Journal of Cellular Biochemistry 55S:66–82 (1994)

Regulation of Hepatocyte Adenylate Cyclase by Amylin and CGRP: A Single Receptor Displaying Apparent Negative Cooperativity Towards CGRP and Simple Saturation Kinetics for Amylin, a Requirement for Phosphodiesterase Inhibition to Observe Elevated Hepatocyte Cyclic AMP Levels and the Phosphorylation of G_i-2

Miles D. Houslay, Nicholas J. Morris, Anne Savage, Alison Marker, and Mark Bushfield

Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland

Abstract Challenge of intact hepatocytes with amylin only succeeded in elevating intracellular cyclic AMP levels and activating phosphorylase in the presence of the cAMP phosphodiesterase inhibitor IBMX. Both amylin and CGRP similarly activated adenylate cyclase, around 5-fold, although ~400-fold higher levels of amylin were required to elicit half maximal activation. Amylin activated adenylate cyclase though apparently simple Michaelien kinetics whereas CGRP elicited activation by kinetics indicative of apparent negative co-operativity. Use of the antagonist CGPP(8–37) showed that both CGRP and amylin activated hepatocyte adenylate cyclase through a common receptor by a mnemonical mechanism where it was proposed that the receptor co-existed in interconvertible high and low affinity states for CGRP. It is suggested that this model may serve as a paradigm for G-protein linked receptors in general. Amylin failed to both stimulate inositol phospholipid metabolism in hepatocytes and to elicit the desensitization of glucagon-stimulated adenylate cyclase. Amylin did, however, elicit the phosphorylation of the inhibitory guanine nucleotide regulatory protein G_i-2 in hepatocytes and prevented the action of insulin in reducing the level of phosphorylation of this G-protein.

Key words: amylin, adenylate cyclase, kinetics, mnemonical, negative co-operativity, cyclic AMP, hepatocytes, CGRP, G-protein, G_i-2, guanine nucleotide regulatory protein

INTRODUCTION

Amylin

The pancreatic β-cell secretes a number of peptides and proteins in addition to the polypeptide hormone insulin [Nishi et al., 1990]. One of these is the 37-amino acid, "neuropeptide-like" molecule called amylin (IAPP; islet amyloid polypeptide). This peptide forms the major constituent of the so-called "amyloid" extracellular deposits that are found in the islets of non-insulin-dependent diabetic (NIDMM) subjects [Cooper et al., 1987]. Such amyloid deposits have also been noted to occur in certain benign insulinomas and have been recorded in the normal pancreas of aging human beings [Leighton and Cooper, 1990].

Amylin appears to be co-secreted with insulin from isolated islets of Langerhans [Kanatsuka et al., 1989], although the functional significance and molecular mechanisms related to this remain to be ascertained. Intriguingly, amylin also appears to be capable of eliciting insulin

Abbreviations: CGRP, calcitonin gene-related neuropeptide; EC_{50}, concentration of activator at which 50% stimulation was observed; G_i-2, inhibitory guanine nucleotide regulatory protein; G_s, stimulatory guanine nucleotide regulatory protein; IBMX, 3-isobutyl-1-methylxanthine; IC_{50}, concentration of inhibitor at which 50% inhibition was observed; PDE, cyclic AMP phosphodiesterase; PKC, protein kinase C.

Received October 19, 1993; accepted February 28, 1994.

Mark Bushfield's present address is Discovery Biology Department, Pfizer Central Research, Sandwich, Kent, England.

Address reprint requests to Dr. Miles D. Houslay, Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland.

resistance, as demonstrated most clearly in skeletal muscle preparations [Leighton and Cooper, 1988, 1990; Molina et al., 1990]. Despite such a fascinating observation, the molecular mechanism(s) through which it achieves such an action is not understood.

Sequence analysis of both rat and human amylin shows that amylin is about 40–50% homologous to the 37-amino acid calcitonin gene related neuropeptide, CGRP [Cooper et al., 1987; Westermark et al., 1987; Leffert et al., 1989]. CGRP is a potent vasodilator which can exert positive chronotropic and inotropic effects in atrial tissue [Yamaguchi et al., 1988]. Like amylin, it can inhibit insulin-stimulated rates of glycogen synthesis in skeletal muscle [Leighton et al., 1989] and glucose utilisation in the liver [Leighton and Cooper, 1990]. There are thus the possibilities that amylin and CGRP might exert their actions through either the same receptor or very similar ones.

Both CGRP and amylin are similarly capable of causing insulin resistance [Cooper et al., 1988; Leighton and Cooper, 1988, 1990; Molina et al., 1990], although the underlying molecular mechanism(s) for such an action remains to be identified. However, both peptides are able to stimulate adenylate cyclase activity in a variety of different cell types [see, e.g., Saito et al., 1986; Chiba et al., 1989; Zhu et al., 1991; Bushfield et al., 1993]. The physiological significance of such an action, certainly as far as amylin is concerned, again remains to be ascertained. Especially so as we have shown that in intact hepatocytes amylin can only increase intracellular cyclic AMP levels, and thus activate phosphorylase a, when a cyclic AMP phosphodiesterase (PDE) inhibitor is present [Bushfield et al., 1993] (vide infra) to prevent the breakdown of cyclic AMP. This suggests that, at least in hepatocytes, the small activation of adenylate cyclase which can be elicited by amylin might require the simultaneous inhibition of PDE activity for any perceptible response to be observed. This situation might occur when PDE inhibitors are given therapeutically or if other cell signaling systems alter PDE activity, which has been noted with various hormones [Houslay and Kilgour, 1990] and upon activation of protein kinase C (PKC) [Irvine et al., 1986]. It is extremely unlikely, however, that amylin's ability to elicit insulin-resistance is mediated, at least primarily or solely, by an increase in cyclic AMP [Leighton and Cooper, 1990; Deems et al., 1991a].

The well-recognised action of both amylin and CGRP in activating adenylate cyclase does provide a means for the analysis of one particular molecular action of these two peptides in defined isolated membrane systems. Irrespective of the physiological significance of the action, such a test system does allow one to address a key question that relates to whether these two peptides can exert such an action through a single common receptor or whether there are distinct receptors for these two peptides. Current evidence indicates, in fact, that both situations may exist, depending on the cell type analysed. In a number of systems, including rat liver, it would appear that specifically bound radiolabeled CGRP can be completely displaced by both unlabeled CGRP and amylin, with the same being true for specifically bound radiolabelled amylin [Morishita et al., 1990; Chantry et al., 1991; Galeazza et al., 1991; Bhogal et al., 1992]. This has led to the suggestion [Chantry et al., 1991] that at least in those situations a common receptor may be able to bind both peptides and elicit intracellular responses. Such a "common" receptor appears to be characterised by having a relatively "low affinity" for amylin compared to that shown for CGRP. As developed below, it also appears to show kinetically distinct differences in its mode of activation of adenylate cyclase dependent on whether amylin or CGRP is bound to it. However, others [Deems et al., 1991b; D'Santos et al., 1992; Bhogal et al., 1992] have suggested that these two peptides may function through different receptors in analyses done on various other cell systems. This would certainly seem to be the case in CHO-K1 cells, where, in contrast to rat liver and skeletal muscle, amylin, but not CGRP, appears to activate adenylate cyclase through an extremely high-affinity receptor [D'Santos et al., 1992]. There is thus the possibility that CGRP and amylin may bind to both common and to separate receptors, dependent on the cell type analysed. This situation would be analogous to that of insulin and IGF [Pilch et al., 1989]. It may be that species and even cell-specific forms of the amylin/CGRP receptor gene family exist that are capable of showing very different kinetic mechanisms, and perhaps even a breadth of responses tailored to their biological situation. Certainly from transfection studies done with a number of G-protein linked receptors it is abundantly clear that the receptor signalling responses, as regards both the magnitude and the

type of signal, can be influenced by the level of receptor expression and that of particular G-proteins in the plasma membrane environment of the cell. The resolution of these issues will ultimately depend on the cloning of the genes for these receptors and the analysis of their expressed protein in a variety of different cell systems.

Adenylate Cyclase Regulation by G-Proteins

Adenylate cyclase activity is now known to be expressed in all mammalian tissues by a family of structurally related transmembrane enzymes [see e.g., Krupinski et al., 1992]. To date, at least eight forms have been identified. The physiological significance of such variation has yet to be established although they appear to differ in their regulatory properties such as the ability of Ca^{2+}/calmodulin to elicit activation and the ability of PKC to effect phosphorylation and modulation of activity [Yoshimura and Cooper, 1993; Jacobowitz et al., 1993]. Receptor-mediated modulation of the activity of this enzyme can be both positive and negative, the enzyme being under dual control [see e.g., Houslay, 1992; Conklin and Bourne, 1993]. Agonist occupied receptors achieve the regulation of adenylate cyclase indirectly through the action of G-proteins that can then exert either stimulatory or inhibitory effects on this enzyme. The stimulatory G-protein is called G_s and the inhibitory G-protein is called G_i. The components of this system appear to be mobile in the plane of the membrane and interact through functional collisions with productive associations occurring only when an appropriate conformation of the receptor has been attained [Houslay et al., 1980]. Such a receptor then interacts with the G-protein, allows this species to bind GTP and adopt an activated state that leads to the dissociation of the G-protein into a GTP-bound α-subunit together with a $\beta\gamma$ complex. It is the GTP-bound α-subunit that is then capable of activating adenylate cyclase. This scheme allows for amplification as one occupied receptor can activate more than one G_s molecule, which can then activate more than one cyclase molecule during its lifetime. For example, with the glucagon receptor this has been calculated as leading to an amplification factor of around 4-fold [Houslay et al., 1980]. The "turn-off" reaction is then supplied by the ability of these G-proteins to effect the hydrolysis of bound GTP to GDP. The GDP bound G-protein fails to alter adenylate cyclase activity and re-associates with its $\beta\gamma$ complex. There are three members of the G_i family, which, when overexpressed in cell transfection studies, all appear capable of inhibiting adenylate cyclase activity. However, in native systems only for G_i-2 has this action been fully established [see e.g., Houslay, 1991a,b]. The mechanism whereby G_i achieves inhibition is complex and appears to be mediated in two distinct ways. Thus agonist-occupied receptors can interact with a member of the G_i family, causing it to bind GTP and dissociate, the released GTP-bound α–G_i can inhibit adenylate cyclase directly and the released $\beta\gamma$ complex itself can effect inhibition of the action of stimulatory receptors by attenuating the dissociation, and hence activation, of the stimulatory G-protein G_s. Thus mechanisms of inhibition can be directed both at the catalytic unit itself and also the stimulatory G-protein input and a method has been devised that may allow the relative magnitude of these two pathways to be gauged [Spence and Houslay, 1989].

