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Insulin and glucagon regulate the activation of two distinct membrane-bound cyclic AMP phosphodiesterases in hepatocytes

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Glucagon (10nM) caused a transient elevation of intracellular cyclic AMP concentrations, which reached a peak in around 5min, and slowly returned to basal values in around 30min. When 1mM-3-isobutyl-1-methylxanthine (IBMX) was present, this process yielded a K_a of 1nM for glucagon. The addition of insulin (10nM) after 5min exposure to glucagon (10nM) caused intracellular cyclic AMP concentrations to fall dramatically, attaining basal values within 10min. The regulation of this process was dose-dependent, exhibiting a K_a of 0.4nM for insulin. If insulin and glucagon were added together to hepatocytes, then insulin decreased the magnitude of the cyclic AMP response to glucagon. IBMX (1mM) prevented insulin antagonizing the action of glucagon in both of these instances. A gentle homogenization procedure followed by a rapid subcellular fractionation of hepatocytes on a Percoll gradient was developed. This was used to resolve subcellular membrane fractions and to identify cyclic AMP phosphodiesterase activity in both membrane and cytosol fractions. Glucagon and insulin only affected the activity of two distinct membrane-bound species, a plasma-membrane enzyme and a 'dense vesicle' enzyme. Glucagon (10nM), insulin (10nM), IBMX (1mM), dibutyryl cyclic AMP (10μM) and cholera toxin (1μg/ml) all elicited the activation of the 'dense vesicle' enzyme. The plasma-membrane enzyme was not activated by glucagon, IBMX or dibutyryl cyclic AMP, although insulin and cholera toxin both led to its activation. The degree of activation of the plasma-membrane enzyme produced by insulin was increased in the presence of IBMX or dibutyryl cyclic AMP. Glucagon pretreatment (5min) of hepatocytes blocked the ability of insulin to activate the plasma-membrane enzyme. The activity state of these phosphodiesterases is discussed in relation to the observed changes in intracellular cyclic AMP concentrations. It is suggested that insulin exerts its action on the plasma-membrane phosphodiesterase through a mechanism involving a guanine nucleotide-regulatory protein.

Hepatocytes express cell-surface receptors for both insulin and glucagon (Cuatrecasas, 1974; Kahn, 1976). Whereas glucagon exerts its effect on the cell by activating adenylate cyclase (Birnbaumer, 1973; Houslay *et al.*, 1980; Ross & Gilman, 1980), which increases intracellular cyclic AMP concentrations (Johnson *et al.*, 1972; Sonne *et al.*, 1978), the molecular mechanism whereby insulin exerts its effect is at present unknown (Czech, 1977; Houslay, 1981; Denton *et al.*, 1981). Glucagon, through its second messenger cyclic AMP, stimulates both glycogenolysis and gluconeogenesis in hepatocytes, whereas insulin can serve to antagonize this action by presumably depressing intracellular cyclic AMP concentrations (see, e.g., Pilkis *et al.*, 1975; Blackmore *et al.*, 1979). Loten *et al.* (1978) demonstrated that a particulate cyclic AMP phosphodiesterase is stimulated in intact hepatocytes by both insulin and glucagon, whereas we (Marchmont & Houslay, 1980*b*,*c*; Houslay *et al.*, 1983*a*,*b*) have shown that a plasma-membrane-associated cyclic AMP phosphodiesterase can be activated, through a phosphorylation mechanism (Marchmont & Houslay, 1981), by insulin in a broken membrane system. Here, in intact hepato-

Abbreviation used: IBMX, 3-isobutyl-1-methylxanthine.

cytes, we examine the role of cyclic AMP phosphodiesterase activation in controlling intracellular cyclic AMP concentrations and demonstrate the rapid, co-ordinate, hormonal control exerted on two distinct membrane-bound phosphodiesterases, by insulin and glucagon.

Materials and methods

Collagenase, cytochrome *c*, cyclic AMP, ATP and 5'-AMP were from Boehringer (U.K.) Ltd., Lewes, East Sussex, U.K. Calf intestine alkaline phosphatase was from Calbiochem, Bishops Stortford, Herts., U.K. All other biochemicals were from Sigma Chemical Co., Poole, Dorset, U.K. Percoll was from Pharmacia. IBMX was from Aldrich Chemical Co., Gillingham, Dorset, U.K. RO-20-1724 was kindly given by Roche, Welwyn Garden City, Herts., U.K. Glucagon was kindly given by Dr. W. W. Bromer, Eli Lilly & Co., Indianapolis, IN, U.S.A. All other chemicals were of A.R. grade from BDH Chemicals, Poole, Dorset, U.K. All radiochemicals were from Amersham International, Amersham, Bucks., U.K.

Preparation of hepatocytes and determination of their intracellular cyclic AMP content

Isolated hepatocytes were prepared from 225–250g male Sprague–Dawley rats (Elliott *et al.*, 1976) and incubated as previously described (Smith *et al.*, 1978). Cells (4–5mg dry wt./ml) were preincubated for 20min at 37°C before use. When IBMX (final concn. 1mM) was added, the preincubation was continued for a further 10min. After incubation, the cell suspension was centrifuged through bromododecane into a $HClO_4$ (0.62M)/sucrose (0.25M) mixture (Cornell, 1980). The supernatant was decanted, the $HClO_4$ neutralized and a sample taken for cyclic AMP determination as described previously (Whetton *et al.*, 1983). In all experiments cell viability was checked by intracellular ATP determination. This was in the range of 9–10nmol/mg dry wt. for viable cells.

Preparation of Percoll for use

A stock Percoll solution was made as a 90% (v/v) suspension in 0.25M-sucrose. Immediately before use, 2vol. of 0.25M-sucrose containing 6mM-imidazole was added to 1vol. of the stock Percoll solution and 1vol. of 0.25M-sucrose. The pH of this mixture was adjusted to pH7.4 with dilute HCl to give a final colloidal suspension which was 22.5% (v/v) Percoll.

