The insulin-stimulated cyclic AMP phosphodiesterase binds to a single class of protein sites on the liver plasma membrane

Miles D. HOUSLAY* and Robert J. MARCHMONT

Department of Biochemistry, University of Manchester Institute of Science and Technology, P.O. Box 88, Manchester M60 1QD, U.K.

(Received 27 April 1981/Accepted 26 May 1981)

The peripheral cyclic AMP phosphodiesterase from rat liver plasma membranes binds with high affinity (2.4 nM) to a single class of receptor sites on the liver plasma membrane. These receptor sites appear to be proteins, as they are trypsin- and heat-labile. The sensitivity of these sites to denaturation by trypsin and heat is a first-order process. The presence of Ca^{2+} (5 mM) increases the affinity of these sites for the enzyme, but does not alter their total number. The receptor sites and the cyclic AMP phosphodiesterase occur in similar numbers, at around 2 pmol/mg of plasma-membrane protein. It is proposed that the peripheral, liver plasma-membrane cyclic AMP phosphodiesterase is attached to a specific site on the insulin receptor and that the binding of insulin to the receptor site triggers a conformational change in the enzyme such that the enzyme can be phosphorylated and activated by an endogenous cyclic AMP-dependent protein kinase.

Liver plasma membranes contain two distinct enzymes capable of hydrolysing cyclic AMP; these are a low-affinity integral enzyme and a high-affinity peripheral enzyme (Marchmont & Houslay, 1980a). The peripheral enzyme can be activated some 2-fold by pre-treatment of the plasma membranes with insulin together with cyclic AMP and MgATP (Marchmont & Houslay, 1980b,c). The activation is due to insulin triggering the phosphorylation by cyclic AMP of this peripheral enzyme (Marchmont & Houslay, 1980b,d), whereby 1 molecule of alkali-labile phosphate is incorporated into each molecule of the enzyme (Marchmont & Houslay, 1981).

Relatively little is known about the interaction of peripheral proteins with biological membranes; presumably they could bind to lipid, protein or carbohydrate components. However, it has been suggested that, in order to localize a peripheral protein to a specific membrane site, it should bind efficaciously to an integral membrane protein (Warren & Houslay, 1980; Houslay, 1981). This would immediately confer a particular localization and asymmetry on the protein.

The present paper describes the interaction of the purified peripheral cyclic AMP phosphodiesterase with liver plasma membranes whose peripheral proteins have been removed.

* To whom reprint requests should be addressed.

Materials and methods

Venom from the snake *Ophiophagus hannah*, Dowex 1 anion-exchange resin, trypsin and trypsin inhibitor were from Sigma. Cyclic AMP, cyclic GMP, ATP and all enzymes were from Boehringer. All other chemicals were of A.R. grade from BDH.

Rat liver plasma membranes

These were prepared from 200–300 g male Sprague–Dawley rats by a modification (Marchmont *et al.*, 1981) of the method of Pilkis *et al.* (1974). Membranes were always rapidly frozen in liquid N_2 and stored therein at a concentration of 10 mg of protein/ml in 1 mM- KHCO_3 , pH 7.4.

Washed high-salt-treated plasma membranes

Plasma membranes were treated with 0.4 M-NaCl under conditions described in detail previously (Marchmont & Houslay, 1980a) in order to remove peripheral proteins (Marchmont & Houslay, 1980d). These membranes were washed and finally resuspended in 1 mM- KHCO_3 , pH 7.4, and used either fresh or after storage as above.

Purification of the peripheral cyclic AMP phosphodiesterase from rat liver plasma membranes

This enzyme was purified to apparent homogeneity by the method described previously in some detail (Marchmont *et al.*, 1981).

Assay of cyclic AMP phosphodiesterase

This was a two-step radio-assay done by a modification of the method of Thompson & Appleman (1971) as described previously in some detail (Marchmont & Houslay, 1980a). As detailed in Marchmont & Houslay (1980a), corrections were made for the binding of [^3H]adenosine to the resin. All assays were carried out at 30°C, utilized less than 10% of the substrate and produced linear time courses.

Protein determination

Protein in the membrane fractions was determined by a modification (Houslay & Palmer, 1978) of the microbiuret method of Goa (1953) with bovine serum albumin as standard. For the purified cyclic AMP phosphodiesterase, the Bio-Rad Protein Assay Kit (see Bradford, 1976; Spector, 1978) was used, again with bovine serum albumin as standard.

Trypsin treatment of washed high-salt-treated plasma membranes

Portions (2 mg of protein) of washed high-salt-treated plasma membranes prepared as described by Marchmont & Houslay (1980a) in 0.5 ml of 1 mM-KHCO₃, pH 7.2, were added to a mixture containing (final concentrations) 20 mM-Tris/HCl, pH 7.2, 5 mM-MgCl₂ and 0.04 mg of trypsin/ml. This mixture was vortex-mixed and incubated in a water bath equilibrated at 30°C. The reaction was terminated by the addition of 500 μl of soya-bean trypsin inhibitor (0.2 mg/ml) in 5 mM-MgCl₂/20 mM-Tris/HCl, pH 7.2. Membranes were then washed twice with ice-cold 1 mM-KHCO₃, pH 7.2, by centrifugation at 14000 g_{av} for 5 min at 4°C before final resuspension in the same buffer. A number of samples were prepared for the rebinding studies by incubating them for different intervals of time.