The degree by which various agonist occupied receptors acting upon adenylate cyclase can increase intracellular cyclic AMP concentrations will depend on a variety of factors. These might include their effectiveness in achieving and prolonging the activation of G_s, the splice variant of G_s expressed in the cells, the type of adenylate cyclase species expressed and the level and type of cyclic AMP phosphodiesterase species as well as any compartmentalisation of receptors and these interacting systems. Modulation of this signalling system has been noted in various pathological states [see e.g., Bushfield et al., 1990b], where alterations in expression and phosphorylation of components has been noted. In particular, protein kinase C action can elicit the phosphorylation of α-G_i–2, whereupon its ability to inhibit adenylate cyclase tonically by virtue of the action of GTP alone is attenuated, although receptor-mediated inhibition is sustained [Houslay, 1991a,b].

AMYLIN ONLY INCREASES HEPATOCYTE CYCLIC AMP LEVELS IN THE PRESENCE OF A PHOSPHODIESTERASE INHIBITOR

Challenge of intact hepatocytes with amylin failed to produce any significant increase in the intracellular concentration of cyclic AMP or any activation of phosphorylase a (Fig. 1a,b). By

contrast, however, when cells were incubated with the cyclic AMP phosphodiesterase inhibitor IBMX (50 μM; higher concentrations can lead to the inhibition of protein kinase A) we found that amylin was able to increase intracellular cyclic AMP accumulation in intact hepatocytes in a time and dose-dependent fashion (Fig. 1). This may offer a explanation for the observations reported by Stephens et al. [1991], who have questioned whether functional amylin receptors occur on hepatocytes on the basis that nonparenchymal cells exhibited much greater specific receptor binding than parenchymal cells and also that amylin failed to stimulate glucose metabolism directly. As their metabolic experiments were done in the absence of any phosphodiesterase inhibitor, it is not surprising that Stephens et al. [1991] did not observe any affect of amylin on glycogen metabolism in isolated hepatocytes. The reason amylin alone fails to increase hepatocyte intracellular cyclic AMP levels, and hence fails to activate protein kinase A, as deduced from the lack of activation of downstream processes mediated by this protein kinase, is presumably because amylin is a poor activator of hepatocyte adenylate cyclase (vide infra). Certainly its action bears no comparison to the dramatic effect that glucagon can achieve in the same cells [Heyworth and Houslay, 1983a,b]. Indeed, there is considerable cyclic AMP phosphodiesterase activity in hepatocytes [Houslay, 1990; Houslay and Kilgour, 1990], and this clearly prevents any net accumulation of cyclic AMP in cells challenged with amylin. This may be further compounded if amylin was able to also effect the activation of one of the numerous isoforms of cyclic AMP phosphodiesterases (PDE) present in hepatocytes. Our experiments indicate that functional amylin receptors do exist on hepatocytes. Certainly such a contention is consistent with the fact that amylin can stimulate glucose production in a hepatocyte-derived cell line, namely Hep-G2 cells [Ciaraldi et al., 1990]. Presumably in HEP-G2 cells either the PDE activity is much lower than in native hepatocytes or that amylin is a much more potent activator of adenylate cyclase activity. Indeed, Gomez-Foix et al. [1991] observed that both amylin and CGRP were able to impair the action of insulin on hepatic glycogen metabolism, again suggesting that functional receptors occur on hepatocytes although, in this case, they indicated that such an action was independent of cyclic AMP, be consistent with their observing such changes in the absence of a PDE inhibitor.

Stephens et al. [1991] have clearly shown that, in liver, the most abundant source of specific CGRP and amylin binding is supplied by the nonparenchymal cells. However, our functional studies, coupled with the requirement for PDE inhibition, indicate that there are functional amylin/CGRP receptors on hepatocytes (parenchymal cells). The physiological relevance of these, if any, requires definition. Certainly, it would seem that under normal conditions they will unable to mediate responses due to their ability to activate adenylate cyclase, as this effect is too small to allow cyclic AMP levels to rise high enough to trigger the activation of protein kinase A. However, they may also exert effects through other signaling systems or even their action upon adenylate cyclase may be productive under conditions where PDE activity in hepatocytes is suppressed. This might be achieved physiologically, for example through protein kinase C activation, which, in hepatocytes, can lower PDE activity [Houslay, 1991a; Irvine et al., 1986]. Also, in man, PDE inhibitors have and are used therapeutically and this treatment may also engender conditions under which such a response of amylin and CGRP on the parenchymal cells of the liver can be exhibited.

In the experiments we have done investigating amylin and CGRP action we used a standard method to produce hepatocytes *that* produces cells of extremely high purity [see Heyworth and Houslay, 1983a] and which has been used by us for a number of years. However, in order to determine the levels of contamination of our hepatocyte preparations with nonparenchymal cells in our current preparations we have undertaken a variety of histological examinations. These showed them to be $>99\%$ hepatocytes by virtue of positive identification of these parenchymal cells, as demonstrated by activity staining for the hepatocyte-specific marker glucose-6-phosphatase [Borges et al., 1991] and by immunological staining for the hepatocyte specific nuclear transcription factor LFB-1 (Fig. 2a) [Frain et al., 1989]. Contamination with Kupffer cells was assessed at $<1\%$ by immunostaining with the antiserum ED-1 (Fig. 2b) [Dijkstra et al., 1985].

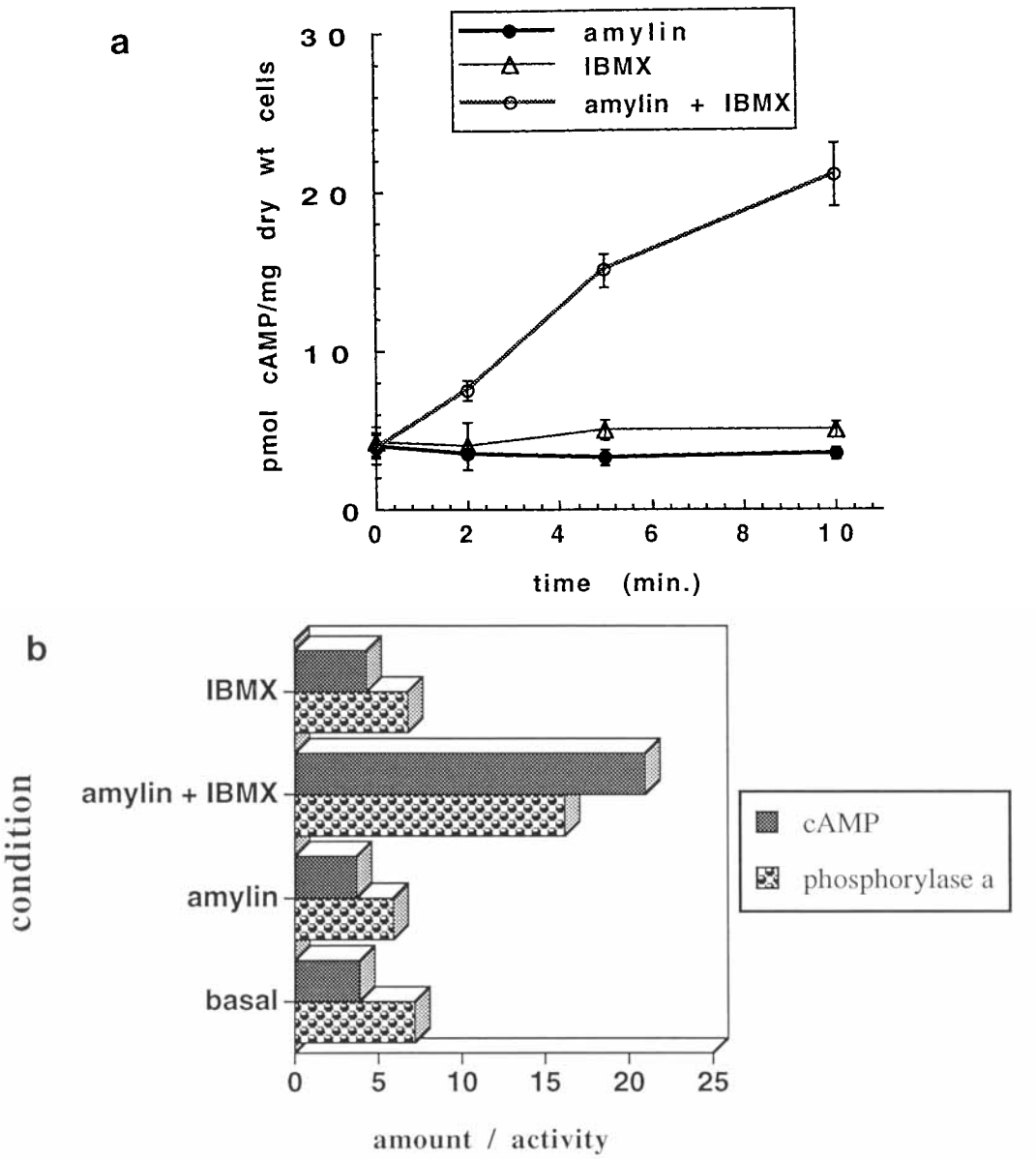

Fig. 1. Inhibition of cAMP phosphodiesterase activity is required for amylin to raise intracellular cyclic AMP levels and activate phosphorylase A. **a:** Intact hepatocytes were challenged with amylin (1 μM) in the presence and absence of 50 μM-IBMX. **b:** Intact hepatocytes were challenged with amylin (1 μM) in the presence and absence of 50 μM-IBMX for 10 min with assessments done of both the levels of cAMP and activity of phosphorylase a. cAMP levels and hepatocye preparations and incubations were done as described by Heyworth and Houslay [1983a] and phosphorylase a assays done as described in Bushfield et al. [1993]. Errors are SD for three separate experiments.