Incubation and homogenization of hepatocytes for Percoll fractionation

Cells (60–75mg dry wt.) were distributed between three 25ml silicone-treated conical flasks. Incubations were done in Krebs–Henseleit buffer containing 2.5% bovine serum albumin and 2.5mM-$CaCl_2$ at 37°C (see Smith *et al.*, 1978). They were then, after appropriate incubation, stopped by immediately adding an equal volume of ice-cold 'stopping' buffer, consisting of freshly prepared 0.25M-sucrose/3mM-imidazole, final pH7.4, before transfer to an ice bath. The cells were centrifuged in plastic tubes at 50*g* for 2min at 4°C. The supernatant was aspirated and the cells were washed twice by resuspension in cold 'stopping' buffer and centrifugation (50*g* for 2min at 4°C). Finally, the cells were resuspended to 20–25mg dry wt. in freshly prepared 'stopping' buffer, and 2ml of this cell suspension was placed in plastic scintillation mini-vial inserts. These were then placed in a pressure cell, which was pressurized to 138kPa (20lb/in^2) for 10min with O_2-free N_2. The vessel was rapidly depressurized and the cells were homogenized gently in a glass vessel by using a Teflon pestle (two up-and-down strokes by hand). The homogenate was centrifuged, at 4°C, for 5min at 1500$g_{max.}$. The supernatant (1.5ml) was removed and the pellet resuspended to half the original homogenate volume in freshly prepared 'stopping' buffer. The whole disruption procedure, including gassing, homogenization and centrifugation, was repeated with 1ml of the resuspended pellet. Then 1ml of the first supernatant was mixed with 0.5ml of the second supernatant. This mixture is the 'final homogenate'.

Percoll fractionation of the homogenate

Centrifugation was performed on an MSE 65 Prepsin ultracentrifuge, with the 8 × 14ml fixed-angle rotor. Into the centrifuge tubes (14ml) were layered first 9ml of the 22.5% (v/v) Percoll mixture, then 0.5ml of 'stopping' buffer and then 0.75ml of the final homogenate. Centrifugation at 4°C was for 1h at 25000*g*. The tubes were fractionated by pumping out material from the bottom of the gradient and collecting 250μl fractions. The entire homogenization and fractionation procedure could be completed in about 2h.

Assay methods

Cyclic AMP phosphodiesterase activity was measured by a modification (Marchmont & Houslay, 1980*a*) of the two-step procedure of Thompson & Appleman (1971). However, 2-mercaptoethanol was omitted from the assays; 0.25% (w/v) Triton X-100 was added to the assays and rates were determined from linear time courses obtained by incubation for 15–20min at 37°C with 1μM-cyclic AMP. To compensate for any underestimation of activity owing to adenosine deaminase activity being present (Rutten *et al.*, 1973), the Dowex resin was freshly prepared as a 1:1:1 (by vol.) slurry of

resin/water/ethanol (Londesborough, 1976; Bublitz, 1978). Percoll was shown not to have any effect on cyclic AMP phosphodiesterase activity in either homogenate or membrane preparations.

To avoid latency problems, we routinely added Triton X-100 (0.25%) to all of the Percoll fractions obtained. However, similar results, for insulin and glucagon, were obtained by using Lubrol PX and Nonidet P40.

Protein in cells and homogenates was determined by a modified (Houslay & Palmer, 1978) of the micro-biuret method of Goa (1953). For gradient fractions, and whenever Percoll was present, a fluorescamine method was employed (Bohlen *et al.*, 1973). RNA was determined with calf liver RNA as a standard (Herbert *et al.*, 1971). DNA was determined with calf thymus DNA as standard (Burton, 1956). 5'-Nucleotidase was assayed as described by Newby *et al.* (1975), succinate dehydrogenase as described by Pennington (1961), galactosyltransferase as described by Morré (1971), NADPH–cytochrome *c* reductase as described by Beaufay *et al.* (1974), and leucine aminopeptidase as described by Peters *et al.* (1972). Glucose 6-phosphatase, lactate dehydrogenase and malate dehydrogenase were all assayed as described by Houslay & Palmer (1978). ATP was measured by the luciferase method (Stanley & Williams, 1969). Acid β-galactosidase and β-*N*-acetylglucosaminidase were assayed as described by Sellinger *et al.* (1960). Glucagon-stimulated adenylate cyclase was measured as described by Houslay *et al.* (1976).

The density profile of Percoll gradients was determined by using standard Percoll solutions of a known density and an Abbé refractometer.

Other methods

The insulin-triggered activation of cyclic AMP phosphodiesterase in the plasma-membrane fraction was performed as described by Marchmont & Houslay (1980*b*), except that 2-mercaptoethanol was omitted from the incubation and assay.

In some instances the membrane fractions (40μl) were treated with 0.5mg of calf intestine alkaline phosphatase/ml in a mixture (50μl) of 10mM-$MgCl_2$/80mM-Tris/HCl, final pH7.4, for 10min at 37°C.

In some instances membrane fractions were treated either as described by Marchmont & Houslay (1980*a*) with 0.3M-NaCl/40mM-Tris/HCl, pH7.4, or as described by Loten *et al.* (1978) with 1mM-EDTA/10mM-Tris/HCl, pH7.4, for 45min on ice, and then centrifuged at 100000*g* for 1h to obtain solubilized cyclic AMP phosphodiesterase activity.

Sucrose-density-gradient centrifugation was performed as described by Marchmont & Houslay (1980*a*).

Peroxisomes were prepared by the method of Appelkvist *et al.* (1981) from rat liver.

Results

Hormonal effects on intracellular cyclic AMP concentrations

The addition of glucagon (10nM) to hepatocytes caused a rapid rise in intracellular cyclic AMP concentrations, which reached a peak at around 5–6min (Fig. 1*a*). After this time the concentration of cyclic AMP fell slowly, taking about 30min to regain its basal value. The extent of this increase in cyclic AMP concentration, achieved at the 5–6min peak, was dependent on the concentration of glucagon employed. When IBMX was present to inhibit phosphodiesterase activity, this process yielded a K_a of $1.2 \times 1.0^{-9} \pm 0.1 \times 10^{-9}$M ($n = 5$, S.E.M.) for glucagon.

