INSULIN CONTROLS THE CYCLIC AMP-DEPENDENT PHOSPHORYLATION OF INTEGRAL AND PERIPHERAL PROTEINS ASSOCIATED WITH THE RAT LIVER PLASMA MEMBRANE

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1. Introduction

The peptide hormones insulin and glucagon are both involved in regulating blood glucose levels. They each interact with specific receptors exposed at the external surface of the plasma membrane [1]. Whilst the molecular action of glucagon at the level of the plasma membrane in activating adenylate cyclase is relatively well understood [2,3], that of insulin is poorly appreciated [1,4]. However, very recently there have been claims that insulins occupancy of its receptor leads to the production of a 'peptide-like' 'second messenger' [5–7]. This fraction can mimic the action of insulin in decreasing the phosphorylation of mitochondrial pyruvate dehydrogenase in adipocytes [5,6], converting the enzyme from an inactive, phosphorylated form into an active, dephosphorylated species [8]. This 'peptide-like' factor is believed to activate a protein phosphatase [7] although it also appears to inhibit a cyclic AMP-dependent protein kinase [7]. Indeed there have been a number of reports that insulin can affect the phosphorylation of adipocyte proteins. The phosphorylation of certain cytosol and endoplasmic reticulum proteins in intact adipocytes was apparently augmented by insulin [9,10] whereas the phosphorylation of other endoplasmic reticulum and plasma membrane proteins may be inhibited [11,12].

As the initial target of insulin's action is the plasma membrane, we have used plasma membranes from rat liver to examine the effects of insulin on the phosphorylation of constituent proteins. The proteins of the plasma membrane have been resolved into peripheral proteins, those binding to the membrane by predominantly electrostatic interactions, and integral proteins, those binding to the membrane through extensive hydrophobic interactions. It would appear that insulin triggers the cyclic AMP-dependent phosphorylation of three peripheral proteins and inhibits the cyclic AMP-dependent phosphorylation of two integral proteins.

2. Materials and methods

Rat liver plasma membranes were prepared from 250–300 g male Sprague-Dawley rats by the method in [13] as modified [14].

Peripheral membrane proteins were separated from integral membrane proteins as in [15]. Briefly this involved incubating for 45 min on ice, plasma membranes (4 mg protein) in a 3 ml cocktail containing (final conc.) 0.4 M NaCl, 20 mM Tris–HCl and 10 mM 2-mercaptoethanol, at final pH 7.2. This mixture was then centrifuged at 300 000 × g_{av} for 30 min at 4°C to yield a clear supernatant which contained the peripheral proteins and a pellet containing the integral proteins. The pellet was resuspended in 1 mM $KHCO_3$ (pH 7.2) and washed by re-centrifugation prior to use.

[γ-^{32}P]ATP at spec. act. 200 mCi/mmol was prepared as in [16].

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was done as in [17]. This employed a 3% acrylamide stacking gel with an acrylamide:bis-acrylamide ratio of (30:0.8) and a 9% acrylamide separating gel keeping a similar acrylamide:bis-acrylamide ratio (30:0.8). The stacking gel contained 0.125 M Tris–HCl buffer (pH 6.8) and the running gel 0.375 M Tris–HCl buffer (pH 8.8). As a routine 6 gels 7.5 cm long were electrophoresed at a

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constant 3 mA for ~4 h. Samples were prepared by mixing a 10 μl sample containing 100 μg protein with a cocktail containing 6 mM EDTA, 0.75 M sucrose, 6% SDS, 0.03% bromothymol blue at a final pH 7.4. Dithiothreitol (5 μl, 160 mM) was added and the mixture incubated at 100°C for 5 min. After cooling, 3 μl 0.5 M iodoacetamide was added and the mixture incubated for 15 min at room temperature prior to electrophoresis. Gels were sliced into 1.5 mm sections which were individually incubated overnight in sealed 1.5 ml plastic microfuge tubes containing 100 μl 40 mM Tris–HCl buffer (pH 7.4) containing 5 mM 2-mercaptoethanol and 0.1% SDS. Samples (50 μl) were then taken for counting. These were each added to 4 ml cocktail of toluene:Triton:H_2O (12:6:1.5; by vol.) containing 2.6 gPPO/l and counting was done on a Packard Tricarb 2425 Liquid Scintillation Spectrometer to 1% standard error.