Heat treatment of high-salt-treated plasma membranes

This was carried out by incubating 250 μl samples (2 mg of plasma-membrane protein) at 65°C as described in detail previously (Marchmont & Houslay, 1980a).

Rebinding of the purified cyclic AMP phosphodiesterase to high-salt-treated plasma membranes

Portions (2 mg of protein) of washed high-salt-treated membranes in 250 μl of 1 mM-KHCO₃, pH 7.2, were mixed with 100 μl of a solution containing (final concentrations) 25 mM-MgCl₂ and 100 mM-Tris/HCl, final pH 7.2. To this was added 150 μl of 1 mM-KHCO₃, pH 7.2, containing the purified peripheral cyclic AMP phosphodiesterase. In the rebinding studies using heat- or trypsin-treated membranes, 20 pmol of the purified enzyme was added, whereas for Scatchard analysis, between 0.5

and 50 pmol of the purified enzyme was added. This mixture (final vol. 0.5 ml) was incubated for 1 h at 4°C with occasional vortex-mixing. The membrane fraction was pelleted by centrifugation at 14000 g_{av} for 10 min at 4°C. The membranes were re-suspended and washed by centrifugation (as above) in 1 mM-KHCO₃, pH 7.2, before final resuspension in this solution (250 μl). Both the initial supernatant and the final pellet were assayed for cyclic AMP phosphodiesterase activity (total recoveries of activities were 100% in all experiments).

Results

Binding to native plasma membranes

Rebinding of the purified enzyme to washed high-salt-treated plasma membranes indicated that it bound to a single class of sites on the membrane (Fig. 1). The K_D for binding was 2.4 ± 0.4 nM, and this decreased to 1.5 ± 0.4 nM when 5 mM-Ca²⁺ was present in the rebinding mixture. The maximum capacity of the membrane for binding this peripheral enzyme was 2.1 ± 0.3 pmol/mg of membrane protein (S.D., $n =$ three different membrane preparations) in both the presence and the absence of Ca²⁺.

Fig. 1. Binding of the peripheral cyclic AMP phosphodiesterase to washed high-salt-treated liver plasma membranes

Scatchard analysis of the rebinding experiment in the absence (●) or presence (O) of 5 mM-Ca²⁺.

Fig. 2. *Trypsin-sensitivity of the binding site for the peripheral cyclic AMP phosphodiesterase*

This demonstrates the percentage (plotted as log) of the peripheral cyclic AMP phosphodiesterase that can be re-bound (at 4°C) to trypsin-treated washed high-salt-treated liver plasma membranes in the absence (●) or presence (○) of 5 mM- Ca^{2+} . Membranes were preincubated with trypsin for the various times shown before its action was stopped with trypsin inhibitor.

Fig. 3. *Heat-lability of the binding site for the peripheral cyclic AMP phosphodiesterase*

This demonstrates the percentage (plotted as log) of the peripheral cyclic AMP phosphodiesterase that can be re-bound (at 4°C) to heat-treated washed high-salt-treated liver plasma membranes in the absence (●) or presence (○) of 5 mM- Ca^{2+} . The membranes were preincubated at 65°C for the times shown before binding.

Binding to trypsin- and heat-treated plasma membranes

Exposing the washed high-salt-treated membranes either to low concentrations of trypsin (Fig. 2) or to heat (Fig. 3) decreased the ability of the membranes to bind the enzyme. This loss of binding was a first-order process, with a half-time ($t_{0.5}$) of $13.5 \pm 2.0 \text{ min}^{-1}$ for heat denaturation and $11.0 \pm 2.0 \text{ min}^{-1}$ for trypsin treatment (s.d., $n =$ three different preparations), indicating a single class of protein sites whether or not Ca^{2+} was present in the rebinding assay.

In these experiments corrections were made at each time-point for the residual activity of the 'high- K_m ' integral enzyme associated with the high-salt-washed membranes (Marchmont & Houslay, 1980a). The addition of trypsin inhibitor in the manner described did not itself affect the binding of the peripheral enzyme to the membrane.

Discussion

Binding of the peripheral cyclic AMP phosphodiesterase to liver plasma membranes, pretreated to remove the peripheral proteins (Marchmont & Houslay, 1980a,d), was lost (Figs. 2 and 3) after treatment either with the proteolytic enzyme trypsin or with heat (65°C). The loss of binding was a first-order process, indicating that the enzyme binds specifically to a single class of receptor protein sites that are presumably integral in nature. Scatchard analysis (Fig. 1) demonstrates that indeed the peripheral cyclic AMP phosphodiesterase binds to a single class of receptor sites, and with high affinity (2.4 nM).

