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AN INSULIN MEDIATOR PREPARATION SERVES TO STIMULATE THE CYCLIC GMP ACTIVATED CYCLIC AMP PHOSPHODIESTERASE RATHER THAN OTHER PURIFIED INSULIN-ACTIVATED CYCLIC AMP PHOSPHODIESTERASES

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SUMMARY An insulin mediator preparation was obtained from rat hepatocytes which had been treated with insulin. This preparation inhibited adenylate cyclase activity. It stimulated the activity of homogeneous preparations of both the cytosolic and membrane-bound forms of rat liver cyclic GMP-activated cyclic AMP phosphodiesterase. It failed to activate homogeneous preparations of both the peripheral plasma membrane and 'dense-vesicle' cyclic AMP phosphodiesterases. The insulin mediator preparation stimulated cyclic GMP-activated cyclic AMP phosphodiesterase activity in a dose-dependent fashion with a hill coefficient of 0.46. Insulin caused the dose-dependent production of mediator activity in intact hepatocytes with a K_a of 9 pM, although concentrations of insulin greater than 10 nM progressively reduced stimulatory activity.

INTRODUCTION. It is well-established that the purified insulin receptor exhibits a functional tyrosine kinase activity and there is compelling evidence to suggest that this plays a pivotal role in the signal transduction mechanism (1-3). However, there is, as yet, no clear indication as to the nature and function of the physiological substrates for this activity other than the insulin receptor itself. Furthermore, no well-defined sequence of events appear to connect this function of the receptor with biochemical changes within the cell. For some time now a number of laboratories (see e.g 4-6) have provided tantalizing evidence which suggests that insulin may act on target cells to release soluble 'second messengers' or 'mediators' within the cell. These have yet to be fully characterized, although recent evidence suggests that such species may include inositol glycans, perhaps with associated peptide material, and also particular forms of diacyl glycerol (7-9). Such species have been suggested to be capable of activating pyruvate dehydrogenase activity , inhibiting cyclic AMP-dependent protein kinase inhibiting adenylate cyclase activity and activating cyclic AMP phosphodiesterase activity (see 10).

In hepatocytes, insulin antagonizes the ability of glucagon to increase cyclic AMP production (11). At subnanomolar glucagon concentrations this effect is achieved by both

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inhibiting adenylate cyclase activity and by activating cyclic AMP phosphodiesterase activity (12,13). However, at nanomolar concentrations and above, the predominant effect of insulin is to increase cyclic AMP phosphodiesterase activity (11). We (11,12) have shown that in hepatocytes insulin can elicit the stable activation of two membrane-bound cyclic AMP phosphodiesterases. In one instance at least this effect seems to be carried out by insulin eliciting the phosphorylation of the enzyme (11). However, by selectively preventing insulin activating these two enzymes we have been able to show that insulin's activation of these two enzymes cannot account in full for the ability of insulin to decrease intracellular cyclic AMP concentrations (14). Thus insulin must activate at least one other cyclic AMP phosphodiesterase which can exert a major effect in lowering intracellular cyclic AMP concentrations. Hepatocytes, which have been pre-treated with insulin, yield subcellular fractions which show evidence of activation of only the two previously described enzymes. This suggests that the additional enzyme(s) must be activated by a rapidly reversible process. We have purified to homogeneity both cytosolic and membrane bound forms of the other major cyclic AMP metabolizing phosphodiesterase in hepatocytes, the so-called cyclic GMP-activated cyclic AMP phosphodiesterase (15). This particular enzyme is characterise by the unique ability of low (μM) concentrations of cyclic GMP which serve to stimulate the cyclic AMP hydrolysis effected by this enzyme. Here we show that this enzyme, and not the other insulin-regulated phosphodiesterases, is activated by a soluble 'mediator' preparation.

