase C; P'lipid – phospholipid; Phos *a* – phosphorylase *a*; Phos *b* – phosphorylase *b*; PK – protein kinase; R – receptor; T6P – trehalose 6-phosphate.

tion of BPB completely blocked the increase in linoleic acid normally induced by HTH-I, without otherwise interfering with the normal level of linoleic acid in the trophocyte. Significantly, BPB had a concentration-dependent inhibitory effect on trehalose efflux from intact fat body, a concentration of 10^{-5} M BPB completely eliminating the stimulatory effect of HTH (Ali et al., 1998). These findings suggest that release of unsaturated fatty acid from position-2 of phospholipid appears to be necessary for the full expression of the hypertrehalosemic effect of HTH.

The demonstration that fatty acids released from position-2 of phospholipid appear to be essential for the hypertrehalosemic effect of HTH is not evidence that the fatty acid is acting unmodified to promote the action of HTH. Although a role for the unmodified fatty acid cannot be ruled out it seems likely that the additional fatty acids might have been converted to arachidonic acid and then metabolized via the cyclooxygenase or leukotriene pathway. The possibility that an arachidonic acid metabolite generated by the cyclooxygenase reaction is necessary for the action of HTH was tested by determining the effect of the cyclooxygenase inhibitor Diclofenac on the efflux of trehalose induced by HTH from intact fat body. The inhibitory effect of Diclofenac between 10^{-4} and 10^{-3} M was concentration dependent. In the higher range of concentration the stimulatory effect of 10 nM HTH, which normally causes a doubling of trehalose efflux from the trophocytes (Ali et al., 1998), was completely eliminated. Interestingly, the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) also inhibited HTH-stimulated trehalose efflux in a manner that was indistinguishable from that of Diclofenac (Ali et al., 1998). Although these data suggest that prostaglandins and leukotrienes are together involved in the hypertrehalosemic response, this interpretation of the data is possibly incorrect. When used with mammalian tissues the inhibitors are not absolutely specific for either of the cyclooxygenase or lipoxygenase reactions (Ku et al., 1986). Both compounds are known to have inhibitory effects on each pathway, although not to the same extent. It is possible therefore that a similar situation occurs in fat body. The correct interpretation may be that only one, and not both, of the pathways is important in providing an essential factor for the hypertrehalosemic response.

The finding that BPB, an inhibitor of PLA_2, not only blocked the increase in fatty acids normally associated with the action of HTH, but also efflux of trehalose induced by the hormone, is circumstantial evidence that fatty acids participate in the response of the trophocyte to HTH. More substantive evidence for the relationship between fatty acids and trehalose efflux has been obtained by incubating trophocytes with those fatty acids whose concentration is increased by HTH, to determine whether the fatty acids alter the normal pattern of trehalose efflux in the absence of HTH. Of the four fatty acids tested, palmitic acid alone had no effect on trehalose efflux. Each of the remaining fatty acids stimulated the efflux of trehalose by as much as 100%, the sensitivity increasing in the order: stearic acid < oleic acid < linoleic acid (Ali & Steele, 1997c). Notably, at concentrations higher than 20 μM, linoleic acid depressed the efflux of trehalose below that of trophocytes not treated with the fatty acid. Because many tissues have the capacity to synthesize arachidonic acid from linoleic acid, it was of interest to know whether arachidonic acid could also stimulate trehalose efflux from the trophocytes even though it had not proven possible to demonstrate the presence of arachidonic acid in our samples. The data (Ali & Steele, 1997c)

show that arachidonic acid was as effective as linoleic acid in stimulating efflux of trehalose and more effective in turning off the efflux of trehalose when the concentration of arachidonic acid exceeded 10 μM.

The demonstration that Diclofenac and NDGA completely block the hypertrehalosemic effect of HTH in intact fat body suggests that either a prostaglandin or a leukotriene may exert a permissive effect on the release of trehalose from the fat body. If this assumption is correct, it is reasonably certain that arachidonic acid must be the precursor. In this context it is interesting to note that arachidonic acid increased the efflux of trehalose from the fat body. Our efforts to demonstrate arachidonic acid in the trophocytes were unsuccessful, possibly because of the small samples of tissue available. It was therefore of interest to know whether linoleic acid, whose concentration increases in the presence of HTH, could be converted into arachidonic acid by the fat body. Using [^{14}C]-linoleic acid we were able to show that HTH stimulated the formation of arachidonic acid, but only if the cyclooxygenase inhibitor indomethacin was present in the incubation medium (Ali & Steele, 1997c). It seems likely that the failure to show an increase in the synthesis of arachidonic acid in the absence of indomethacin could be attributed to rapid utilization of the newly formed arachidonic acid. These data suggest that arachidonic acid may be the key fatty acid formed under the influence of HTH and is the precursor of an eicosanoid essential for the hypertrehalosemic response. This interpretation is supported by the finding that, in common with the enhanced efflux of trehalose initiated by arachidonic acid and certain other fatty acids, a similar increase in trehalose efflux occurs when the trophocytes are incubated with prostaglandin $F_{2\alpha}$ (Ali & Steele, 1997c). This effect is not a general effect of prostaglandins since prostaglandin E_2 was ineffective in modulating the efflux of trehalose from the trophocytres.

A tentative model to explain the HTH signalling mechanism in the cockroach, *Periplaneta americana*, is shown in Fig. 2. The model postulates that HTH triggers the activation of phospholipase C and PLA_2. Activation of phospholipase C ultimately leads to an increase in the supply of glucosyl residues required for the synthesis of trehalose whereas activation of PLA_2 results in the production of fatty acids and metabolites of fatty acids that appear to regulate the synthesis of free trehalose and its release from the fat body. It is possible that activation of phospholipase C and PLA_2 is mediated by a G protein common to both enzymes although this view must be considered speculative. IP_3 generated by the phosphatidylinositol pathway, in common with its action in many other tissues, probably acts on the endoplasmic reticulum to release calcium and this in turn may activate a protein kinase. A protein kinase so activated might promote the entry of Ca^{2+} into the cell by causing a conformational change in the calcium channel protein. However, it is equally possible that a store-activated mechanism could initiate an influx of Ca^{2+}. The increase in intracellular Ca^{2+} that results is then responsible for the activation of phosphorylase which increases the release of glucosyl residues from glycogen. Activation of PLA_2 increases the production of fatty acids from phospholipid and these are subsequently presumed to be converted to arachidonic acid. This arachidonic acid is then available for metabolism via the cyclooxygenase or lipoxygenase pathway or both. The products of these reactions are potential regulators of the trehalose biosynthetic pathway where, it is suggested, they might control the rate of trehalose synthesis and its efflux from the trophocyte by regulating the activity of trehalose 6-phosphatase or the trehalose transport mechanism in the plasma membrane. Other sites of action cannot, of course, be excluded at this time.

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