AMYLIN AND CGRP ACTIVATE HEPATOCYTE ADENYLATE CYCLASE IN KINETICALLY DISTINCT FASHIONS

Both amylin and CGRP induced a similar degree of activation of adenylate cyclase activity, of around 4- to 5-fold, as assessed in the presence of GTP (100 μM). These actions occurred *in a* concentration-dependent fashion (Fig. 3), although ~400-fold higher concentrations of amylin (EC_{50} ~120 nM) were required to achieve half-maximal activation compared to those for CGRP (EC_{50} ~0.3 nM). The kinetics of activation by amylin were representative of those of simple saturation (hill coefficient, h ~1.0), whereas those for CGRP were not, taking around four orders of magnitude to achieve maximal activation (hill coefficient, h ~0.3). Such data for the ability of CGRP to activate adenylate cyclase indicate either a single population of

receptors showing apparent negative cooperativity or multiple receptors having differing affinity for CGRP.

AT HALF-MAXIMALLY ACTIVATING CONCENTRATIONS OF AGONISTS, THE COMPOUND CGRP-(8-37) INHIBITS THE STIMULATION OF ADENYLATE CYCLASE BY BOTH AMYLIN AND CGRP IN VERY DIFFERENT FASHIONS

Inhibitors (antagonists) purporting to show selective actions have often been used in attempts to identify multiple enzyme and receptor forms. However, the design of the experiments used to address these issues needs careful evaluation. The critical issue is that inhibition be assessed on a "level playing field": that is, when different substrates (agonists) are used, it is crucial to analyse inhibition at concentrations that are not necessarily identical in absolute levels but that are identical as regards their value relative to that of the K_m for the substrate or the K_a for activation. Thus, equal relative concentrations of substrates (agonists) must be used. Failure to do so will lead to the appearance of apparent selectivity and the possibly erroneous conclusion that multiple enzymes (receptors) exist able to bind substrates (agonists) selectively [see Houslay et al., 1974].

Taking this into account, a degree of caution must be taken as regards the conclusion drawn by Deems et al. [1991b] that CGRP and amylin function through distinct receptors in soleus muscle. This is because whereas they looked for, and found, differences in the ability of CGRP(8–37) to inhibit glycogen metabolism when it was stimulated using equal concentrations of either amylin or CGRP as agonists. For their conclusion that separate receptors for these two peptides exist on soleus muscle to be correct, both CGRP and amylin would have to exhibit identical EC_{50} values to stimulate glycogen metabolism, which does not seem to be the case. Thus, we would maintain that the issue as to whether amylin and CGRP act through separate or the same receptor in soleus muscle is still an open one.

Here then we evaluated the action of the competitive CGRP antagonist, CGRP(8–37) to inhibit both the CGRP- and amylin-stimulated adenylate cyclase activities in hepatocyte membranes by determining inhibition occurring at concentrations of CGRP and amylin, which reflect levels of these agonists where half maximal activation ensued, i.e., at their [EC_{50}]. Under such conditions, CGRP(8–37) caused the dose-dependent inhibition of both amylin and CGRP-stimulated adenylate cyclase activities (Fig. 4). However, considerably higher concentrations of CGRP(8–37) were required to inhibit CGRP-stimulated adenylate cyclase activity (IC_{50} ~ 119 nM) compared to that seen when amylin was used as the stimulatory agonist (IC_{50} ~ 3 nM).

When amylin, the agonist that yielded simple saturation kinetics for stimulation, was used (Fig. 4) in such experiments with CGRP(8–37), we found an anomalous dose dependency for antagonist action, with CGRP(8–37) requiring around four orders of magnitude increase in concentration to complete inhibition (h ~0.2). By contrast, for CGRP-stimulated activity, CGRP(8–37) showed inhibition obeying simple saturation kinetics (h ~1.0).

The action of this antagonist was specific in that at high concentrations (1 μM), CGRP(8–37) had no effect (<5%) on the stimulation of adenylate cyclase by 100 nM glucagon, which elicited a ~14.2-fold increase in adenylate cyclase activity.

MODELS FOR THE ACTIVATION OF ADENYLATE CYCLASE BY AMYLIN AND CGRP

The kinetics of activation of adenylate cyclase by CGRP are clearly aberrant. They could, however, be reconciled either by envisaging two distinct receptor populations having different affinities for CGRP or one homogenous receptor population showing apparent negative cooperativity. As none of the many G-protein linked receptors that have been identified have multiple binding sites for agonists [Houslay, 1992], it is unlikely that this will be the case for CGRP. Criteria for negative cooperativity could be met then by a single receptor type expressing a single site for CGRP but that interacted to form functional aggregates. Alternatively, negative cooperativity could be attained by a single receptor type with a single agonist site but which functioned through a mnemonical mechanism. Whichever of these models is correct, it must take into account the fact that amylin displays normal saturation kinetics for adenylate cyclase activation but aberrant kinetics of inhibition by the antagonist CGRP(8–37). We believe that this would most likely seem to be satisfied through a model in which both agonists bind to a common CGRP receptor.

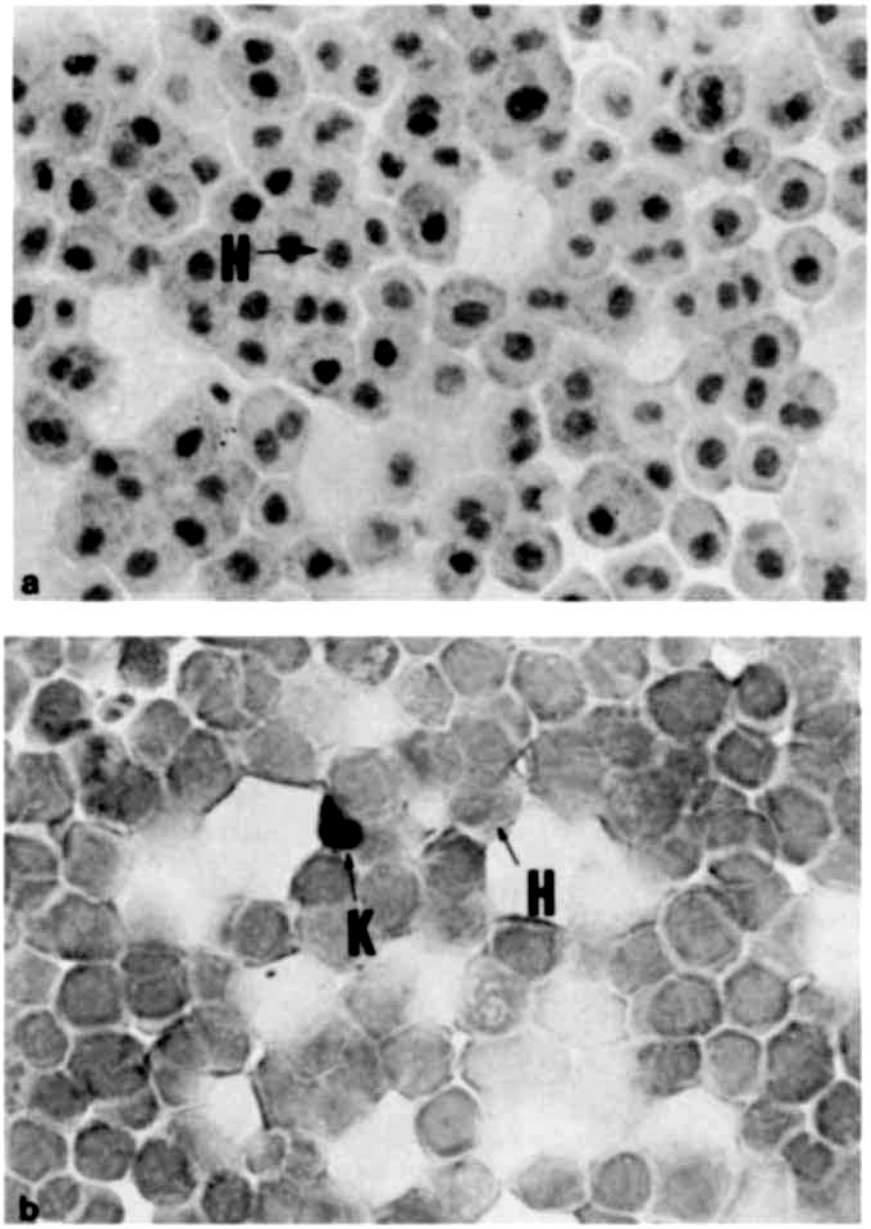

Fig. 2. Purity of hepatocyte preparations. **a:** Staining of hepatocyte nuclei by anti LFB-1 antibody. Use of an antiserum to the parenchymal cell (hepatocyte)-specific transcription factor LFB-1 shows that >99% of the cells in our preparations have nuclei that are recognized by this antiserum. Similar data can be obtained while activity staining using the parenchymal cell-specific marker (in liver) enzyme glucose-6-phosphatase. **b:** Staining of Kupfer cells using an anti-ED-1 antibody. Use of an antiserum to the Kupffer cell surface marker protein ED1 shows contamination of <1% with these cells.