If, however, insulin (10nM) was added at the peak of cyclic AMP accumulation achieved by glucagon, then the cyclic AMP concentration fell dramatically (Fig. 1*a*); basal concentrations were reached some 10min after application. The magnitude of this effect of insulin, in decreasing intracellular cyclic AMP concentrations, was dependent on insulin concentration (Fig. 2). A K_a of $4.1 \times 10^{-10} \pm 2.2 \times 10^{-10}$M ($n = 4$, S.E.M.) was found for the process.

In contrast with these observations, marked differences were observed if the cells were pretreated with IBMX (1mM), to inhibit cyclic AMP phosphodiesterase within the cell, before exposure to the hormones. Under such conditions, glucagon (10nM) alone elicited a much greater increase in intracellular cyclic AMP concentrations, with, again, a peak occurring at around 5–6min. However, the fall in the cyclic AMP concentrations after this time was much less obvious than was observed in the absence of IBMX (Fig. 1*b*). The addition of insulin (10nM), after a 5min exposure to glucagon (10nM), had no observable effect on the rate of fall of intracellular cyclic AMP concentrations (Fig. 1*b*).

The addition of both glucagon (10nM) and insulin (10nM) together at the beginning ($t = 0$) of the experiment yielded a profoundly different result (Fig. 1*c*). In this case it appeared that the extent of the increase in intracellular cyclic AMP, i.e. the height of the peak occurring at 5–6min, was decreased by the presence of insulin. However, the rate of decrease in intracellular cyclic AMP concentrations appeared to be similar, whether or not insulin was present. If the cells were pretreated for 10min with IBMX before the addition of hormones, then the intracellular cyclic AMP-accumulation curves were almost identical, irrespective of whether or not insulin was present (Fig. 1*d*).

When insulin (10nM) was added to hepatocytes not exposed to glucagon (Fig. 3), we consistently

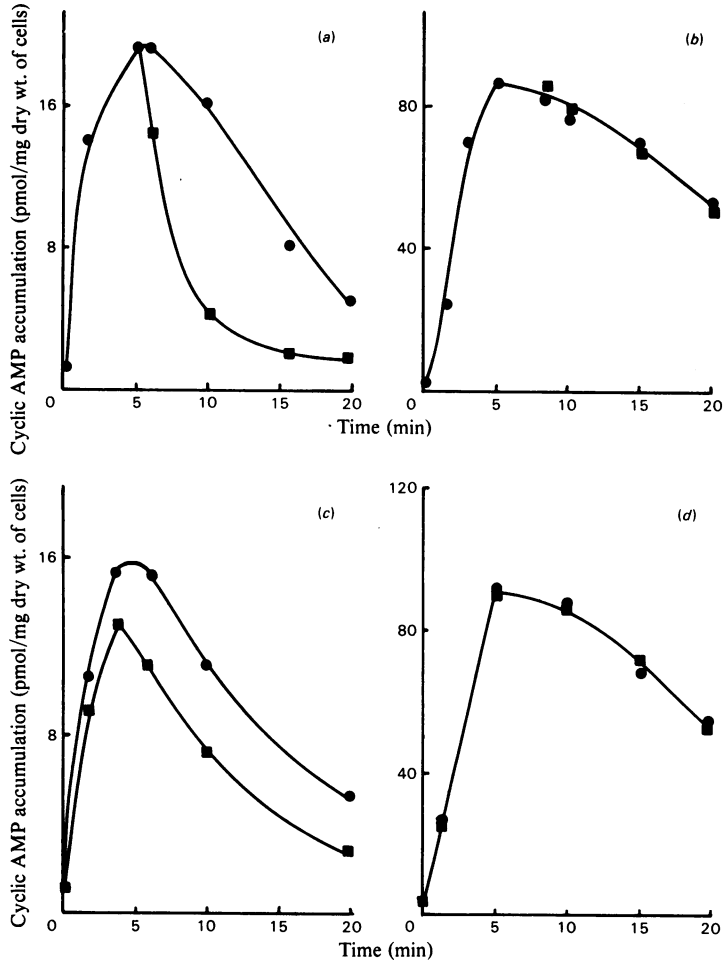

Fig. 1. *Effect of insulin on the glucagon-stimulated rise in hepatocyte intracellular cyclic AMP concentrations*
(*a*) Addition of glucagon (10nM) at $t = 0$ (●), with subsequent addition of insulin (10nM) at $t = 5$min (■). (*b*) As in (*a*), but IBMX (1mM) was added to the cells 10min before the addition of glucagon. (*c*) Addition of either (●) glucagon (10nM) or (■) glucagon (10nM) plus insulin (10nM) together at $t = 0$. (*d*) As (*c*), but IBMX was added 10min before hormone addition. All incubations were performed at 37°C as described in the Materials and methods section. These data are averages for cell incubations in triplicate with duplicate determinations of intracellular cyclic AMP. Errors in all cases were less than 10%. The experiment here is typical of results obtained with cell preparations from three different animals.

observed a 30–50% (range, $n = 3$) decrease in intracellular cyclic AMP concentrations after 5min exposure to the hormone. These concentrations then appeared slowly to re-attain basal values. In contrast, the presence of IBMX (1mM) abolished this effect of insulin (Fig. 3).