The phosphorylation reactions were done by incubating intact plasma membranes (500 μg protein/ml) for 5 min at 30°C in a mixture containing (final conc.) 1 mM [γ-^{32}P]ATP spec. act. 200 mCi/mmol, 5 mM $MgCl_2$, 100 μM $CaCl_2$ and 20 mM Tris–HCl at final pH 7.4 in 250 μl final vol. To this mixture was also added various ligands and in some cases a protein kinase inhibitor prepared as in [18]. The reaction was then either:

(i) Quenched rapidly on ice and samples taken for electrophoresis; or

(ii) Quenched by adding 1 ml ice-cold 1 mM $KHCO_3$ (pH 7.2) and centrifuging for 6 min at 14 000 × g_{av} at 4°C prior to separation of the peripheral and integral proteins for electrophoresis; or

(iii) Quenched by adding 0.5 ml 10% cold trichloroacetic acid and the pellet resuspended in 0.15 ml 20 mM Tris–HCl (pH 7.4) and taken for electrophoresis.

All biochemicals were from Boehringer. Bovine insulin was from Sigma. Radiochemicals were from Amersham. Other chemicals were of the highest quality available from BDH.

3. Results

Incubation of rat liver plasma membranes with [γ-^{32}P]ATP and various ligands for 5 min at 30°C followed by rapid quenching in trichloroacetic acid

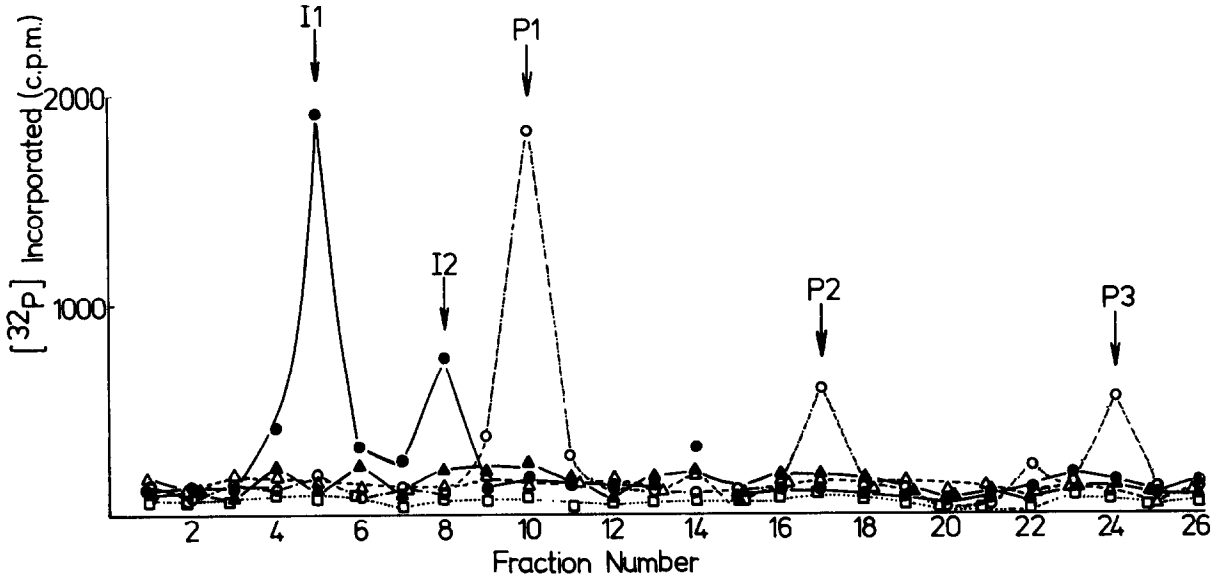

Fig.1. The effect of insulin and cyclic AMP on the phosphorylation of proteins in rat liver plasma membranes. The incorporation of ^{32}P from [γ-^{32}P]ATP into protein resolved by SDS – PAGE of intact rat liver plasma membranes. Labelling was done as in section 2 and quenching was by trichloroacetic acid. Experiments were done with ATP alone (△); ATP + 10^{-9} M insulin (▲); ATP + 4 × 10^{-6} M cyclic AMP (●); ATP + insulin + cyclic AMP (○). Experiments were also done in the presence of 4 μg protein/ml pure cyclic AMP protein kinase inhibitor prepared as in [18]. This was a kind gift from Dr H. G. Nimmo. For clarity the results obtained using this inhibitor and either ATP + cyclic AMP or ATP + cyclic AMP + insulin have been averaged and are shown as (□). This is because in both instances they are indistinguishable from background. We show here a typical experiment. Essentially similar results were obtained by quenching on ice prior to the preparation of samples for SDS–PAGE.