Negative Cooperativity: Functional Aggregates

Agonist binding to both β-adrenoceptors [Limbird and Lefkowitz, 1976] and to glucagon receptors [Sonne et al., 1978] has been shown to indicate apparent negative cooperativity. The molecular basis of such kinetic phenomena, as regards these G-protein linked receptors, remains to be ascertained. However, it is possible that functional aggregates of these receptors can be formed based upon analyses done using fluorescent probes with β-adrenoceptors [Henis et al., 1982] and from irradiation inactivation studies done with glucagon receptors either in situ [Houslay et al., 1977] or in solubilized material [Herberg et al., 1984]. Envisaging a model of negative cooperativity where a common receptor accounts for the action of both amylin and CGRP on adenylate cyclase activation, we would

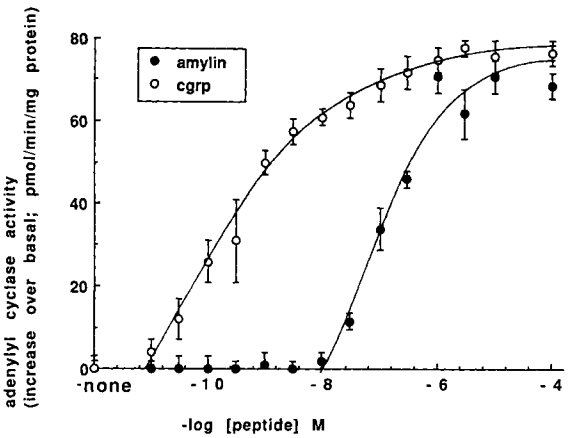

Fig. 3. Amylin and CGRP activate hepatocyte adenylate cyclase activity to the same extent but show very different kinetics of activation. This shows that amylin activates adenylate cyclase activity in membrane fractions through simple saturation kinetics whereas CGRP shows apparent negative cooperativity. (Data adapted from Bushfield et al. [1993] with permission of The Biochemical Society and Portland Press.)

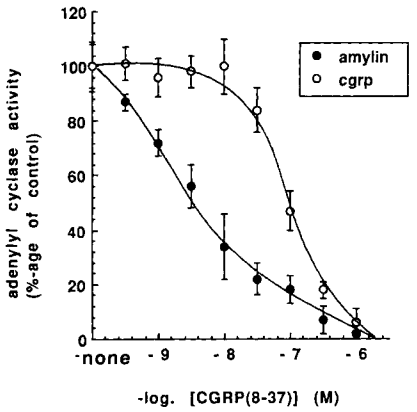

Fig. 4. CGRP(8–37) acts as an antagonist against the stimulatory action of both CGRP and amylin on adenylate cyclase but shows very different kinetic properties in each instance. This shows that CGRP(8–37) exhibits simple saturation kinetics against CGRP but those of negative cooperativity against amylin. (Data adapted from Bushfield et al. [1993] with permission of The Biochemical Society and Portland Press.)

expect to find two or more interacting sites. These could either be provided by a single receptor or by the formation of functional aggregates of receptors having a single agonist binding site. As the binding amylin and CGRP has been shown to be influenced by GTP analogues [Bhogal et al., 1992] and G-protein linked receptors fall into a structurally related family in which members have but a single binding site [Houslay, 1992], we would suggest that it would be extremely unlikely that the CGRP/amylin receptor showed multiple agonist binding sites. One

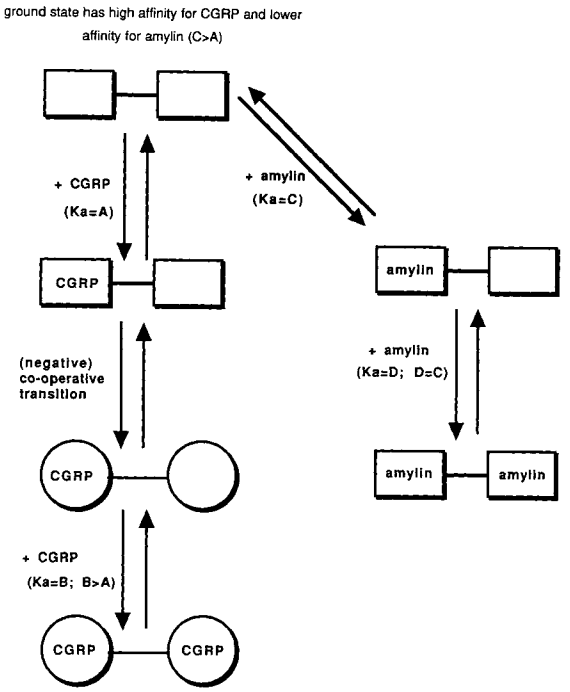

Fig. 5. Negative cooperativity: model envisaging functional aggregates of receptors. This, for simplicity, shows a dimer but, of course, the functional aggregates could be larger. Only CGRP, and not amylin, elicits the cooperative transition. (Adapted from Bushfield et al. [1993] with permission of The Biochemical Society and Portland Press.)

possibility is that the receptor forms functional aggregates of a complex of units which each can bind but a single molecule of agonist.

Such a model is shown in Figure 5 where, in the "resting" state (squares), the receptor would take up a "high-affinity state" for CGRP. The binding of CGRP to the site on one receptor molecule in the aggregate would engender a conformational change (shown as a shift from squares to circles) whose action was to lower the affinity of CGRP to bind to sites on associated receptor molecules in the complex, thus providing kinetics indicative of negative cooperativity. Schematically this is shown (Fig. 5) for simplicity as a complex formed between two receptor molecules only. Amylin binding would not trigger such a conformational change in the complex and would merely bind with low affinity to the receptor species. In this model, then for CGRP binding we would suggest that both agonist-bound conformational states of the receptor (squares and circles) would be capable of activating adenylate cyclase.

The action of the antagonist CGRP(8–37) would then have to, like CGRP itself, induce the conformational change to a "low-affinity" state,

indicative of negative cooperativity. However, in its case, antagonist-bound receptor would have a specifically distinct conformation (squares* and circles*; not shown in the scheme) that would not be capable of stimulating adenylate cyclase.

We can then make predictions about the kinetics expected for CGRP(8–37) inhibition, when the agonists are present at half-maximally activating concentrations. With CGRP, the receptor would be, essentially, in the "low-affinity" state (circles); thus, the antagonist addition should show normal inhibition kinetics as it would engender a functionally inactive "low-affinity" state. Amylin, being unable to trigger the negative cooperative transition would leave the receptor in the "resting," "high-affinity" state (squares) as regards the binding of either (8–37)CGRP or CGRP itself. Thus dose-dependent increases in CGRP(8–37) concentration would cause the receptor to take up a "lower-affinity" state (circle*), yielding kinetics of negative cooperativity (Fig. 3).

Negative Cooperativity: Mnemonical Mechanism

Analysis of the kinetics of a number of homogeneous enzyme preparations in which the protein clearly existed in a monomeric state and exhibited but a single substrate binding site, showed evidence of apparent negative cooperativity [Meunier et al., 1974; Storer and Cornish-Bowden, 1977; Marchmont et al., 1981]. Such systems clearly could not be accommodated by "traditional" theories of negative cooperativity involving either multiple interacting substrate sites upon a single protein or functionally interacting aggregates. However, Ricard et al. [1974] developed a rather simple and elegant concept, called a "mnemonical mechanism" that not only could explain such kinetics of apparent negative cooperativity in a monomeric enzyme possessing a single substrate binding site but would also explain situations where positive cooperativity occur. Such a mechanism can provide explanations for the apparently bizarre discrepancies of action of the agonists amylin and CGRP as well as the antagonist (8–37)CGRP. We would also suggest that such a mechanism may in fact be typical of all G-protein linked receptors and could explain the variety of anomalous kinetic observations that have been made over the years. Furthermore, it would provide a ready explanation for the recent observations that unoccupied receptors under certain circumstances, for example, when overexpressed, can in fact lead to the triggering of a response [Adie and Milligan, 1993; Schultz and Freissmuth, 1992]. Thus, a "mnemonical mechanism" does not place the structural constraint for aggregation of functional units to occur in this system in order to account for the observed apparent negative cooperativity.

So what defines a mnemonical system? The core of the argument centres around two proposals: (1) here, the receptor should exist in two conformational distinct states (circles and octagons) that are in equilibrium in the absence of any agonist (Fig. 6). Importantly, each of these is able to bind agonist but when they do they both change their conformation to yield a single common form of the occupied receptor (square). In the case of an enzyme this would be equivalent to forming an enzyme–substrate complex (ES). The next stage is crucial, and was termed by Ricard et al. [1974] as the "mnemonical change," in that this complex underwent isomerisation to form a product-bound complex whose conformation reflected that of one of the forms of the enzyme in the resting (ground) state. The subsequent release of product would then drive the reaction forward in an essentially irreversible fashion under initial rate conditions and release free enzyme in a conformation that reflected this one form of the enzyme in the ground state. While equilibration between the two ground state conformations will occur, clearly the speed at which this happens will depend upon the rate constants which govern this reaction. However, it is clear that as substrate levels rise and reaction rates increase, proportionately more of one of the forms of the enzyme will be available to bind substrate. In other words, a "memory" is built into the system which is the essence of the mnemonical event. If the two conformationally distinct forms of the enzyme available have different affinities for substrate, the proportion of high/low-affinity enzyme available will alter, as does the substrate conformation, i.e., depending on the system then either negative or positive cooperativity can be observed. If a substrate binds equally well or similarly to both forms, apparently normal kinetics will ensue.

In the case examined here, we would suggest that it is CGRP that shows different affinities for the two conformations of the receptor, whereas amylin does not. We can now develop these arguments for the two agonists, with that for the antagonist (8–37)CGRP being identical to CGRP except that the occupied states are incapable of activating G_s (Fig. 6). Binding of

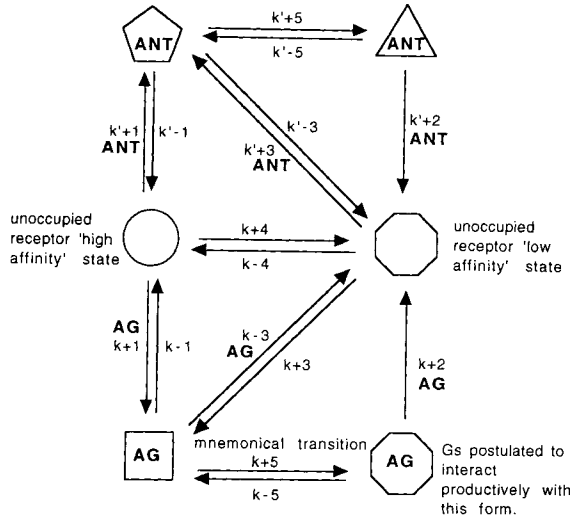

Fig. 6. Negative cooperativity: mnemonical model. This has at its basis the concept of two states of the receptor being in equilibrium in the absence of agonist and the mnemonical transition being characterised by a change in conformation of the agonist bound receptor to adopt that of one of the original forms. For negative cooperativity this form would be the low-affinity species. The G-protein G_s would presumably bind to this latter agonist bound form. (Adapted from Bushfield et al. [1993] with permission of The Biochemical Society and Portland Press.)