Fractionation of the hepatocyte homogenate on a Percoll gradient

After cell breakage, under iso-osmotic conditions, the homogenate was fractionated on a Percoll gradient precisely as described in the Materials and methods section, as the cell density during homogenization was found to be critical. The density profile is shown in Fig. 4, as are the profiles for protein, RNA and various 'marker' enzymes. We identified cytosol (soluble fractions) with lactate dehydrogenase, soluble malate dehydrogenase and soluble RNA as markers, plasma membranes by using leucine aminopeptidase (Peters *et al.*, 1972), 5′-nucleotidase (Gurd & Evans, 1974; Riemer & Widnell, 1975) and glucagon-stimulated adenylate cyclase (Pilkis *et al.*, 1974), Golgi with galactosyltransferase (Morré, 1971), lysosomes with acid

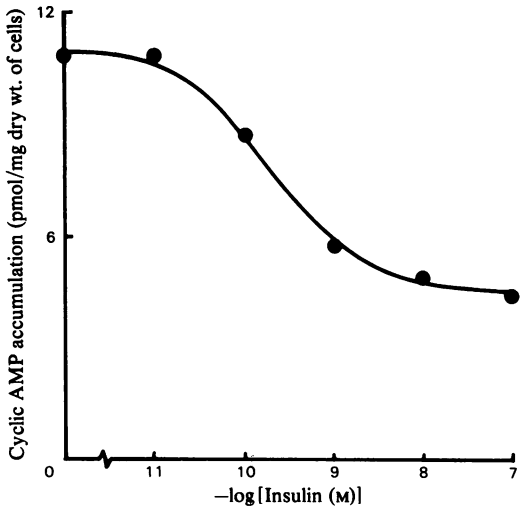

Fig. 2. *Dose-dependent effect of insulin in lowering intracellular cyclic AMP concentrations elevated by glucagon*

Cells were incubated at 37°C with glucagon (10nM) for 5min and then challenged with various concentrations of insulin. After 5min incubation with insulin, the intracellular cyclic AMP concentration was assessed. The data obtained had errors less than 10%; see legend to Fig. 1.

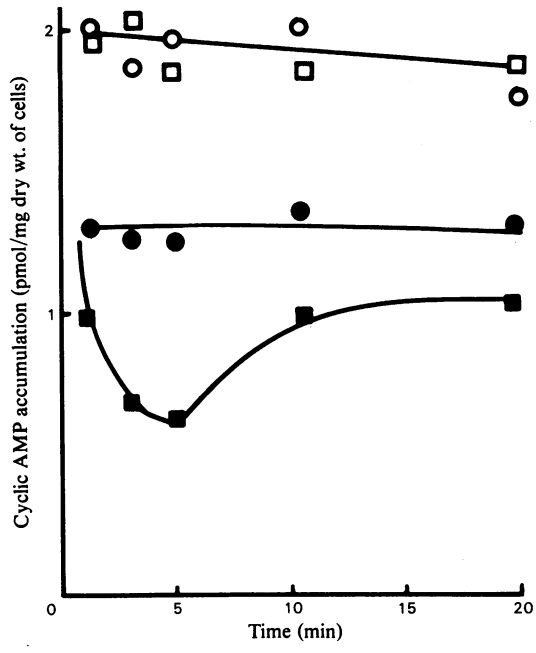

Fig. 3. *Effect of insulin on basal intracellular cyclic AMP concentrations*

Insulin (10nM) was (■, □) or was not (●, ○) added to cells that either had not (■, ●) or had (□, ○) been pre-treated for 10min with IBMX (1mM) before the start of the experiment. Errors were less than 10%, with the results assessed as in the legend to Fig. 1.

β-galactosidase (Sellinger *et al.*, 1960), with β-*N*-acetylglucosaminidase showing an identical distribution (results not shown; Sellinger *et al.*, 1960), mitochondria with latent malate dehydrogenase, with succinate dehydrogenase (Bachman *et al.*, 1966) showing a distribution identical with that of this latent activity, and endoplasmic reticulum by using glucose 6-phosphatase activity, with NADPH–cytochrome *c* reductase (De Pierre & Ernster, 1977) showing a very similar distribution (results not shown).

We note that the 5′-nucleotidase distribution is apparently split between a plasma-membrane fraction and a Golgi fraction. This is in accord with the findings of Stanley *et al.* (1980), who have shown that a significant fraction of hepatocyte 5′-nucleotidase activity is in an internalized, Golgi-associated, vesicle pool. In contrast with the other markers, that for the endoplasmic reticulum, glucose 6-phosphatase, is well distributed through the membrane fractions. This reflects the heterogeneity of this system (De Pierre & Ernster, 1977). We note that there are low- and high-density populations separated by a trough at about fractions with an R_F of 0.3 (density approx. 1.06g/cm^3). Presumably the high-density fractions reflect the rough endoplasmic reticulum, as they co-migrate with the membrane-bound RNA in the gradient.

We noted that the percentage recovery, with respect to the intact cell, for all of the various marker enzymes was of the order of 70–80% in the final homogenate fraction. This indicates that 70–80% of the cells were disrupted by our homogenization procedure. The remaining 20–30% of unbroken cells were discarded in the second pellet. The recovery of DNA was >98% in the second pellet, indicating that all of the nuclei were removed by centrifugation at this stage. After centrifugation of the final homogenate fraction on Percoll gradients, with subsequent fractionation, we recovered of the order of 100% of the activities of all of the marker enzymes.

Phosphodiesterase distribution in hepatocytes

After fractionation of the final homogenate, cyclic AMP phosphodiesterase activity was found throughout the gradient (Fig. 5). Activity was concentrated in the endoplasmic-reticulum and plasma-membrane areas of the gradient, which is consistent with our observations with purified fractions (Marchmont & Houslay, 1980*a*; Cercek & Houslay, 1982; Cercek *et al.*, 1983).

