***N*⁶-(Phenylisopropyl)adenosine prevents glucagon both blocking insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase and uncoupling hormonal stimulation of adenylate cyclase activity in hepatocytes**

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Glucagon (10 nM) prevented insulin (10 nM) from activating the plasma-membrane cyclic AMP phosphodiesterase. This effect of glucagon was abolished by either PIA [*N*⁶-(phenylisopropyl)adenosine] (100 nM) or adenosine (10 μM). Neither PIA nor adenosine exerted any effect on the plasma-membrane cyclic AMP phosphodiesterase activity either alone or in combination with glucagon. Furthermore, PIA and adenosine did not potentiate the action of insulin in activating this enzyme. 2-Deoxyadenosine (10 μM) was ineffective in mimicking the action of adenosine. The effect of PIA in preventing the blockade by glucagon of insulin's action was inhibited by low concentrations of theophylline. Half-maximal effects of PIA were elicited at around 6 nM-PIA. It is suggested that adenosine is exerting its effects on this system through an R-type receptor. This receptor does not appear to be directly coupled to adenylate cyclase, however, as PIA did not affect either the activity of adenylate cyclase or intracellular cyclic AMP concentrations. Insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase, in the presence of both glucagon and PIA, was augmented by increasing intracellular cyclic AMP concentrations with either dibutyryl cyclic AMP or the cyclic AMP phosphodiesterase inhibitor Ro-20-1724. PIA also inhibited the ability of glucagon to uncouple (desensitize) adenylate cyclase activity in intact hepatocytes. This occurred at a half-maximal concentration of around 3 μM-PIA. However, if insulin (10 nM) was also present in the incubation medium, PIA exerted its action at a much lower concentration, with a half-maximal effect occurring at around 4 nM.

There is a high-affinity cyclic AMP phosphodiesterase associated as a peripheral protein with liver plasma membranes (Marchmont & Houslay, 1980a). Both in a broken-membrane system (Marchmont & Houslay, 1980b) and in intact hepatocytes (Heyworth *et al.*, 1983b) the activity of this enzyme can be increased by insulin. Such activation is achieved by insulin triggering the cyclic AMP-dependent phosphorylation of the plasma-membrane phosphodiesterase (Marchmont & Houslay, 1980b, 1981). It has been suggested (Heyworth *et al.*, 1983a,b; Heyworth & Houslay, 1983b; Houslay & Heyworth, 1983) that insulin's

effect on this enzyme and its inhibitory action on adenylate cyclase might be mediated by a distinct species of guanine nucleotide regulatory protein.

Exposure of intact hepatocytes, but not broken membranes, to glucagon causes a rapid 'uncoupling' of hormonally stimulated adenylate cyclase activity (Heyworth & Houslay, 1983a). Similarly, pretreatment of hepatocytes with glucagon blocks insulin's ability to activate the plasma-membrane cyclic AMP phosphodiesterase (Heyworth *et al.*, 1983b). This action of glucagon in blocking a response to insulin has also been observed in adipocytes. There glucagon was seen to block insulin-stimulated glucose transport (Green, 1983a). However, such an effect was not apparent if either adenosine or its non-metabolizable analogue PIA was present in the incubations.

PIA is believed to bind specifically to R-type adenosine receptors exposed at the external surface

Abbreviation used: PIA, *N*⁶-(phenylisopropyl)-adenosine.

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of the cell plasma membrane, where their binding and action is antagonized by methylxanthines. In contrast with this, neither PIA nor methylxanthines interact with P-type adenosine receptors, which are exposed at the cytosol surface of the plasma membrane (Fain & Malbon, 1979; Cooper, 1982; Londos *et al.*, 1983).

Here we demonstrate that in intact hepatocytes both adenosine and its non-hydrolysable analogue PIA can relieve the block imposed on insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase by glucagon. They also relieve the 'uncoupling' of adenylate cyclase effected by glucagon.

Materials and methods

All methods, including the preparation of hepatocytes, incubation of hepatocytes with various ligands, Percoll fractionation of homogenates, assay of cyclic AMP phosphodiesterase and determination of intracellular cyclic AMP concentrations were precisely as described in some detail by Heyworth *et al.* (1983b).