MATERIALS AND METHODS. All biochemicals, including insulin (porcine) were from either Sigma (Poole, UK) or from Boehringer (Lewes, UK); general chemicals were of AR grade or higher (BDH, UK) and radiochemicals were from Amersham International (Amersham, UK). The 'dense-vesicle' (16,17) and peripheral plasma membrane (17) cyclic AMP phosphodiesterases and the cytosolic and membrane bound forms of the cyclic GMP-activated cyclic AMP phosphodiesterase (15) were purified to apparent homogeneity as described in detail before by us. Assay of cyclic AMP phosphodiesterase activity was performed as before (15-17) and initial rates were taken from linear time-courses. Assays were done in a total volume of 100μl and, unless specified otherwise, at 1μM cyclic AMP substrate. Adenylate cyclase assays were carried out for 10min. at 30°C in a total volume of 100μl as described before by us (13). A mediator preparation was obtained essentially as described by Larner et al. (4). Briefly this involved the preparation and pre-incubation of intact hepatocytes, as done before by us (13). Five flasks, each containing 5ml. of Krebs-Hensleit buffer and 5-6 mg. dry weight of cells were incubated for 10min. at 37°C in either the presence or absence of insulin. At the end of this time 5ml. of 'stopping' buffer (0.25M-sucrose, 1mM-EDTA, 10mM-tris HCl final pH7.4) was added to each flask and the cells were harvested by centrifugation at 4°C at 500rpm for 2min. in an MSE Minor centrifuge. The resultant pellets were each resuspended in 5ml. of 'stopping' buffer prior to centrifugation as above. This washing procedure was repeated one further time. The final pellets were pooled and resuspended in 1ml of 'stopping' buffer. 2ml. of 'extraction' solution at 100°C (0.1mM-EDTA, 0.1mM-cysteine, 50mM-formic acid final pH3.5) was added to the resuspended pellets and samples kept at this temperature for 4min. prior to cooling and centrifugation at 14,000g_{av} for 10min. to yield a supernatant fraction (S1). This fraction was treated with activated charcoal (5mg/ml), vortexed for 2min and centrifuged again. The resultant supernatant (S2) was lyophilized and then resuspended in 0.5ml of sample buffer (0.1mM-2-mercaptoethanol, 0.1mM-EDTA, 50mM-formic acid, final

pH3.5). This material was applied, at a flow rate of 0.3ml/min to a 25x1.5 column of Sephadex G25 which had been equilibriated in sample buffer. Fractions (1ml) were collected and immediately lyophilized prior to resuspension in 20µl of 20mM-tris-HCl buffer final pH7.4. Routinely 4/5 fractions were obtained which activated the cyclic GMP-activated cyclic AMP phosphodiesterase. These were pooled to give 80-100µl per preparation and it is with respect to this volume that 'activity' of the mediator is expressed. Comparable fractions from the column were taken for examining 'basal' effects. For chloroform treatment, 20µl fractions were vortexed for 15min with 20µl chloroform. Phases were separated by centrifugation, lyophilized and resuspended in 20mM-tris-HCl buffer final pH7.4.

RESULTS AND DISCUSSION. Mediator preparations were extracted from hepatocytes which had been pre-treated with insulin (10nM) for 10min. at 37°C. Extracts were also made from untreated cells to provide control extracts. The method of extraction and partial purification employed was essentially the same as that described by Larner et al. (4,18). These investigators showed that such a 'mediator' preparation could inhibit adenylate cyclase activity. We confirm this here as the addition of 10µl of such a mediator preparation to adenylate cyclase assays (100µl) gave rise to a 21.6-34.0% (range, n=6) inhibition of the glucagon (10nM) plus GTP (0.1mM) stimulated activity.

The addition of either such a 'mediator' preparation or control extracts, to assays containing either the 'dense-vesicle' or peripheral plasma membrane cyclic AMP phosphodiesterases elicited no change in their activities (<7%) when assayed over a range of mediator concentrations(1-10µl/100µl assay volume) and over a range of substrate concentrations (0.1-1.0µM cyclic AMP). In contrast this 'mediator' preparation elicited a profound activation of both the soluble and membrane-bound forms of cyclic GMP activated cyclic AMP phosphodiesterase (table 1). These two enzymes are almost identical and appear to differ only in the expression of a what is presumed to be a small hydrophobic region which serves to anchor this enzyme to the plasma membrane

Table 1. Selective stimulation of the cyclic GMP activated cyclic AMP phosphodiesterase by insulin 'mediator'

cell treatment	cG-act-sol. PDE	cG-act-membr PDE	'dense-vesicle' PDE	PPM PDE
none	35±5	28±6	3±5	3±6
insulin (10nM)	114±10	173±12	-4±4	5±8
glucagon (10nM)	30±7	25±8	-2±6	3±4
insulin + glucagon	109±12	177±14	1±3	-2±6

Intact hepatocytes were incubated for 10 min. in the presence or absence of the above ligands prior to a mediator fraction being prepared. A portion (10µl) of this fraction was added to assays (100µl)containing the specific phosphodiesterases (PDE) in the presence of 1µM cyclic AMP as substrate. PPM-peripheral plasma membrane. The cytosolic (sol) and membrane (membr) forms of the cyclic GMP activated cyclic AMP phosphodiesterase were used here. Activities are expressed as a percentage increase of the control activity. Errors are S.D., n=4.