The recovery of cyclic AMP phosphodiesterase,

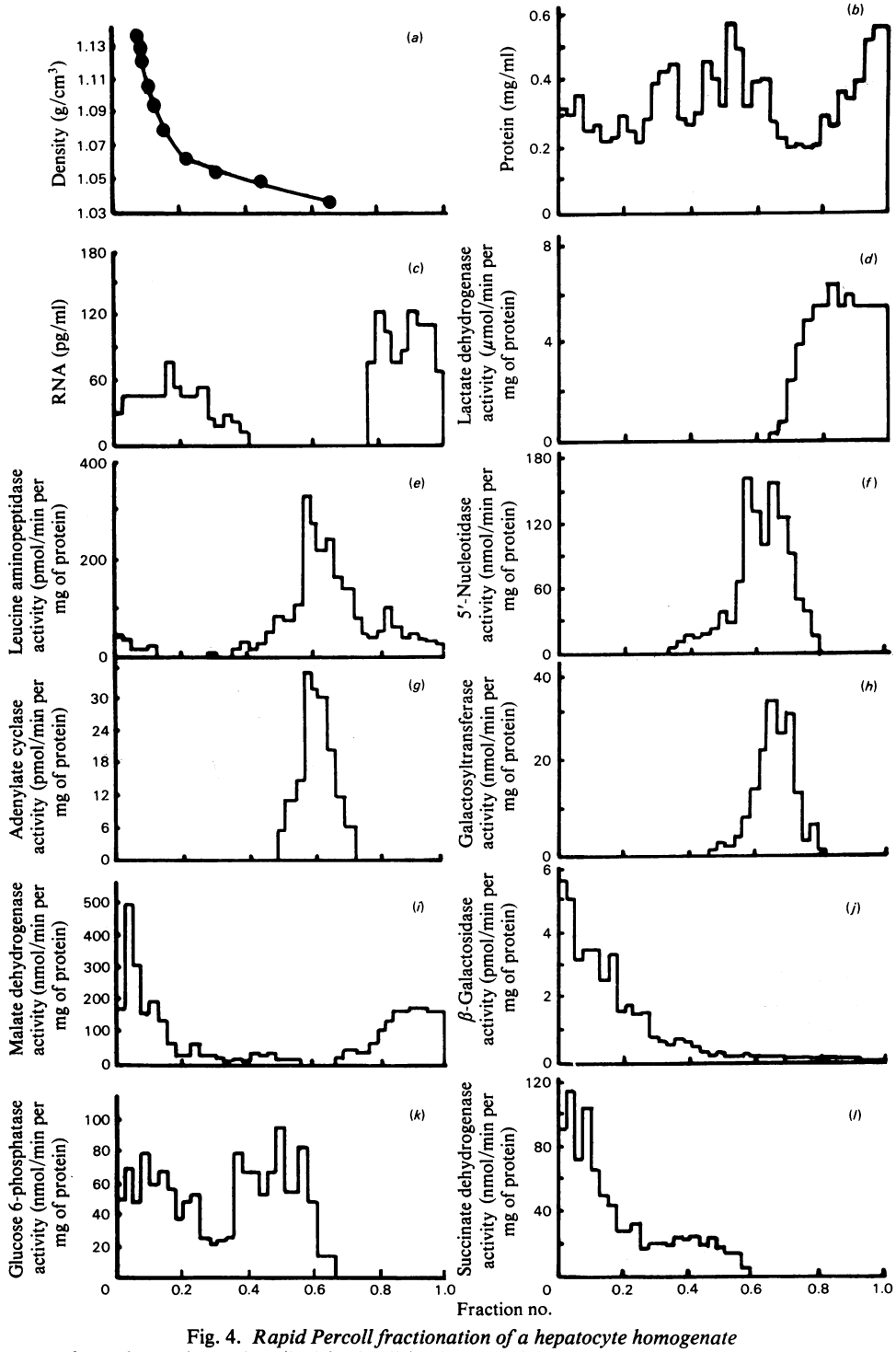

Fig. 4. *Rapid Percoll fractionation of a hepatocyte homogenate*

This was performed exactly as described in detail in the Materials and methods section. The distributions shown are typical of those that can be obtained by this procedure. For enzyme markers, specific activities are shown. (*a*) Density; (*b*) protein; (*c*) RNA; (*d*) lactate dehydrogenase; (*e*) leucine aminopeptidase; (*f*) 5'-nucleotidase; (*g*) glucagon-stimulated adenylate cyclase; (*h*) galactosyltransferase; (*i*) malate dehydrogenase [this distribution is shown after detergent (Triton X-100) addition to the fractions; in the absence of detergent, no 'high-density' peak is found in assays under iso-osmotic conditions]; (*j*) β-galactosidase; (*k*) glucose 6-phosphatase, (*l*) succinate dehydrogenase. R_F is defined as the inverse of the distance moved from the meniscus over the total height of the gradient.

and percentage breakage of the cells based on this, followed that observed for all of the marker enzymes. As during this fractionation procedure we lost all of the nuclei (into the second pellet), this implies that there is negligible phosphodiesterase activity, associated with the nuclear fraction. Indeed, isolated purified nuclei from hepatocytes exhibited <5% of total phosphodiesterase activity, and the specific activity of this fraction remained unchanged after challenge of the hepatocytes with hormones (S. R. Wilson & M. D. Houslay, unpublished work).

This rapid and gentle procedure, which failed to cause lysosomal breakage (Fig. 4), appeared to prevent any modification of phosphodiesterase activity after homogenization. We found that highly reproducible results were obtained (Table 1) by 'stopping' the incubations with ice-cold 'stopping' buffer, with immediate transfer to an ice bath and subsequent homogenization. It was found to be unnecessary to add NaF (15 mM) to inhibit phosphatase action, and important not to add EGTA (1 mM), which caused the release of peripheral proteins from membranes (Houslay, 1981).

If IBMX (1 mM) was present in assays of homogenate cyclic AMP phosphodiesterase activity (1 μM-cyclic AMP), then over 90% of the total enzyme activity was inhibited. The ID_{50} (concentration giving 50% of maximal inhibition) for IBMX, under such conditions, was 7.1 μM, and for the phosphodiesterase inhibitor RO-20-1724 was 80 μM. When IBMX (1 mM) was added to the assays of the gradient fractions, no activity was observed in the membrane fractions ($R_F = 0$–0.7), the residual activity being found associated with the soluble species.