As previously (Heyworth *et al.*, 1983b), hepatocytes were prepared from fed adult male Sprague-Dawley rats (200–300 g body wt.) by the method of Elliott *et al.* (1976). They were then preincubated at 37°C in Krebs–Henseleit (1932) buffer containing 2.5% bovine serum albumin and 2.5 mM CaCl_2 for at least 20 min before use. All subsequent incubations were performed at the same temperature.

The assessment of the degree of 'uncoupling' or desensitization of (glucagon + GTP)-stimulated adenylate cyclase activity in membranes from glucagon-treated hepatocytes was performed exactly as described by Heyworth & Houslay (1983a). Briefly, a membrane preparation was prepared from hepatocytes incubated for 5 min, as above, with glucagon (10 nM). This membrane preparation was then assayed for adenylate cyclase activity in the presence of glucagon (10 nM) + GTP (0.1 mM). Adenylate cyclase activity was obtained from linear time-courses observed over a 10 min incubation performed at 30°C. The degree of desensitization was assessed as the difference between the activities expressed between membranes from control and glucagon-treated hepatocytes.

As previously (Whetton *et al.*, 1983), intracellular cyclic AMP was determined in hepatocytes, which were centrifuged through bromododecane into a mixture of HClO_4 /sucrose. A neutralized sample was taken for the determination of cyclic AMP by the heart muscle binding-protein assay of Rubin *et al.* (1975).

All materials were from sources cited in the

papers mentioned above. Adenosine, insulin, phosphocreatine, bovine serum albumin and 2-deoxyadenosine were from Sigma Chemical Co., Poole, Dorset, U.K. All enzymes, other biochemicals and PIA were from Boehringer (U.K.) Ltd., Lewes, East Sussex, U.K. As previously, we thank Dr. W. W. Bromer of Eli Lilly and Co., Indianapolis, IN, U.S.A. for kindly giving us glucagon. (+)-4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone, known as Ro-20-1724, was kindly given by Roche Products, Welwyn Garden City, Herts., U.K.

Results

It has been demonstrated previously (Heyworth *et al.*, 1983b) that treatment of intact hepatocytes with insulin (10 nM) caused a pronounced activation of the plasma-membrane cyclic AMP phosphodiesterase (see also Table 1). As previously, this effect was blocked by pre-treating the cells with glucagon (10 nM) for 5 min before addition of insulin (Table 1). We show here, however, that if, 1 min before the addition of glucagon, adenosine (10 μM) was added to the hepatocytes, activation of the plasma-membrane cyclic AMP phosphodiesterase was indeed observed on their exposure to insulin (Table 1). The non-metabolizable analogue of adenosine, PIA, also had a similar effect (Table 1). Thus both adenosine and PIA are actually able to relieve the glucagon-mediated block on insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase. In this context we note that adenosine alone did not activate the plasma-membrane cyclic AMP phosphodiesterase; adenosine did not potentiate the ability of insulin to activate this phosphodiesterase, and did not elicit the activation of the enzyme in the presence of glucagon alone. The effect exerted by PIA probably reflects its ability to reverse, rather than to block, this action of glucagon. This is because, if PIA was added 5 min after exposure to glucagon, insulin was still able to activate the plasma-membrane cyclic AMP phosphodiesterase (Table 1).

Although the non-hydrolysable analogue of adenosine, PIA (100 nM), mimicked these actions of adenosine, 2-deoxyadenosine (10 μM) did not (Table 1). Analysis of the dose-dependency of this action of PIA (Fig. 1) showed that the concentration at which it exerted half-maximal effects (EC_{50}) was 5.6 ± 2.9 nM.

Over the range of PIA concentrations examined in Fig. 1, we did not observe any effects (A. V. Wallace & M. D. Houslay, unpublished work) on the activation of the 'dense-vesicle' cyclic AMP phosphodiesterase that we have shown to be activated in hepatocytes by both insulin and glucagon (Heyworth *et al.*, 1983b).

Table 1. Adenosine and *N*⁶-(phenylisopropyl)adenosine prevent glucagon from blocking insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase in intact hepatocytes

Results are means $\pm$ S.E.M. for three separate experiments using different hepatocyte preparations. As detailed previously (Heyworth *et al.*, 1983b), hepatocytes were prepared from fed rats and preincubated for 20 min before the start of the experiment. Incubations were performed at 70°C and terminated by rapidly cooling the cells on ice, homogenizing them and fractionating them on a Percoll gradient. Activation of the plasma membrane was assessed by expressing its activity as a percentage of that observed in a control gradient, without ligand addition, as previously. Such a control gradient was run every time. Control activity is taken as 100%.