CGRP to either form of the receptor then yields an occupied state of common conformation which then undergoes a "mnemonical transition" that allows it to adopt a conformation that reflects that of one of the original forms of the receptor when it was in the unoccupied, "ground" state (octagon). We would suggest that it is this form of the agonist occupied receptor that can interact with the stimulatory G-protein G_s and hence to the activation of adenylate cyclase. The subsequent step, leading to the release of agonist, yielding the free form of the receptor in one particular ground state conformation (Fig. 6) can thus appear as a quasi-irreversible step, reminiscent of the release of product from an enzyme under initial rate conditions, as we would expect GTP hydrolysis, by G_s, to drive this "turn-off" event.

Kinetics, then, indicative of negative cooperativity, can then be expected to be observed under conditions in which the "remembered" or "mnemonical" conformation of the receptor, i.e., that adopted by the form proposed here to interact with G_s, is of lower affinity than the species with which it is in equilibrium. Formally, analysis of the rate equations for this system shows that the curvature of such a plot will be defined by the second derivative [Bushfield et al., 1993]; from this it can readily be seen that kinetics of negative cooperativity will be displayed if the value of the rate constant is $k_1 > k_3$. This has the effect of providing interconvertable "high-affinity" (circle) and "low-affinity" (octagon) forms of the receptor (Fig. 6). The core of the "mnemonical transition" displaying apparent negative cooperativity is a system driven through a final, common stage that involves the release of agonist (product) from the "low-affinity" (octagon) form of the receptor (enzyme). Thus, after release of agonist, the concentration of the "low-affinity" (octagon) receptor species will be disproportionately higher due to the finite time for re-equilibration to occur between the "low"- and the "high"-affinity (circle) states. In such a fashion, the system will be poised to "remember" [Ricard et al., 1974] the "low-affinity" state. This effect will become more pronounced as concentrations of agonist become higher, leading to kinetics of apparent negative cooperativity. This is presumed to be the situation with CGRP as agonist.

In the case of amylin, presumably, it can bind to both conformations of the receptor in the ground state but these show similar affinities for this agonist, hence normal saturation kinetics ensue. Nevertheless, it is tied into a system that can show aberrant kinetics, most dramatically exemplified by experiments using the antagonist (8–37) CGRP, which clearly triggers a mnemonical transition and shows aberrant inhibition kinetics with amylin as agonist (vide infra). Thus, analyses done with this antagonist perhaps provide the most striking evidence for a common amylin/CGRP receptor in this membrane system, which obeys this particular kinetic model.

Inhibition With the Antagonist CGRP(8–37)

CGRP(8–37) is a truncated form of CGRP that can act as an antagonist of it's ability to stimulate adenylate cyclase. Using hepatocyte membranes, we can see that it will inhibit not only the ability of CGRP to stimulate adenylate cyclase, but also that of amylin (Fig. 7). The antagonist CGRP(8–37) can be expected to act in a parallel fashion to CGRP, either eliciting negative cooperativity by promoting a "high"- to "low"-affinity transition between function aggregates or by triggering a mnemonical event leading to apparent negative cooperativity ($k'_1 > k'_3$). The difference between its action and that of CGRP would be that antagonist occupied receptor would be unable to activate G_s. Thus, both the "mnemonical" and "functional

aggregate" models would yield symmetrical kinetic schemes to those for CGRP.

Such systems would then account for the fact that the antagonist CGRP(8–37) showed simple saturation kinetics for inhibition of CGRP-stimulated adenylate cyclase under conditions in which agonist (CGRP) was present at concentrations that half-maximally activated the enzyme (Fig. 4). In other words, at such concentrations of agonist, the receptor would now essentially be functioning as a low-affinity system, thus, the presence of antagonist would merely compete this out in a simple competitive fashion while itself perpetuating the low-affinity state.

In marked contrast, with amylin as agonist, in the functional aggregate model the receptor would not have undergone a negative cooperative transition to a "low-affinity" state as regards CGRP (Fig. 5). Thus, the binding of CGRP(8–37) would progressively engender such a state and lead to inhibition kinetics following those of negative cooperativity (Figs. 4, 5). With the mnemonical model, the presence of increasing antagonist concentration would proportionately increase the amount of low-affinity receptor and again yield inhibition kinetics of apparent negative cooperativity (Fig. 6).

Apparent Negative Cooperativity: Elimination of a Two-Receptor Model

Considering the hypothesis that the kinetics of CGRP activation of adenylate cyclase indicated the presence of multiple receptor populations, one could attempt to resolve the CGRP activation data to account for this. Such a model could be accommondated by considering the presence of two populations of receptors, namely one with a high-affinity receptor for CGRP (EC_{50} ~ 0.1 nM) and one with a low affinity for CGRP (EC_{50} ~ 100 nM). A number of investigators [Morishita et al., 1990; Chantry et al., 1991; Galeazza et al., 1991] have demonstrated that amylin can displace all specifically bound CGRP from liver membranes. On this basis, one would envisage that amylin would to both putative receptor species with a similar low affinity, hence exhibiting simple saturation kinetics for the activation of adenylate cyclase. CGRP(8–37) can apparently displace all radiolabeled CGRP from liver membranes with an EC_{50} or ~ 40 nM [Chiba et al., 1989]. Under such an hypothesis, our analyses using the antagonist CGRP(8–37) were done at a concentration of CGRP at a level (0.3 nM) that predominantly the functioning of the putative "high-affinity" CGRP receptor would have been analysed. For this situation, we would then envisage that CGRP(8–37) would, as it does, show normal saturation inhibition kinetics. This agonist concentration of is a little larger than the presumed EC_{50} for the high-affinity receptor, suggesting that the IC_{50} value for CGRP(8–37) that we might be expected to record for the antagonist would be higher than 40 nM, which might account for the value of ~ 120 nM that we noted.

In this model, amylin is suggested to activate both receptor populations with similar EC_{50} values; this would imply that if CGRP(8–37), as CGRP, had very different affinities for the two putative receptors, a two-component dose–effect curve might result. An essential requirement of this two receptor model is that amylin must bind to both the putative "low" and "high"-affinity CGRP receptors and activate adenylate cyclase. If this was not the case, a monocomponent plot for CGRP(8–37) inhibition of amylin-stimulated adenylate cyclase would not have resulted. Thus, our data would indicate that for the system studied here, it would be impossible to have a two-receptor model in which amylin and CGRP do not bind to both receptor species.

Experimentally, however, we can show that it is possible to exclude such a two-receptor model. This can be done by analysing the action of the antagonist CGRP(8–37) at concentrations of agonist that are below those at which half-maximal activation are achieved ([EC_{50}]). On the basis of the two-receptor model propounded above, it would be predicted that normal saturation inhibition kinetics would always be evident with CGRP as an agonist, even when CGRP concentrations were very much below this value. This is because under all such circumstances the functioning of the putative "high-affinity" receptor would predominate. However, this situation is in dramatic contrast to either the mnemonical or functional aggregate models proposed. For example, with the functional aggregate model, when concentrations of CGRP were insufficient to sustain the receptor in the lower-affinity state, that is as they fall below the EC_{50} concentration, a stage will be reached when a fraction of high-affinity state receptors accrues. When this situation occurs, the addition of CGRP(8–37) will now trigger the negative cooperative transition, causing a change in inhibition characteristics from those of simple saturation kinetics to those of apparent negative cooperativity. This, in fact, is precisely what we observe (Fig. 7a), thus

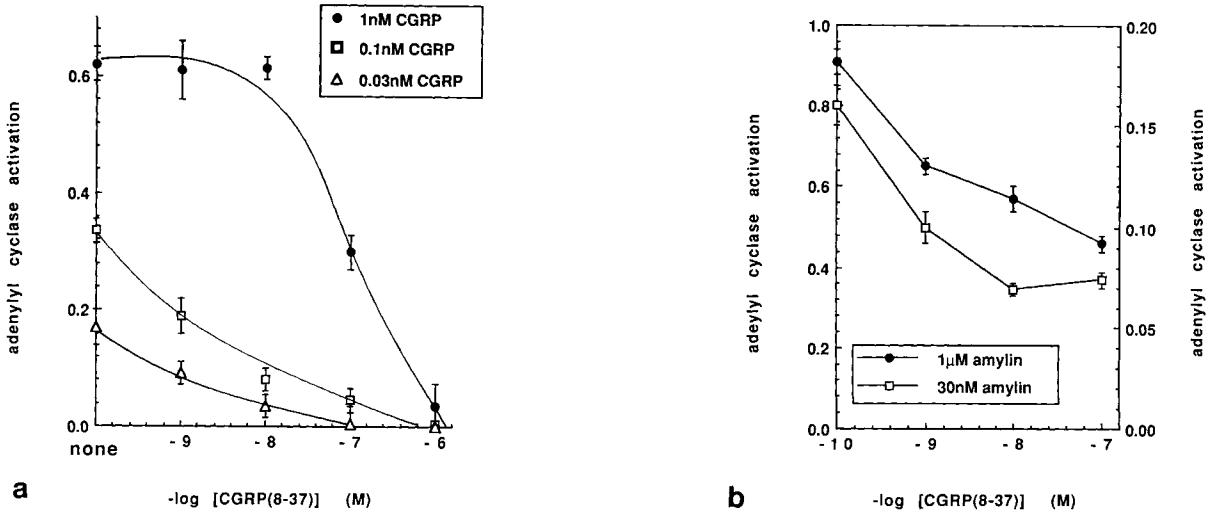

Fig. 7. At concentrations of CGRP below those effecting half-maximal concentration, the simple saturation inhibitory kinetics of CGRP(8–37) action change to those of negative cooperativity. **a:** With CGRP as agonist. **b:** With amylin as agonist. (Adapted from Bushfield et al. [1993] with permission of The Biochemical Society and Portland Press.)

refuting the two receptor model. A similar change in the characteristics of inhibition by CGRP(8–37) would also be predicted for a system obeying the mnemonical model. In this instance, when agonist concentrations are well below the EC_{50} concentration, the system would function primarily in the "high-affinity" state, but this would change with the addition of more antagonist, as this would engender an increase in the amount of the "low-affinity" receptor state relative to that of the "high-affinity" state.