Intact hepatocytes were incubated with various ligands before fractionation on Percoll gradients. Glucagon and insulin were found to influence the phosphodiesterase activity occurring at two positions only on the gradient (Fig. 5). The cyclic AMP phosphodiesterase activity displayed by these fractions was membrane-bound, as it could be readily sedimented by centrifugation at 100000 **g** for 1 h at 4°C. One of these activated species migrated with the plasma-membrane markers (density 1.04 g/cm^3) and will be termed the 'plasma-membrane' enzyme. The other did not migrate (density, 1.06 g/cm^3) as any of the markers tested, occurring on the high-density shoulder of the 'microsomal' fraction, and hence will be termed the 'dense-vesicle' enzyme. Although peroxisomes have been shown to migrate to a density of about 1.06 g/cm^3 on Percoll gradients

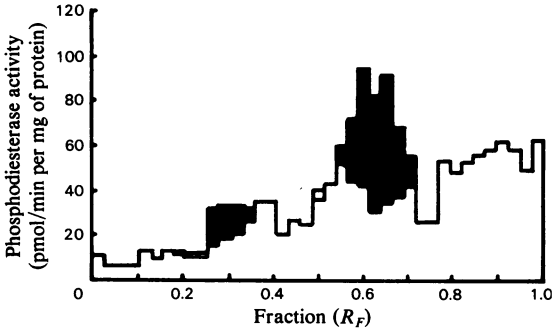

Fig. 5. *Cyclic AMP phosphodiesterase distribution, and activation of selective species, in hepatocytes*
This demonstrates the distribution (specific activity) of cyclic AMP phosphodiesterase activity in Percoll gradient of a hepatocyte homogenate fractionated as in Fig. 4. The black areas represent the changes in specific activity that occur after hepatocytes were incubated at 37°C with dibutyryl cyclic AMP (10 μM) added at $t = 0$ and then insulin (10 nM) added at $t = 5$ min. Reactions were stopped at $t = 10$ min and treated as in the Materials and methods section. R_F is defined in the legend to Fig. 4.

Table 1. *Regulation of plasma-membrane and 'dense-vesicle' cyclic AMP phosphodiesterase activity in hepatocytes*
Results are means ± S.E.M., calculated from three separate experiments on different animals. Activation over control activities (100%) were calculated as in the Materials and methods section from plots as in Fig. 5.

	Activation (%)	
Incubation conditions (total incubation time)	'Dense-vesicle' enzyme	Plasma-membrane enzyme
10 nM-glucagon (10 min)	162 ± 10	98 ± 7
10 nM-insulin (5 min)	126 ± 5	203 ± 15
10 nM-glucagon, then at 5 min 10 nM-insulin (10 min)	258 ± 12	105 ± 5
0.5 nM-glucagon, then at 5 min 10 nM-insulin (10 min)	144 ± 8	152 ± 17
10 nM-insulin + 10 nM-glucagon together (10 min)	124 ± 5	127 ± 12
Cholera toxin (1 μg/ml) (35 min)	175 ± 31	149 ± 9
Cholera toxin (1 μg/ml), then at 30 min 10 nM-insulin (35 min)	159 ± 7	162 ± 11
10 μM-dibutyryl cyclic AMP (10 min)	160 ± 2	104 ± 3
10 μM-dibutyryl cyclic AMP, then at 5 min 10 nM-insulin (10 min)	183 ± 23	283 ± 16
1 mM-IBMX (30 min)	158 ± 3	97 ± 4
1 mM-IBMX, then at 25 min 10 nM-insulin (30 min)	103 ± 10	288 ± 18

(Appelkvist *et al.*, 1981), we found no detectable cyclic AMP phosphodiesterase activity (<0.02 pmol/min per mg of protein) associated with a purified preparation of peroxisomes.

Fig. 5 demonstrates the difference between a control gradient and an 'activated' gradient, where the hepatocytes had been preincubated with dibutyryl cyclic AMP (10 μM) for 5 min and then with insulin as well for a further 5 min. Table 1 summarizes the changes in activity of these two enzymes observed by incubating hepatocytes with a variety of ligands. In each case activation was calculated by comparing the specific phosphodiesterase activity of the 'activated' and control gradients over the peak fractions. Glucagon, insulin, IBMX, dibutyryl cyclic AMP and cholera toxin all elicited the activation of the 'dense-vesicle' enzyme. The plasma-membrane enzyme was not activated by glucagon, IBMX or dibutyryl cyclic AMP, although insulin and cholera toxin both led to its activation. The degree of activation of the plasma-membrane enzyme achieved by insulin was increased in the presence of IBMX or dibutyryl cyclic AMP. However, glucagon pretreatment of hepatocytes blocked the ability of insulin to activate the plasma-membrane enzyme.

In no instance did we observe any activation of soluble enzyme activities that migrated at the top of the gradient. We also performed parallel experiments in which homogenized cells were immediately centrifuged at 100000 *g* for 1 h at 4°C, to obtain a high-speed supernatant. Using this as a source of soluble enzyme, we again saw no effect of these ligands on the activity of such enzymes; however, we did observe activations of total particulate phosphodiesterase activity (results not shown).

Nature of the activated phosphodiesterase activities

Phosphodiesterase activity in the 'plasma-membrane' fraction could be solubilized by using the high-ionic-strength procedure of Marchmont & Houslay (1980*a*), but not by the hypo-osmotic shock method of Loten *et al.* (1978) or by 1 mM-$KHCO_3$, pH 7.4. The solubilized activity ran as a 3.8 ± 0.2 S ($n = 3$, S.D.) peak on a sucrose-density gradient.

Incubation of membranes from this fraction, obtained from control cells, with alkaline phosphatase had no effect (>99% of control) on the phosphodiesterase activity expressed by these membranes. A fraction of plasma membranes derived from insulin-treated hepatocytes, however, exhibited a phosphodiesterase activity some 194 ± 13% of that of their control. In this instance, alkaline phosphatase treatment decreased the activity to 107 ± 7% of that of the control ($n = 3$, S.E.M.).

We also noted that a plasma-membrane fraction purified by this Percoll-gradient procedure could be activated, *in situ*, with insulin to 155 ± 8% ($n = 3$, S.E.M.) of control activity in the presence of cyclic AMP (0.1 mM) and ATP (4 mM).

The phosphodiesterase activity in the 'dense-vesicle' fraction was not solubilized by the high-ionic-strength procedure of Marchmont & Houslay (1980*a*), but was solubilized by the hypo-osmotic-shock method of Loten *et al.* (1978). The solubilized activity yielded a peak at around 6S on a sucrose-density gradient.