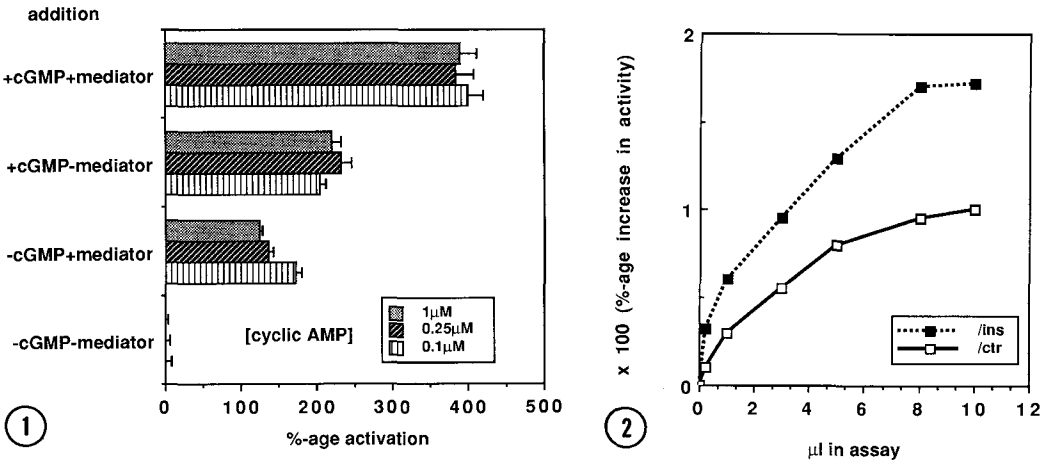

Figure 1. Stimulation of the cyclic GMP activated cyclic AMP phosphodiesterase by mediator fractions occurs in both the presence and absence of cyclic GMP. Cyclic GMP activated cyclic AMP phosphodiesterase was assayed using the indicated concentrations of cyclic AMP as substrate. In some instances mediator (10μl/100μl assay) and maximally stimulating (unlabelled) cyclic GMP (2.5μM) were added to these assays. The %-age increase in activity elicited by these compounds is shown compared to enzyme activity monitored in the absence of either ligand. Errors are S.D., n=4).

Figure 2. Alteration in mediator concentration on cyclic GMP activated cyclic AMP phosphodiesterase activity. Data showing the effect of increasing concentration of 'mediator' extracts on enzyme activity. This is done for 'mediator' produced in response to insulin (10nM) treatment of hepatocytes and also for extracts from control incubations done in the absence of insulin. The 'concentration' indicated is that volume (μl) of extract added to phosphodiesterase assays (100μl) done with 1μM cyclic AMP as substrate. The data is shown for a typical experiment (see text for analysis).

(15). Further studies focussed on the soluble enzyme where we noted that in the absence of cyclic GMP then stimulation by the mediator preparation was somewhat larger at low substrate concentrations (fig 1). In the presence of maximally activating concentrations of cyclic GMP (2.5μM) then mediator preparation yield further activation which appeared to be additive to that elicited by cyclic GMP (fig 1). This indicates that the 'mediator' was not cyclic GMP, which indeed we could show to be completely removed by the charcoal absorption step of the purification. Activation, by the 'mediator' preparation in the presence of cyclic GMP was of a similar magnitude over the range of substrate concentrations examined (0.1-1.0μM cyclic AMP).

Increasing amounts of mediator preparation in the enzyme assay increased activity in an apparently saturatable fashion which was reversible upon dilution (fig. 2). Hill plot analysis of this process showed that control extracts stimulated the enzyme with an EC_{50} of 0.24±0.04 μl/100μl assay whilst observing Michaelis kinetics yielding a Hill coefficient of 1.07±0.05. In contrast, the insulin-stimulated 'mediator' fraction exhibited a Hill coefficient of 0.72±0.06 and an EC_{50} of 0.29±0.05 μl/100μl assay. A hill coefficient of less than unity could indicate either heterogeneity of binding sites or apparent negative co-operativity. In this regard it is of note that