Such experiments showed that reducing CGRP concentrations to a level (0.1 nM) that was about three times below its EC_{50} value led to the dramatic appearance of apparent negative cooperative inhibition kinetics (h ~ 0.2). Indeed, such an action was magnified even more (h ~ 0.1) when CGRP concentrations dropped further to 0.03 nM, a value some 10-fold below the EC_{50} value (Fig. 7a).

This change in kinetics of inhibition, which leads to the production of a transition in which enhanced sensitivity to the action of the antagonist CGRP(8–37) occurs at CGRP concentrations below the EC_{50} value, can be observed from another perspective. This can be done by examining the dose-dependent activation of adenylate cyclase by CGRP in both the presence and the absence of 10 nM CGRP(8–37). Such analyses [Bushfield et al., 1993], instead of yielding a simple "right-shifted" parallel curve for dose responses done in the presence of antagonist, in fact identify an abrupt transition that occurs at a concentration of agonist just below that at which half-maximal activation occurred. This shows that CGRP(8–37) can serve as a relatively more potent inhibitor at the lower CGRP concentrations because it is binding there to a "high-affinity" state of the receptor.

Such changes in the kinetics of inhibition by the antagonist CGRP(8–37) were not observed when amylin was used as an agonist (Fig. 7b). This is because, in the models expounded in the previous sections, amylin is not suggested to elicit any conformational change. Consistent with such proposals, CGRP(8–37) continued to exhibit apparent negative cooperative inhibition kinetics at concentrations of amylin both below and above (Fig. 7b) those at which this agonist attained half-maximal activation of adenylate cyclase. Furthermore, as expected, in contrast to our observations with CGRP, no abrupt change in the form of the amylin dose–effect responses were seen in the presence of the antagonist CGRP(8–37).

Negative Cooperativity: Data From Other Investigators

The two models we propose here to account for the negative cooperativity seen in this system are consistent with data obtained by Chatterjee and Fisher [1991], who studied CGRP binding in the cerebellum. On the basis of such investigations, they too proposed that the CGRP receptor existed in two interconvertable conformational states. They believed that this transformation was driven primarily through the association of the receptor with the stimulatory

G-protein G_s. However, these investigators identified a limitation of this model in that it failed to explain the presence of marked heterogeneity occurring in the dissociation kinetics of CGRP seen in the absence of added guanine nucleotides. On the basis of the mnemonical model proposed by us [Bushfield et al., 1993], a prediction would be the presence of two such interconvertible forms of the receptor even in the presence of guanine nucleotides. Furthermore, our model (Figs. 5, 6) would again predict the subsequent potentiation of the proportion of the low-affinity component in the presence of guanine nucleotides, as this would be caused by coupling to G_s. Their striking observation that kinetics of dissociation of CGRP from membranes is dramatically biphasic, showing an initial fast (low-affinity) component followed by a slow (high-affinity) component would thus be entirely explainable by our proposals that CGRP causes a concentration-dependent change in the conformation of the receptor.

That such biphasic dissociation kinetics have been recorded for the glucagon receptor [Horwitz et al., 1986], the β-adrenoceptor [Limbird and Lefkowitz, 1976] and the thyroid stimulating hormone (TSH) receptor [Kohn and Winand, 1975] suggests to us that a similar system to that detailed here may provide a mechanism which is in fact a characteristic of the family of G_s-linked receptors. In this we envisage either a monomeric species obeying a mnemonical mechanism or a system in which functional aggregates of receptors are formed. For reasons threaded through this discussion, our prejudice is to favour the mnemonical mechanism. Interestingly, this mechanism also offers an explanation for a rather bizarre phenomenon noted over recent years by a number of investigators [Schultz and Freissmuth, 1992; Adie and Milligan, 1993]. This is the phenomenon whereby apparently unoccupied receptors can activate adenylate cyclase. This is seemingly most obvious in conditions in which G_s-linked receptors are overexpressed in cells. In the mnemonical mechanism proposed, a key feature of this is that the "low-affinity" conformational state of the receptor is found under agonist-free "resting" conditions [Bushfield et al., 1993] (Fig. 6). This is by definition of the mnemonical model proposed a conformation that can interact with G_s, which is proposed to drive agonist release. One might presume, however, that even in its agonist-free condition, this "low-affinity" state would have a finite affinity for G_s and a finite ability to activate it. On this basis, as the concentration of receptors was increased in cell membranes, the levels of this state of the receptor may achieve a concentration sufficiently high to lead to significant activation of G_s. Such a situation would envisage that any circumstances that would drive the equilibrium to favor this state of the receptor could lead to receptor-mediated agonist-independent activation of adenylate cyclase. If this applies to G-protein-linked receptors as a family, one can expect to see similar observations with other effector systems.

ACTION OF AMYLIN ON OTHER SIGNALING SYSTEMS IN HEPATOCYTES

A substantial body of evidence would suggest that amylin can exert actions through signal-generating systems other than adenylate cyclase. These may be mediated by G_s activation. In this instance, such a G-protein is believed to have further bioactive properties such as have been exemplified by its α-subunit, which, in certain cell types can affect Ca^{2+} movements; there is also the possibility that βγ-subunits released from this G-protein may exert bioactive effects. Nevertheless, it is also apparent that at least certain subgroups of G-protein-linked receptors can activate more than one G-protein species and hence elicit signals through a variety of systems [Houslay, 1991, 1992]. However, few systematic analyses of possible actions of amylin on signaling reactions have been reported. This apparent silence may indicate a dearth of positive responses.

Inositol Phospholipid Metabolism

We have addressed the possibility that amylin may stimulate inositol phospholipid metabolism in hepatocytes. However, as can be seen in Figure 8, under conditions in which vasopressin gave a marked stimulation of inositol phospholipid metabolism, amylin quite clearly failed to do so.

Glucagon Desensitization as an Index of Protein Kinase C Activation

While amylin does not appear to elicit the stimulation of inositol phospholipid metabolism, it is possible that it could cause the stimulation of PKC, perhaps by stimulating other lipid-signaling pathways. This would be a potentially interesting signaling pathway for amylin to stimulate, as amylin is believed to elicit insulin resistance, and there is some evidence to

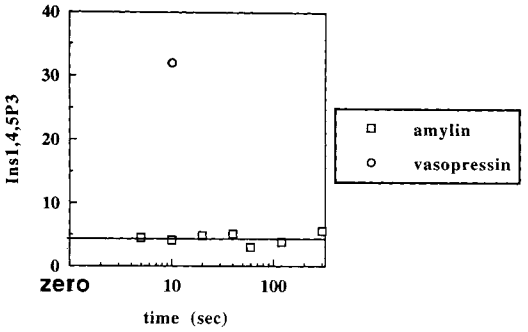

Fig. 8. Amylin fails to stimulate the production of INS1,4,5P_3 formation in hepatocytes. Hepatocye preparations and incubations were done as described by Heyworth and Houslay [1983a] with determinations of Ins1,4,5P_3 done by the binding assay method of Palmer et al. [1989]. This shows a typical experiment of one done three times using amylin (1 μM) and also with vasopressin as a positive control. Ins 1,4,5P_3 was determined using a mass assay [Palmer et al., 1989].

suggest that PKC activation could modify the insulin receptor and cause just such action [Houslay, 1991a]. Challenge of intact hepatocytes with hormones such as vasopressin and angiotensin, which stimulate inositol phospholipid metabolism, leads to the desensitization of glucagon-stimulated adenylate cyclase [Heyworth and Houslay, 1983; Houslay, 1991a]. This action can be mimicked by treatment of hepatocytes with either phorbol esters or synthetic diacylglycerol species [Newlands and Houslay, 1991], indicating that it is mediated by PKC activation. Indeed, the time course of glucagon desensitization closely follows that of an initial, rapid increase in PKC activity [Tang and Houslay, 1992]. The molecular mechanism of desensitization takes the form of an uncoupling of the glucagon receptor from G_s and is believed to be attributable to the phosphorylation of the glucagon receptor [Houslay, 1991a], rather than of G_s, which has been shown not to occur [Bushfield et al., 1990a]. Thus, the ability of a hormone (ligand) to elicit glucagon desensitization would indicate the possibility that it could activate protein kinase C in these cells. We routinely assess such a desensitization function by challenging intact cells with hormone for ~3 min, quench the cells in ice cold buffer, disrupt the cells under hypotonic conditions, and prepare a washed membrane fraction for assay of glucagon-stimulated adenylate cyclase activity [Heyworth and Houslay, 1983a]. However, under conditions in which vasopressin achieved the desensitization of glucagon-stimulated adenylate cyclase identified by a reduction in the action of glucagon of ~50%, there was no change (<5%) elicited by amylin over a wide range of concentrations. To state unequivocally that amylin does not activate PKC in these cells would be improper, as there are a range of PKC isoenzymes found in hepatocytes (M.D. Houslay, unpublished observations), and we do not know whether all or just certain forms actually mediate glucagon desensitization. However, it would seem that amylin cannot activate the PKC isoform(s) responsible for glucagon desensitization.