Discussion

Exposure of intact hepatocytes to glucagon caused a transient rise in intracellular cyclic AMP concentrations (see Johnson *et al.*, 1972; Sonne *et al.*, 1978; the present work), which attained a maximum after 5–6 min and fell thereafter (Fig. 1). This effect exhibited a K_a of about 10^{-9} M for glucagon, reflecting the action of glucagon in activating adenylate cyclase in isolated plasma membranes (Rodbell *et al.*, 1971*a*; Iyengar *et al.*, 1979) and of specific binding of glucagon to liver plasma membranes (Rodbell *et al.*, 1971*b*).

It has been shown by others, in hepatocytes, that insulin can decrease the cyclic AMP response induced by low glucagon concentrations (see, e.g., Blackmore *et al.*, 1979; Pilkis *et al.*, 1975). We show here that, even when glucagon is present at a concentration 10 times its K_a, insulin can decrease these elevated cyclic AMP concentrations (Fig. 1) in a dose-dependent fashion (Fig. 2). The K_a for this action of insulin is of the order of 10^{-10} M, which reflects the high-affinity component of specific receptor binding (Cuatrecasas, 1974; Kahn, 1976).

Insulin could exert this effect through inhibition of adenylate cyclase, by increase in cyclic AMP exit or by activation of cyclic AMP phosphodiesterase activity. Others (Pilkis *et al.*, 1975) have demonstrated that insulin does not cause an increase in cyclic AMP exit from hepatocytes, and we can confirm this (C. M. Heyworth, unpublished work). Inhibition of adenylate cyclase by insulin is a controversial issue, with certain groups observing a small effect (see, e.g., Hepp & Renner, 1972; Illiano & Cuatrecasas, 1972; Kiss, 1978) and others none at all (Pilkis *et al.*, 1974; Bitensky *et al.*, 1972). However, in the presence of IBMX, the ability of insulin to decrease intracellular cyclic AMP concentrations was abolished (Figs. 1 and 3). It is likely that insulin exerts such effects by the activation of cyclic AMP phosphodiesterase activity.

Liver contains multiple forms of cyclic AMP phosphodiesterase activity (see, e.g., Thompson & Strada, 1978), with their activities approximately equally expressed by membrane-bound and soluble species (Marchmont & Houslay, 1980*a*). We have previously shown that distinct forms are found in isolated plasma membranes (Marchmont & Hous-

lay, 1980*a*), mitochondria (Cercek & Houslay, 1982) and endoplasmic reticulum (Cercek *et al.*, 1983), but no detectable activity is found associated with the Golgi and lysosomal fractions (Cercek & Houslay, 1982; Cercek *et al.*, 1983). Thompson *et al.* (1973) and Loten *et al.* (1978) have demonstrated that treatment of whole animals or hepatocytes with insulin elicited an activation of an undefined cyclic AMP phosphodiesterase activity in a crude particulate fraction. We (Marchmont & Houslay, 1980*b*,*d*), using a purified plasma-membrane preparation, have shown that insulin elicited the activation of the peripheral cyclic AMP phosphodiesterase via a phosphorylation mechanism (Marchmont & Houslay, 1980*b*,*c*, 1981; Houslay *et al.*, 1983*a*,*b*). This insulin-activated enzyme was, however, distinct from that observed by Loten *et al.* (1978) by a number of criteria [see Marchmont & Houslay (1981) and Houslay *et al.* (1983*a*,*b*) for discussion].

In order to define the effects of glucagon and insulin on cyclic AMP phosphodiesterase activity in intact cells, we have devised a rapid and gentle method of subcellular fractionation. In the variety of conditions tested (Table 1) no activation of cyclic AMP phosphodiesterase activity occurred in the soluble fractions. However, activations were observed at two distinct positions in the gradient. These were at the plasma-membrane fraction (density 1.04 g/cm^3) and at a point in the gradient of density 1.06 g/cm^3, which was not identified by any of the markers employed in this study. We note, however, that Khan *et al.* (1981, 1982) have observed that insulin is rapidly internalized into a unique vesicular fraction migrating to an identical density on a Percoll gradient. We shall thus refer to this enzyme as the 'dense-vesicle' cyclic AMP phosphodiesterase. The 'dense-vesicle' phosphodiesterase is apparently the enzyme described by Loten *et al.* (1978), as it is released from the membrane by hypo-osmotic shock, has a similar sedimentation coefficient, and is activated by both glucagon and insulin (Table 1), in agreement with their studies. Although markers for the endoplasmic reticulum migrate nearby, this enzyme exhibits characteristics which are distinct from the cyclic AMP phosphodiesterases associated with the rough and smooth fractions (Cercek *et al.*, 1983; Wilson & Houslay, 1983).

Agents that increase intracellular cyclic AMP concentrations, such as IBMX, dibutyryl cyclic AMP and cholera toxin, all activated this 'dense-vesicle' enzyme to a similar extent to that elicited by glucagon (Table 1). Insulin, however, activated the enzyme to a much lesser extent. However, when the cells were given both glucagon and insulin together, the activity state reflected that observed when insulin alone was added. In sharp contrast with this, if cells were pretreated with glucagon, before exposure to insulin, a synergistic increase in activity was observed. It seems that there is a complex interplay between insulin and glucagon in controlling the activity of the 'dense-vesicle' phosphodiesterase. This interaction presumably lies at the plasma-membrane receptor level, for the elevation of cyclic AMP concentrations achieved by IBMX, dibutyryl cyclic AMP or cholera toxin failed to mimic glucagon in eliciting a synergistic increase in cyclic AMP phosphodiesterase activity on subsequent exposure to insulin (Table 1).