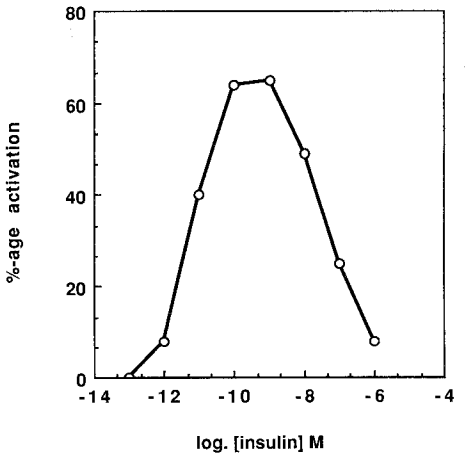

Figure 3. Insulin causes a dose-dependent effect on 'mediator' production. A dose-effect curve for treatment of hepatocytes with increasing concentrations of insulin. Activation has been corrected for 'basal' stimulatory effects and shows the percentage increase in cyclic GMP activated cyclic AMP phosphodiesterase activity. In this experiment material was used which had not been subjected to gel filtration. The data is shown for a typical experiment (see text for analysis).

substrate and activator regulation of this enzyme does not obey Michaelis kinetics (15), indicating that it is subject to complex multisite regulation. In any event, such observations show that the basal activating substance(s) is distinct from the insulin elicited 'mediator'. Indeed, this is all too apparent from data showing (fig. 2) that at 10µl of extract /100µl assay activation by the basal activating substance(s) has been saturated yet it is still possible to elicit further activation using extracts from insulin-treated cells. Analysis of the net stimulatory effect of insulin then yields an EC_{50} of 0.19±0.06 µl/100µl assay and a Hill coefficient of 0.46±0.07.

Production of mediator by the intact hepatocytes was dose-dependent upon insulin, with an apparent K_a of 0.9 x 10^{-11}M (fig. 3). However, stimulatory activity was progressively reduced when hepatocytes were challenged with concentrations of insulin greater than 10^{-9}M. Such an observation has been noted before with a variety of other mediator controlled systems (6) including the cyclic AMP phosphodiesterase activity of adipocyte membranes (7) and is evident when insulin inhibits adenylate cyclase activity in broken membrane preparations (13).

The mediator activity which stimulated the cyclic GMP activated cyclic AMP phosphodiesterase chromatographed as a single peak of activity on Sephadex G25 with an apparent M_r in the range of 1000-2000. This result is similar to that reported by others (18) using adenylate cyclase inhibition to follow mediator activity. We noted that this mediator preparation was essentially hydrophilic in character and was not extracted into chloroform. Analysis of the mediator by isoelectric focussing indicated that the isoelectric point (P_I) was in the range 6.4-6.6 and showed that the mediator activity did not co-chromatograph with the bulk of the ninhydrin reacting material in the extract, which was more acidic. The nature of the biologically active

substances in the mediator fraction has not been identified. However, our previous studies (15) on the cyclic GMP activated cyclic AMP phosphodiesterase showed that its activity was not affected by inositol trisphosphate, inositol bisphosphate and fructose 2,6-bisphosphate. Furthermore, the polyamines spermine, spermidine and putrescine, which have been suggested might mediate certain of insulin's actions (see 1), cause inhibition of both forms of this enzyme (15).

It has been demonstrated (6,7) that mediator/inositol glycan preparations could activate cyclic AMP phosphodiesterase activity in crude membrane preparations but were unable to exert any effect on a phosphodiesterase activity purified from such membranes (19). However, it appears that the enzyme isolated by these investigators was akin to the 'dense-vesicle' enzyme from hepatocytes which is activated in a stable fashion by insulin and not one which is reversed by dilution (see 10, 20 for discussion). Thus failure to observe any effect of a mediator on this species would be in accord with the observations made here. As adipocyte membrane preparations contain cyclic GMP activated cyclic AMP phosphodiesterase activity (20), such an enzyme may have provided the target for activation by the insulin produced mediator.

We also noted that treatment of hepatocytes with glucagon failed to elicit the production of mediator activity (table 1), indicating a specificity for insulin. This is of importance for two reasons, firstly because it clearly shows a difference with the 'dense vesicle' enzyme which can be activated by both insulin and glucagon (12), albeit by distinct mechanisms (21). Secondly, it was clear from our earlier studies that insulin must be capable of activating a cyclic AMP phosphodiesterase which is distinct from both the 'dense vesicle' and peripheral plasma membrane forms (14). The properties of such a enzyme demanded that it be capable of being activated by insulin in the presence of glucagon. Clearly, stimulation of the cyclic GMP activated cyclic AMP phosphodiesterase by the mediator preparation satisfies such criteria and indicates that this may be the prime route through which insulin decreases intracellular cyclic AMP concentrations in hepatocytes.

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