Binding of the cyclic AMP phosphodiesterase to the plasma membrane appears to be predominantly electrostatic in nature, as the enzyme is progressively released as the ionic strength of the medium is raised (Marchmont & Houslay, 1980a). Furthermore, this release is inhibited by the presence of the bivalent cation Ca^{2+} and augmented by the presence of the chelating agent EGTA (Houslay, 1981). We note here that Ca^{2+} increases the affinity of the receptor protein for this cyclic AMP phosphodiesterase, but does not alter the total number of sites available to the enzyme (Fig. 1). This is confirmed by the rebinding studies carried out on heat- or trypsin-treated membranes (Figs. 2 and 3), which yield identical results whether or not Ca^{2+} is included in the rebinding medium.

Attachment to these sites does not, however, alter either the kinetic properties of the cyclic AMP phosphodiesterase or its thermostability (Marchmont & Houslay, 1980a). The only other peripheral enzyme that has so far been demonstrated to bind specifically with high affinity (100 nM) to a single class of integral protein receptor sites is glycer-

aldehyde 3-phosphate dehydrogenase from erythrocytes (Kant & Steck, 1973). Similarly, binding had no significant effect on the kinetic properties of that enzyme.

There appears to be no great excess of receptor sites on the liver plasma membrane for the cyclic AMP phosphodiesterase, as the value for the total number of binding sites for the peripheral cyclic AMP phosphodiesterase (2.15 pmol/mg of plasma-membrane protein) is very similar to the amount of enzyme (1.6 pmol/mg of plasma-membrane protein, assuming a 100% yield) that can be released and purified to homogeneity (Marchmont *et al.*, 1981). Assuming that about 1.2% of the total cell protein is in the plasma membrane (Seglen, 1973; Toda *et al.*, 1975), then each liver cell would have about 26000 copies of this protein associated with its plasma membrane.

In view of the observations that insulin can activate this cyclic AMP phosphodiesterase in isolated plasma membranes (Marchmont & Houslay, 1980b,c) by triggering its cyclic AMP-dependent phosphorylation (Marchmont & Houslay, 1981), it is perhaps not unreasonable to suggest that the integral protein, which acts as a receptor for the enzyme, is connected with the activation process. Studies on the insulin receptor in liver plasma membranes have indicated that binding exhibits apparent negative co-operativity (De Meyts *et al.*, 1976), identifying a total number of insulin-receptor sites of 1.5–2.0 pmol/mg of plasma-membrane protein (Kahn *et al.*, 1974). This value is very similar to the number of receptor sites on the liver plasma membrane for the cyclic AMP phosphodiesterase. It may be that the peripheral enzyme binds to the insulin receptor itself. As the purified enzyme in the soluble state is not activated by the catalytic unit of cyclic AMP-dependent protein kinase (R. J. Marchmont, unpublished work), it is possible that insulin, upon binding to its receptor, elicits a change in the conformation of the cyclic

AMP phosphodiesterase which allows this enzyme to be phosphorylated and activated.

This work was supported by a project grant from the S.R.C. to M.D.H. and by a grant from the California Metabolic Research Association to M. D. H.

References

- Bradford, M. (1976) *Anal. Biochem.* **72**, 248–256
- De Meyts, P., Bianco, A. R. & Roth, J. (1976) *J. Biol. Chem.* **251**, 1877–1888
- Goa, J. (1953) *Scand. J. Clin. Lab. Invest.* **5**, 218–222
- Houslay, M. D. (1981) *Biosci. Rep.* **1**, 19–34
- Houslay, M. D. & Palmer, R. J. (1978) *Biochem. J.* **174**, 909–919
- Kahn, C. R., Freychet, P., Roth, J. & Neville, D. M. (1974) *J. Biol. Chem.* **249**, 2249–2257
- Kant, J. A. & Steck, T. L. (1973) *J. Biol. Chem.* **248**, 8457–8464
- Marchmont, R. J. & Houslay, M. D. (1980a) *Biochem. J.* **187**, 381–392
- Marchmont, R. J. & Houslay, M. D. (1980b) *Nature (London)* **286**, 904–906
- Marchmont, R. J. & Houslay, M. D. (1980c) *Biochem. Soc. Trans.* **8**, 537–538
- Marchmont, R. J. & Houslay, M. D. (1980d) *FEBS Lett.* **118**, 18–24
- Marchmont, R. J. & Houslay, M. D. (1981) *Biochem. J.* **195**, 653–660
- Marchmont, R. J., Ayad, S. R. & Houslay, M. D. (1981) *Biochem. J.* **195**, 645–652
- Pilkis, S. J., Exton, J. H., Johnson, R. A. & Park, C. R. (1974) *Biochim. Biophys. Acta* **343**, 250–267
- Seglen, P. O. (1973) *Exp. Cell Res.* **82**, 391–398
- Spector, T. (1978) *Anal. Biochem.* **86**, 142–146
- Thompson, W. J. & Appleman, M. M. (1971) *Biochemistry* **10**, 311–316
- Toda, G., Oka, H., Oda, T. & Ikeda, Y. (1975) *Biochim. Biophys. Acta* **413**, 52–64
- Warren, G. B. & Houslay, M. D. (1980) in *Cellular Receptors for Hormones and Neurotransmitters* (Schulster, D. & Levitzki, A., eds.), pp. 29–54, Wiley, London