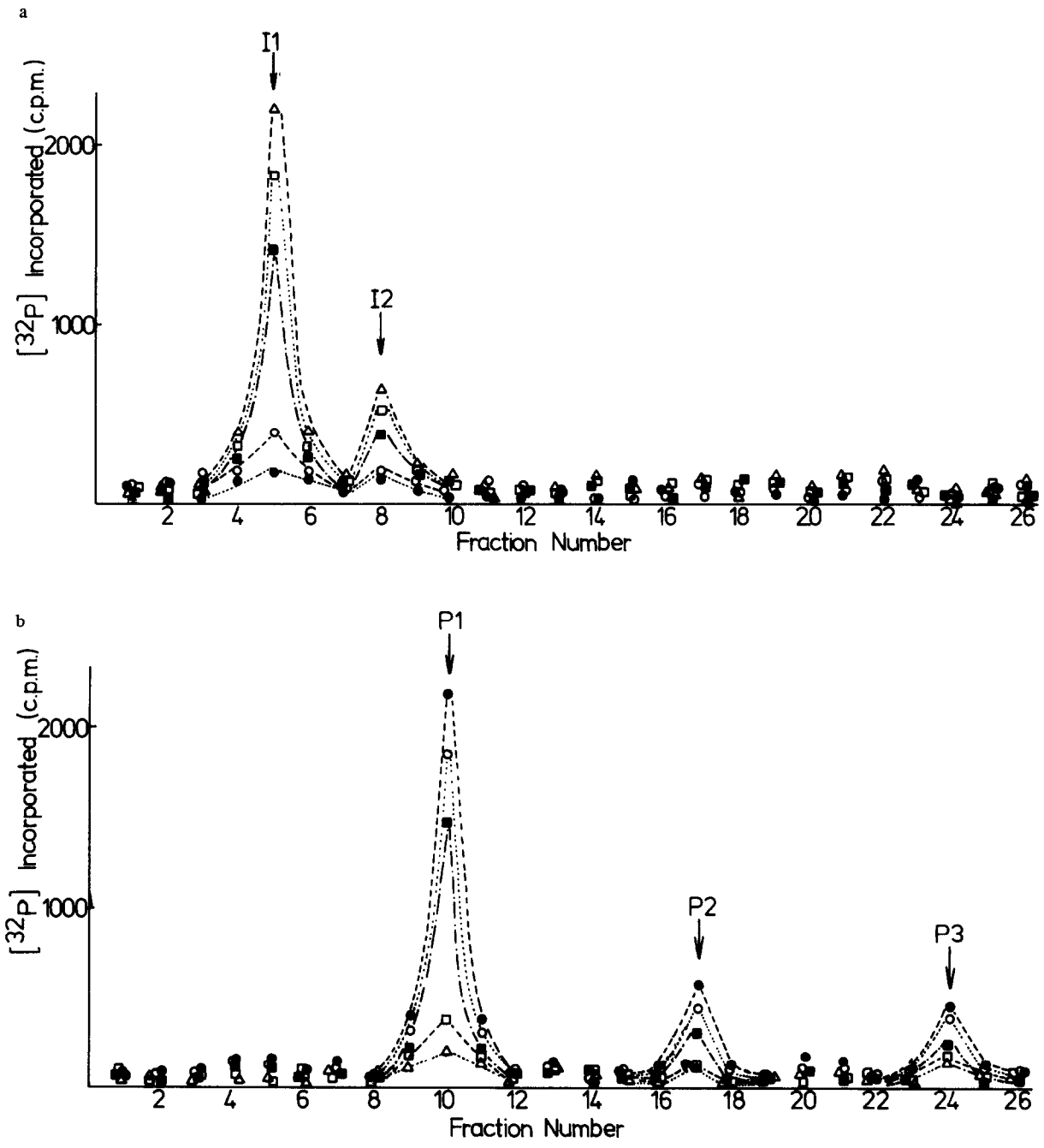

Fig.2. The resolution of the integral and peripheral proteins of rat liver plasma membranes and the effect of insulin and cyclic AMP on their phosphorylation. After the native membranes were incubated with labelled ATP and the various ligands, the membrane proteins were resolved into peripheral and integral species prior to SDS–PAGE.