The activity in the plasma-membrane fraction was not elevated by glucagon treatment of the cells, but was almost doubled after insulin was added (Table 1). We believe that this peripheral enzyme is that identified by us in purified plasma membranes (Marchmont & Houslay, 1980*a*). Consistent with this is its ability to be solubilized by high ionic strength and its identical sedimentation coefficient (Marchmont & Houslay, 1980*a*), which are distinct from that of the endoplasmic-reticulum enzymes (Cercek *et al.*, 1983). The impinging Golgi fraction (Fig. 5) does not exhibit cyclic AMP phosphodiesterase activity (Cercek *et al.*, 1983). The peripheral plasma-membrane phosphodiesterase from control gradients could also be activated in the manner shown by us previously (see Marchmont & Houslay, 1980*b*), with the activity of this enzyme (Marchmont & Houslay, 1981), and that obtained from insulin-treated cells, being decreased to that of the basal enzyme by treatment with alkaline phosphatase.

Cyclic AMP is required for the activation of the plasma-membrane enzyme (Marchmont & Houslay, 1980*b*,*c*,*d*), where it exhibits a K_a of about 1.6 μM. This value is very similar to our observed intracellular cyclic AMP concentrations (approx. 1 μM), explaining why insulin alone triggers activation in the intact cell. Consistent with this notion are our observations that elevation of intracellular cyclic AMP concentrations by using either dibutyryl cyclic AMP or IBMX, which alone have no effect on the activity of the peripheral plasma-membrane enzyme, augment the activation caused by insulin (Table 1).

We show here that the plasma-membrane phosphodiesterase is activated by cholera toxin (Table 1). This is not a cyclic AMP-mediated event, as other agents that elevate cyclic AMP concentrations failed to activate this enzyme (Table 1). We suggest that insulin could mediate its effects on the activation of this phosphodiesterase through a guanine nucleotide-regulatory protein (see Heyworth *et al.*, 1983). This would bear analogy with the light-activated cyclic AMP phosphodiesterase in rod outer segments. Here rhodopsin exerts its action through a guanine nucleotide-regulatory protein (see, e.g., Bitensky *et al.*, 1978, 1982; Kohnken *et al.*, 1981; Hurley &

Stryer, 1982) which can be ADP-ribosylated by cholera toxin, whereupon it triggers phosphodiesterase activation (Abood *et al.*, 1982).

The activity of the cholera-toxin-activated enzyme was not as great as that of the insulin-activated enzyme, and moreover, it was resistant to further activation by insulin (Table 1). Such an effect resembles that of cholera toxin on adenylate cyclase from hepatocytes, where the activated enzyme exhibited very little stimulation by glucagon compared with the native enzyme (Houslay & Elliott, 1979).

Exposure of the cells to glucagon, which elevates intracellular cyclic AMP concentrations, did not elicit any changes in the activity of the plasma-membrane enzyme. However, prior exposure of the cells to glucagon blocked the activation by insulin of this phosphodiesterase (Table 1). A short pre-incubation with glucagon also causes the rapid desensitization of adenylate cyclase (Heyworth & Houslay, 1983). This densitization response appears to be mediated through rather high-affinity ($K_a = 4 \times 10^{-10}$ M) glucagon receptors (Heyworth & Houslay, 1983), which form a small fraction of the total population of somewhat lower-affinity receptors (Rodbell *et al.*, 1971*b*; Sonne *et al.*, 1978). Interestingly, if we pretreated cells with a low glucagon concentration, which reflects this K_a value, insulin was able to elicit an activation of the plasma-membrane enzyme which was about 50% of that observed without glucagon pre-treatment (Table 1). The magnitude of such an effect is as predicted for an event mediated through such receptors. Indeed we have demonstrated that the desensitization response appears to be caused by the receptor-mediated modification of the guanine nucleotide-regulatory system. We suggest that this modification is also capable of blocking activation by insulin of the plasma-membrane phosphodiesterase. Presumably the guanine nucleotide-regulatory system, which we suggest controls the activation of this phosphodiesterase, has properties distinct from that through which glucagon exerts its stimulatory effect on adenylate cyclase. Indeed there is evidence for distinct types of guanine nucleotide-regulatory proteins in liver and other tissues (Rodbell, 1980; Cooper *et al.*, 1981).

By simultaneously monitoring both intracellular cyclic AMP concentrations and cyclic AMP phosphodiesterase activities, we can begin to gain insight into the complex interplay between the actions of glucagon and insulin on hepatocytes. For example, when insulin is added after 5 min exposure to glucagon (Fig. 1), the rapid fall in intracellular cyclic AMP concentrations is due to the pronounced activation of the 'dense-vesicle' phosphodiesterase, as the plasma-membrane enzyme has been switched off (Table 1). In contrast, if insulin is added together with glucagon (Fig. 1), there is a small activation of both the 'dense-vesicle' and plasma-membrane phosphodiesterase (Table 1), which now act in concert to depress the magnitude of the peak of cyclic AMP accumulation triggered by glucagon. Interestingly, after the peak of cyclic AMP accumulation (Fig. 1), in this instance the net stimulation of phosphodiesterase activity must *in situ* approximately equal that triggered by glucagon alone. This is because the rate of fall in the concentration of intracellular cyclic AMP is approximately equal in each case (Fig. 1). It also appears that insulin can regulate basal cyclic AMP concentrations by activating the plasma-membrane enyzme and, to a lesser extent, the 'dense-vesicle' enzyme (Fig. 3). This is consistent with the observations of Exton *et al.* (1971), who observed that insulin lowered basal cyclic AMP concentrations in perfused rat liver.

Owing to compartmentalization and the presence of small molecules regulating their activity, it is difficult either to assess or to predict the activity that the multiple forms of cyclic AMP phosphodiesterase display *in situ* (see Houslay *et al.*, 1983*b*, for discussion). We show here, however, that the pattern of regulation of two specific high-affinity cyclic AMP phosphodiesterases (see Houslay *et al.*, 1983*b*) is consistent with them eliciting corresponding changes in intracellular cyclic AMP concentrations. We do not intend to imply that insulin exerts its regulatory action on the cell by manipulating intracellular cyclic AMP concentrations, but rather that this is merely one reflection of a number of cellular events that can be triggered when insulin binds to its receptor. We suggest that a number of such insulin-triggered events, including the activation of the plasma-membrane phosphodiesterase, may be mediated via a distinct guanine nucleotide-regulatory protein and, presumably, associated protein kinase(s).

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