Amylin Elicits Phosphorylation of G_i-2 and Negates the Inhibitory Effect That Insulin Exerts on Phosphorylation of This G-Protein

α-G_i-2, in hepatocytes, is partially phosphorylated under basal conditions [Rothenberg and Kahn, 1988; Pyne et al., 1989; Bushfield et al., 1990]; this is markedly increased upon challenge of the cells with the phorbol ester TPA or with hormones that stimulate inositol phospholipid metabolism, such as angiotensin and vasopressin [Pyne et al., 1989; Bushfield et al., 1990a,b]. These effects occur in a nonadditive fashion, indicating that they are mediated by the action of PKC. Immunoprecipitation experiments with various antisera showed that whereas α-G_i-2 was modified, there was no labeling of either α-G_i-3 or α-G_s and, furthermore, it was not due to α-G_i-1 as it is not expressed in hepatocytes. That a fraction of α-G_i-2 was found to be phosphorylated under basal conditions, i.e., in the absence of any hormone, and that treatment of cells with the protein phosphatase inhibitor okadaic acid led to a rapid increase in the labeling of this G-protein indicates that an active phosphorylation/dephosphorylation cycle may be ensuing [Houslay, 1991a] (Fig. 9). Thus, under basal conditions, there was some 0.3 mol ^{32}P incorporated per mol of α-G_i-2 that was increased to >1 mol ^{32}P/mol. α-G_i-2 after challenge of intact cells with able to stimulate PKC. In all these cases, only phosphoserine was observed as the labeled amino acid in the immunoprecipitated G_i-2.

Phosphorylation of α-G_i-2 in intact hepatocytes was found to correlate well with the loss of ability of GTP itself to mediate an inhibitory effect on adenylate cyclase [Bushfield et al., 1990a,b]. This led to the formulation of a hypothesis that proposed that the phosphorylation of α-G_i-2 ablates the "tonic" inhibition of adenylate cyclase that occurs in cells due to the high levels of GTP (~500–700 μM) present [see Houslay, 1991a,b] (Fig. 9). Apparently, it does not

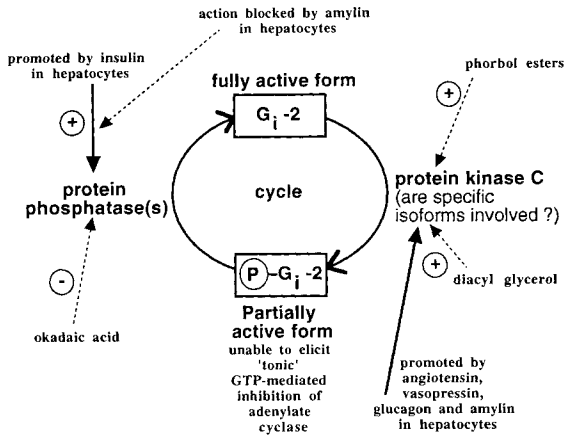

Fig. 9. Scheme for the regulation of GI-2 function in hepatocytes. Amylin can elicit the phosphorylation of this inhibitory G-Protein and prevent insulin from reducing the level of phosphorylation of GI-2.

lead to any loss of receptor-mediated inhibitory action mediated through this system. Presumably, in this instance, the physical coupling of an inhibitory receptor to α-G_i-2 engenders such a powerful conformational change in this G-protein that it overcomes the action of phosphorylation.

We have recently observed that, in hepatocytes, insulin can markedly lower the degree of phosphorylation of G_i-2 effected through PKC (N.J. Morris and M.D. Houslay, unpublished observations). As this action is abolished in the presence of the protein phosphatase inhibitor, okadaic acid, it is possible that such an effect reflects augmented protein phosphatase activity occurring in hepatocytes subsequent to insulin challenge. This insulin-mediated increase in the level of active, dephosphorylated G_i-2 might be expected to lead to a small increase in the tonic GTP-mediated inhibition of adenylate cyclase. As such, insulin could be seen to cause a small inhibition of adenylate cyclase activity in intact hepatocytes, which has been recorded [e.g., Heyworth and Houslay, 1983b; Heyworth et al., 1986]. This observation may offer an explanation for the confusion that has existed in the literature over many years concerning the ability or not of insulin to inhibit adenylate cyclase activity [see Houslay, 1986]. Such a model would thus explain that (1) the process of adenylate cyclase inhibition by insulin is apparently blocked by pertussis toxin yet the insulin receptor is not a member of the G-protein linked family, and (2) the insulin-mediated inhibition of adenylate cyclase is only seen in certain cell types and, even then, under highly defined conditions, as has been reported for G_i-2 phosphorylation [Houslay, 1991a]. This is presumably because on this basis there would be an absolute requirement for phosphorylated G_i-2 to exist under basal conditions in order for any insulin-mediated decrease in phosphorylated G_i-2, therefore an increase in adenylate cyclase inhibition, to occur. Similarly, the concentration of dephosphorylated G_i-2 would not have to be at such a level that any further increase would fail to lead to increased adenylate cyclase inhibition. It is also possible that various reports of successes and failures to observe insulin inhibition of adenylate cyclase in membrane preparations may be attributable to different preparative procedures. This is because for such a function to ensue then associated phosphatase and coupling systems interacting with the insulin receptor would be required. It is easy to envisage that these are capable of being displaced during certain membrane preparations as would be a reduction in the amount of phosphorylated G_i-2. Thus, insulin would seem to be able to alter the status of the ratio of phosphorylated/dephosphorylated G_i-2 in hepatocytes and hence may achieve a small alteration in the activity state of adenylate cyclase.

Intriguingly, we have recently noted that when intact hepatocytes are challenged with amylin, an increase in the level of phosphorylated G_i-2 occurs. The underlying molecular mechanism remains to be determined. We know that PKC activation can elicit such an action [Bushfield et al., 1990a,b], but so too can increases in cyclic AMP levels, although we have eliminated any direct action of protein kinase A on this G-protein [Bushfield et al., 1990a]. However, whereas insulin can decrease the basal level of phosphorylation of G_i-2 and that elevated by vasopressin, it has no power to decrease the stimulatory effect that amylin has on the phosphorylation of G_i-2. In other words, amylin has engendered an insulin-resistant state with this particular process (Fig. 9).

CONCLUSIONS

We have shown that hepatocytes contain functional amylin/CGRP receptors coupled to the activation of adenylate cyclase. The physiological significance of these receptors remains to be elucidated, especially in view of the fact that their occupancy can only lead to a net accumulation of cyclic AMP and to protein kinase A activation if PDE activity is attenuated. Further-

more, very high levels of amylin are required to achieve stimulation of this system. Nevertheless, a single, common receptor species appears to be responsible for mediating the actions of both amylin and CGRP through adenylate cyclase. This receptor shows apparent negative cooperativity for CGRP, but not amylin, and such kinetics may be provided for either by functional aggregates or by a mnemonical mechanism. Such a mnemonical/aggregate mechanism may, in fact, characterise the functioning of the entire G-protein receptor family and may also offer a molecular explanation for the observations that high concentrations of unoccupied G-protein linked receptors can stimulate G-proteins and elicit signal generation.

Amylin does not appear to stimulate inositol phosphate metabolism or to cause glucagon desensitization; if it does cause PKC activation, this must be restricted to a particular class or classes of isoforms. Amylin can, however, cause the phosphorylation of the inhibitory G-protein G_i-2 in hepatocytes and can block the ability of insulin's phosphatase-mediated reduction in G_i-2 phosphorylation, providing an example of an insulin-driven process that amylin can clearly attenuate. The mechanism whereby amylin exerts such an example of insulin resistance remains to be elucidated. However, it has been noted that amylin can apparently exert cyclic AMP-independent effects on liver parenchymal cells [Gomez-Foix et al., 1991].

ACKNOWLEDGMENTS

We thank the MRC and British Diabetic Association for providing financial support for this work.

REFERENCES

Adie EJ, Milligan G (1993): Agonist regulation of cellular levels of Gs in wild-type and transfected NG108-15 cells. Biochem Soc Trans 21:431–435.

Bhogal R, Smith DM, Bloom SR (1992): Investigation and characterisation of binding sites for amylin in rat membranes. Endocrinology 130:906–913.

Borges M, First JA, Stoward PJ, Verheyen A (1991): In Stoward PJ, Everson-Pierce AG (eds): "Histochemistry." Vol. 3. 4th Ed. New York: Churchill-Livingstone, pp 187–218.

Bushfield M, Murphy GJ, Lavan BE, Parker PJ, Hruby VJ, Milligan G, Houslay MD (1990a): Hormonal regulation of G_i-2 α-subunit phosphorylation in intact hepatocytes. Biochem J 268:449–457.

Bushfield M, Griffiths SL, Murphy GJ, Pyne NJ, Knowler JT, Milligan G, Parker PJ, Mollner S, Houslay MD (1990b): Diabetes-induced alterations in the expression, functioning and phosphorylation state of the inhibitory G-protein Gi-2. Biochem J 271:365–372.

Bushfield M, Savage A, Morris NJ, Houslay MD (1993): A mnemonical or negative co-operativity model for the activation of adenylate cyclase by a common G-proterin coupled CGRP/amylin receptor. Biochem J 293:229–236.

Chantry A, Leighton B, Day AJ (1991): Cross-reactivity of amylin with CGRP binding sites in rat liver and skeletal muscle preparations. Biochem J 277:139–143.

Chatterjee TK, Fisher RA (1991): Multiple affinity forms of the CGRP receptor in rat cerebellum. Mol Pharmacol 39:798–804.

Chiba T, Yamaguchi A, Yamatani T, Nakamura A, Moroshita T, Inui T, Fukase M, Noda T, Fujita T (1989): Calcitonin gene related peptide receptor antagonist CGRP(8–37). Am J Physiol 256:E331–E335.

Ciaraldi TP, Cooper GJS, Stolpe M (1990): In vitro effects of amylin on carbohydrate metabolsim. Diabetes 39:149A.

Conklin BR, Bourne HR (1993): Structural elements of G-α subunits which interact with Gβγ, receptors and effectors. Cell 73:631–641.