(a) Integral proteins: The effect of increasing insulin concentrations on the phosphorylation effected by 4×10^{-6} M cyclic AMP. The insulin concentrations were: zero (△); 5×10^{-11} M (□); 2×10^{-10} M (■); 5×10^{-10} M (○); 10^{-9} M (●).

(b) Peripheral proteins: The effect of increasing insulin concentrations in the presence of 4×10^{-6} M cyclic AMP. The insulin concentrations were: 10^{-11} M (△); 5×10^{-11} M (□); 2×10^{-10} M (■); 5×10^{-10} M (○); 10^{-9} M (●).

The concentration dependence of the phosphorylation of both these groups upon cyclic AMP is shown in fig.3. The phosphorylation of the peripheral proteins is dependent upon both cyclic AMP and insulin. These are typical results.

and SDS – PAGE led to the resolution of a number of bands of radioactivity (fig.1). Under the incubation conditions described, the labelling was essentially complete after this 5 min incubation and little significant labelling occurred in the presence of ATP alone. The addition of cyclic AMP (4×10^{-6} M), however, caused two major bands to appear (I1, I2) and when bovine insulin (10^{-9} M) was added together with cyclic AMP, three major new labelled bands appeared (P1–P3) whilst the two bands occurring in the presence of cyclic AMP alone disappeared. Insulin by itself did not elicit the production of labelled bands from [γ-^{32}P]ATP and the addition of a specific inhibitor of cyclic AMP-dependent protein kinase [18] blocked the production of labelled bands in instances where cyclic AMP was added.

If after this incubation the reaction was quenched by ice-cold buffer and the integral and peripheral proteins resolved by a high ionic strength procedure, then upon SDS–PAGE the differences in the effects of insulin and cyclic AMP became apparent. For, in the presence of cyclic AMP alone, we observed that the two proteins labelled were in fact both integral proteins which we have designated I1, I2. These have M_r 140 000 and 80 000, respectively (fig.2a). The labelling of the peripheral proteins of rat liver plasma membranes was only significant if both insulin and cyclic AMP were present together (2b,c). In this instance three major peaks were resolved which we have designated P1, P2 and P3, having M_r 52 000, 28 000 and 14 000, respectively.

When insulin and cyclic AMP were present together in the incubation then we observed that the integral proteins, I1 and I2, did not become labelled. However, these proteins could be labelled by [γ-^{32}P]ATP in the presence of cyclic AMP, then the intact membranes washed by repeated centrifugation and resuspension in ice-cold 1 mM $KHCO_3$ (pH 7.2). After this insulin (10^{-8} M) was added to these labelled, washed membranes suspended in a phosphorylation 'cocktail' lacking ATP. During incubation for 40 min at 30°C the radioactivity associated with I1 and I2 remained constant.

By carrying out experiments at different concentrations of cyclic AMP and insulin, resolving the integral and peripheral components and running SDS–PAGE it was possible to obtain dose response curves for ^{32}P incorporation into these proteins (fig.3). The effect of insulin on triggering the cyclic AMP-dependent phosphorylation of the peripheral pro-

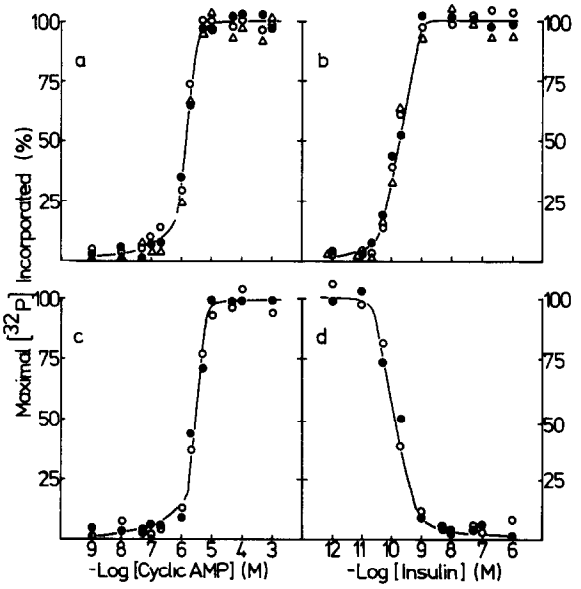

Fig.3. Dose response curves for the effects of insulin and cyclic AMP on the phosphorylation of peripheral and integral proteins of rat liver plasma membranes. These data show typical experiments.
(a) Peripheral proteins: The dose dependence of the phosphorylation of P1 (•), P2 (○) and P3 (△) upon cyclic AMP in the presence of 10^{-9} M insulin.
(b) Peripheral proteins: The dose dependence of the phosphorylation of P1 (•), P2 (○) and P3 (△) upon insulin in the presence of 4×10^{-6} M cyclic AMP.
(c) Integral proteins: The dose dependence of the phosphorylation of I1 (•) and I2 (○) upon cyclic AMP.
(d) Integral proteins: The dose dependence of the phosphorylation of I1 (•) and I2 (○) upon insulin in the presence of 4×10^{-6} M cyclic AMP.

Table 1
The sensitivity of the phosphorylation of rat liver plasma membrane integral and peripheral proteins to insulin and cyclic AMP

Band	K_a insulin (M^{-1})	K_a cyclic AMP (M^{-1})
P1	$2.2 \pm 0.2 \times 10^{-10}$	$1.6 \pm 0.15 \times 10^{-6}$
P2	$2.0 \pm 0.3 \times 10^{-10}$	$1.55 \pm 0.1 \times 10^{-6}$
P3	$2.1 \pm 0.2 \times 10^{-10}$	$1.65 \pm 0.15 \times 10^{-6}$
I1	$2.1 \pm 0.2 \times 10^{-10}$	$2.4 \pm 0.2 \times 10^{-6}$
I2	$2.15 \pm 0.15 \times 10^{-10}$	$2.55 \pm 0.15 \times 10^{-6}$

The labelled bands are designated in fig.1 and fig.2. The data is given ± SD for 4 expt on different preparations

teins and in blocking the cyclic AMP-dependent phosphorylation of the integral proteins, produced identical K_a-values of $\sim 10^{-10}$ M from the dose–response curves (table 1). However there appeared to be a significant difference in the K_a-values for the cyclic AMP dependence of the phosphorylation of the integral and peripheral proteins ($P < .001$). For, whilst the values were in the μM range, those expressed by the integral proteins were slightly higher than those expressed by the peripheral proteins (table 1).

4. Discussion

Unlike glucagon which appears to mediate its cellular effects by simply activating adenylate cyclase, hence elevating intracellular cyclic AMP levels, insulin stimulates a wide variety of cellular phenomena [1,4]. An occupied insulin receptor may express itself at the molecular level by directly acting upon several target proteins within the membrane; it may produce a specific 'second messenger' or it may achieve a combination of both. Our study demonstrates that the cyclic AMP-dependent phosphorylation of plasma membrane proteins is specifically affected as an immediate consequence of insulin binding to its receptor. Interestingly, insulin's action on the integral membrane proteins is quite distinct from its action on the peripheral proteins. The two integral membrane proteins I1 and 12 (M_r 140 000 and 80 000, respectively) which were phosphorylated by a cyclic AMP-dependent mechanism did not incorporate ^{32}P from [γ-^{32}P]ATP when insulin was present. This could be due to insulin either inhibiting the action of a membrane-bound cyclic AMP-dependent protein kinase or to a stimulation of a membrane-bound protein phosphatase. Our experiments demonstrating that labelled I1 and I2 did not lose ^{32}P when incubated with insulin, suggests that insulin inhibits a cyclic AMP-dependent protein kinase. On the other hand, the 3 peripheral proteins that became labelled, only did so when both cyclic AMP and insulin were present together. It would appear that insulin triggers the cyclic AMP-dependent phosphorylation of these 3 peripheral proteins. Whilst the K_a-values were in the μM range, the cyclic AMP dependent of the phosphorylation of the integral and peripheral proteins appeared to be significantly different. This could imply distinct cyclic AMP-dependent protein kinases with different affinities for cyclic AMP and, importantly, a different response to insulin. However, the sensitivity of the phosphorylation of both the integral and peripheral proteins to insulin yielded similar K_a-values of $\sim 10^{-10}$ M, even though insulin blocked the labelling of the integral proteins but triggered that of the peripheral proteins. The K_a-value for this action of insulin is well within the physiological range and reflects the high affinity component observed in receptor binding studies [1]. That obtained for cyclic AMP is rather intriguing, as basal intracellular cyclic AMP levels in the liver are likely to be 0.3–0.5 μM [19,20]: too low to allow these phosphorylation events to occur. However, after stimulation of liver cells by glucagon, cyclic AMP levels rise to 2–4 μM [21,22]. This would allow the phosphorylation of the integral proteins to ensue and would allow the phosphorylation of the peripheral proteins to occur if insulin was present at an appropriate concentration. It would appear that for insulin to achieve the effects observed in this study, intracellular cyclic AMP levels must first be elevated above their basal levels. This can be achieved by exposure of the cells to glucagon, and may reflect aspects of the mechanism by which these two hormones antagonise each other. Liver cells with intracellular cyclic AMP levels sufficiently high to allow the phosphorylation of these proteins such as may occur in some cancer and other abnormal states and during certain stages of the cell cycle [23,24] might be expected to exhibit very different responses to insulin compared with normal, resting liver cells.