Cooper GJS, Willis AC, Clark A, Turner RC, Sim RB, Reid KBM (1987): Purification and characterisation of a peptide from amyloid-rich pancreases of type-2 diabetic patients. Proc Natl Acad Sci USA 84:8628–8632.

Deems RO, Deacon RW, Young DA (1991a): Amylin activates glycogen phosphorylase and inactivates glycogen synthase via a cAMP-independent mechanism. Biochem Biophys Res Commun 174:716–720.

Deems RO, Cardinaus F, Deacon RW, Young DA (1991b): Amylin or CGRP(8–37) fragments reverse amylin-induced inhibition of glycogen accumulation. Biochem Biophys Res Commun 181:116–120.

Dijkstra CD, Dopp EA, Joling P, Kraal G (1985): Kuppfer cell marker ED1. Immunology 54:589–599.

D'Santos CS, Gatti A, Poyner DR, Hanley MR (1992): Stimulation of adenylate cyclase by amylin in CHO-K1 cells. Mol Pharmacol 41:894–899.

Frain M, Swart G, Monaci P, Nicosia A, Stampfil S, Frank R, Cortese R (1989): The liver specific transcription factor LF-B1 contains a highly diverged homeobox region. Cell 59:145–157.

Galeazza MT, O'Brien TD, Johnson KH, Seybold VS (1991): Amylin competes for two binding sites of CGRP. Peptides 12:585–591.

Gomez-Foix AM, Rodriguez-Gil JE, Guinovart JJ (1991): Anti-insulin effects of amylin and CGRP onhepatic glycogen metabolism. Biochem J 276:607–610.

Henis YI, Hekman M, Elson EL, Helmreich EJM (1982): Lateral motion of beta receptors in membranes of cultured liver cells. Proc Natl Acad Sci USA 79:2907–2911.

Herberg JT, Codina J, Rich KA, Rojas FJ, Iyengar R (1984): The hepatic glucagon receptor. J Biol Chem 259:9285–9294.

Heyworth CM, Houslay MD (1983a): Challenge of hepatocytes by glucagon triggers a rapid modulation of adenylate cyclase activity in isolate membranes. Biochem J 214:93–98.

Heyworth CM, Houslay MD (1983b): Insulin exerts actions through a distinct species of guanine nucleotide regulatory protein: Inhibition of adenylate cyclase. Biochem J 214:547–552.

Heyworth CM, Grey A-M, Wilson SR, Hanski E, Houslay MD (1986): The action of islet activating protein (pertussis toxin) in insulin's ability to inhibit adenylate cyclase and activate cyclic AMP phosphodiesterases in hepatocytes. Biochem J 235:145–149.

Houslay MD (1986): Insulin, glucagon and the receptor-mediated control of cyclic AMP concentrations in liver (Colworth Medal Lecture). Biochem Soc Trans 14:183–193.

Houslay MD (1990): The use of selective inhibitors and computer modelling to evaluate the role of specific high affinity cAMP phosphodiesterases in the hormaonal regulation of hepatocyte intracellular cAMP concentrations. Cell Signal 2:95–98.

Houslay MD (1991a): "Crosstalk": A pivotal role for protein kinase C in modulating relationships between signal transduction pathways. Eur J Biochem 195:9–27.

Houslay MD (1991b): Gi-2 is at the centre of an active phosphorylation–dephosphorylation cycle in hepatocytes. Cell Signal 3:1–9.

Houslay MD (1992): G-protein linked receptors. Clin Endocrinol 36:525–534.

Houslay MD, Kilgour E (1990): Cyclic nucleotide phosphodiesterases in rat liver: A review of their characterisation, of their hormonal regulation by insulin and glucagon and their role in controlling intracellular cyclic AMP concentrations. In Beavo J, Houslay MD (eds): "Cyclic Nucleotide Phosphodiesterases." Vol. 2: "Molecular Pharmacology of Cell Regulation." London: John Wiley & Sons, pp 185–224.

Houslay MD, Barrett NJ, Tipton KF (1974): Mixed substrate experiments with human brain monoamine oxidase. Biochem Pharmacol 23:1937–1944.

Houslay MD, Dipple I, Elliott KRF (1980): A collision coupling mechanism for glucagon receptor activation of adenylate cyclase. Biochem J 186:649–658.

Houslay MD, Ellory JC, Smith GA, Hesketh TR, Stein JK, Warren GB, Metcalfe JC (1977): Exchange of partners in glucagon receptor–adenylate cyclase complexes: Physical evidence for the independent mobile receptor model. Biochim Biophys Acta 467:208–219.

Irvine F, Pyne NJ, Houslay MD (1986): The phorbol ester TPA inhibits cyclic AMP phosphodiesterase activity in intact hepatocytes. FEBS Lett 208:455–459.

Jacobowitz O, Chen J, Premont RT, Iyengar R (1993): Stimulation of specific types of Gs-stimulated adenylate cyclase by probol ester treatment. J Biol Chem 268:3829–3832.

Kanatsuka A, Makino H, Ohsawa GT, Tokuyama Y, Yamaguchi T, Yoshida S, Adachi M (1989): Amylin is co-secreted with insulin from islet cells. FEBS Lett 259:199–201.

Krupinski J, Lehman TC, Frankenfeld CD, Zwaagstra JC, Watson PA (1992): Molecular diversity of the adenylate cyclase family. J Biol Chem 267:24858–24862.

Leffert JD, Newgard CB, Okamoto H, Milburn JL, Luskey KL (1989): Rat amylin. Proc Natl Acad Sci USA 86:3127–3130.

Leighton B, Cooper GJS (1988): Pancreatic amylin and CGRP cause resistance to insulin in skeletal muscle in vitro. Nature 335:632–635.

Leighton B, Cooper GJS (1990): The role of amylin in the insulin-resistance of NIDDM. TIBS 15:295–299.

Leighton B, Foot EA, Cooper GJS, King JM (1989): CGRP-1 is a potent regulator of glycogen metabolism in rat skeletal muscle. FEBS Lett 249:357–361.

Limbird LE, Lefkowitz RJ (1976): Negative co-operativity among the beta-adrenergic receptors in frig erythrocyte membranes. J Biol Chem 251:5007–5014.

Marchmont RJ, Ayad SR, Houslay MD (1981): Purification and properties of the insulin-stimulated cyclic AMP phosphodiesterase from rat liver plasma membranes. Biochem J 195:642–652.

Meunier JC, Buc B, Navarro A, Ricard J (1974): Regulatory behavior of monomeric enzymes—A wheat-germ hexokinase as a mnemonical enzyme. Eur J Biochem 49:209–223.

Molina JM, Cooper GJS, Leighton B, Olefsky JM (1990): Induction of insulin resistance in vivo by amylin and CGRP. Diabetes 39:260–265.

Morishita T, Yamaguchi A, Fijita T, Chiba T (1990): Activation of adenylate cyclase by amylin via CGRP receptors on rat liver plasma membranes. Diabetes 39:875–877.

Newlands C, Houslay MD (1991): Treatment of intact hepatocytes with synthetic diacylglycerol mimicks the ability of glucagon to cause the desensitization of adenylate cyclase. FEBS Lett 289:129–132.

Nishi M, Sanke T, Nagamatsu S, Bell GI, Steiner DF (1990): Islet amyloid polypeptide. J Biol Chem 265:4173–4176.

Palmer S, Hughes KT, Lee DY, Wakelam MJO (1989): Development of a novel Ins1,4,5P_3 specific binding assay. Cell Signal 1:147–156.

Pilch PF, Waugh SM, O'Hare T (1989): The structural basis for insulin receptor function. Adv Regul Cell Growth 1:203–232.

Pyne NJ, Murphy GJ, Milligan G, Houslay MD (1989): Treatment of intact hepatocytes with either the phorbol ester TPA or glucagon elicits the phosphorylation and functional inactivation of the inhibitory guanine nucleotide regulatory protein G_i. FEBS Lett 243:77–82.

Richard J, Meunier JC, Buc J, (1974): Regulatory behavior of monomeric enzymes: The mnemonical enzyme concept. Eur J Biochem 49:195–208.

Rothenberg PL, Kahn R (1988): Insulin alters the ADP-ribosylation of G_i by pertussis toxin. J Biol Chem 263: 15546–15552.

Saito A, Kimura S, Goto K (1986): Stimulation of adenylate cyclase by amylin and CGRP. Am J Physiol 250, H693–H696.

Schultz G, Freissmuth M (1992): G-protein linked receptors. TIPS 13:376–380.

Sonne O, Berg T, Christofferson T (1977): Binding of iodinated glucagon and the glucagon-stimulated accummulation of cAMP in hepatocytes. J Biol Chem 253:3205–3210.

Spence S, Houslay MD (1989): The local anaesthetic benzyl alcohol attenuates the α_2-adrenoceptor-mediated inhibition of adenylate cylase when stimulated by prostaglandin E1 but not that activity stimulated by forskolin. Biochem J 264:483–488.

Stephens TW, Heath WF, Hermeling RN (1991): Presence of liver CGRP/amylin receptors in only nonparenchymal cells and the absence of direct regulation of rat liver glucose metabolism by CGRP/amylin. Diabetes 40:395–400.

Storer AC, Cornish-Bowden A (1977): Kinetic evidence for a "mnemonical" mechanism for rat liver glucokinase. Biochem J 165:61–69.

Tang EY, Houslay MD (1992): Glucagon, vasopressin and angiotensin all elicit a rapid, transient increase in hepatocyte protein kinase C activity. Biochem J 283:341–346.

Westermark P, Wernstedt C, O'Brien TD, Hayden DW, Johnson KH (1987): Amyloid fibrils in human insulinoma and islets of the diabetic cat are derived from a neuropeptide-like protein present in normal islets. Am J Pathol 127:414–417.

Yoshimura M, Cooper DMF (1993): Type-specific stimulation of adenylate cyclase by protein kinase. C. J Biol Chem 268:4604–4607.

Zhu G, Dudley DT, Saltiel AR (1991): Amylin increases cAMP formation in L6 myocytes through CGRP–related receptors. Biochem Biophys Res Commun 177:771–776.