Of the proteins labelled in this study we are at present only able to identify one; the M_r 52 000 peripheral species. This is a low K_m cyclic AMP phosphodiesterase [25], which has been shown to be a peripheral enzyme [15] and is activated some 2-fold only when insulin, cyclic AMP and ATP are added together to a plasma membrane preparation [25]. The K_a for activation by insulin and cyclic AMP, at saturating concentrations of the other ligand, reflects that obtained in this study for the phosphorylation of the peripheral species [25]. Presumably the activation of this enzyme, which can only occur after intracellular cyclic AMP levels have been elevated above their basal levels, explains why insulin can depress those elevated cyclic AMP levels achieved by glucagon, but does not affect basal levels [21]. This observation provides a molecular rationale for the proposal [21] that it is first necessary to elevate

cyclic AMP levels before they can be depressed by insulin.

It is tempting to speculate on the nature of the other phosphoproteins, for example, insulin affects fluxes of Na^+ and K^+ across the plasma membrane [1]. This may be due to the proposed effect of insulin preventing the cyclic AMP-mediated inhibition of the Na^+/K^+-ATPase [26]. If this were due to insulin blocking a cyclic AMP-dependent phosphorylation of a component of the Na^+/K^+-ATPase then I1 and I2 would be candidates. Insulin is also reported to affect Na^+:K^+ exchange [27] and Ca^{2+} fluxes [1]. These could be affected either by direct action on the integral protein responsible or via a regulatory protein that is subject to cyclic AMP-dependent phosphorylation like the M_r 22 000 component regulating Ca^{2+} transport in cardiac sarcoplasmic reticulum [28]. The stimulating effect of insulin on amino acid transport may also be effected by a control of plasma membrane cyclic AMP-dependent protein kinases as γ-aminoisobutyrate uptake into fibroblasts has been shown to be inhibited by a cyclic AMP-dependent protein kinase [28]. Indeed, in [29] insulin lowered the phosphorylation state and hence the activity of inhibitor-1 in rat skeletal muscle. This inactivation of inhibitor-1 relieves the inhibition on protein phosphatase-1 which can then carry out reactions which inhibit glycogenolysis or activate glycogen synthesis [30,31]. As inhibitor-1 is phosphorylated by a cyclic AMP-dependent mechanism, this would be consistent with our proposal that insulin can modulate the activity of cyclic AMP-dependent protein kinases.

It is possible that the 'peptide-like' factor in [5–7], presumably released from the plasma membrane after occupancy of the insulin receptor, may be responsible for some or all of the effects observed. Alternatively one of the phosphoproteins we have identified may give rise to the production or release of such a factor.

Our study demonstrates that in plasma membranes from liver, insulin has distinct effects on the cyclic AMP-dependent phosphorylation of specific integral and peripheral proteins. The concentration dependence upon insulin for the 5 proteins studied was identical and reflected that of insulin binding specifically with high affinity to its receptor. Occupancy of the insulin receptor allows the hormone to modulate a variety of cellular processes by initial actions at the plasma membrane.

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