Incubation conditions (total incubation time, min)	Activity (% of control)
10nM-insulin (5)	162 $\pm$ 7
10nM-glucagon (5)	98 $\pm$ 7
10nM-glucagon, then at 5 min 10nM-insulin (10)	105 $\pm$ 6
10 μ M-adenosine (5)	113 $\pm$ 4
10nM-PIA (5)	102 $\pm$ 6
10 μ M-2-deoxyadenosine (5)	109 $\pm$ 5
10 μ M-adenosine, then at 1 min 10nM-glucagon (6)	105 $\pm$ 3
10 μ M-adenosine, then at 1 min 10nM-insulin (6)	170 $\pm$ 5
100nM-PIA, then at 1 min 10nM-glucagon (6)	107 $\pm$ 9
100nM-PIA, then at 1 min 10nM-insulin (6)	164 $\pm$ 8
10 μ M-adenosine, then at 1 min 10nM-glucagon, then at 6 min 10nM-insulin (11)	160 $\pm$ 9
100nM-PIA, then at 1 min 10nM-glucagon, then at 6 min 10nM-insulin (11)	151 $\pm$ 2
10 μ M-2-deoxyadenosine, then at 1 min 10nM-glucagon, then at 6 min 10nM-insulin (11)	109 $\pm$ 8
100 μ M-theophylline, then at 5 min 10nM-glucagon, then at 10 min 10nM-insulin (15)	106 $\pm$ 3
100 μ M-theophylline, then at 4 min 100nM-PIA, then at 5 min 10nM-glucagon, then at 10 min 10nM-insulin (15)	109 $\pm$ 3
10 μ M-theophylline, then at 4 min 100nM-PIA, then at 5 min 10nM-glucagon, then at 10 min 10nM-insulin (15)	142 $\pm$ 7
400 μ M-Ro-20-1724, then at 10 min 100nM-PIA, then at 11 min 10nM-glucagon, then at 16 min 10nM-insulin (21)	196 $\pm$ 6
400 μ M-Ro-20-1724, then at 10 min 100nM-PIA, then at 11 min 10nM-insulin (16)	192 $\pm$ 9
100 μ M-dibutylryl cyclic AMP + 100nM-PIA + 10nM-glucagon, then at 5 min 10nM-insulin (10)	213 $\pm$ 10
10nM-glucagon, then at 4 min 100nM-PIA, then at 5 min 10nM-insulin	157 $\pm$ 17

Treatment of hepatocytes with low concentrations (100 μ M) of theophylline for up to 10 min exerted little effect (<10%) on intracellular cyclic AMP concentrations. However, theophylline (100 μ M) completely prevented PIA (100nM) from relieving the block on insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase that is imposed by glucagon (Table 1). This effect was decreased when a lower concentration (10 μ M) of theophylline was used (Table 1).

Agents that raise intracellular cyclic AMP concentrations have previously been demonstrated to augment insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase (Heyworth *et al.*, 1983b). This can be achieved either by using an agent to inhibit cyclic AMP phosphodiesterase activity in the cell or by using dibutylryl cyclic AMP (Table 1). In this instance the phosphodiesterase inhibitor Ro-20-1724 was employed. This was used rather than either theophylline or 3-isobutyl-1-methylxanthine, which, being methylxanthines, would actually prevent PIA binding to its receptor. The enhanced activation observed when these agents are used to elevate

intracellular cyclic AMP concentrations undoubtedly reflects the rapid fall in intracellular cyclic AMP concentrations that would otherwise have occurred after the addition of insulin (see Heyworth *et al.*, 1984).

We have previously demonstrated (Heyworth & Houslay, 1983a) that treatment of hepatocytes with glucagon (10nM) gives rise to a rapid 'uncoupling' of adenylate cyclase activity. The loss of the response of the adenylate cyclase activity of hepatocyte membranes, from glucagon (10nM)-treated hepatocytes, to stimulation by (glucagon + GTP) was decreased in hepatocytes incubated with PIA (100nM). This is clearly seen in the dose-effect curve (Fig. 1) which plots the degree of 'uncoupling' observed against the concentration of PIA present in the hepatocyte incubation mixture. The EC₅₀ for this effect of PIA was 3.2 $\pm$ 0.2 μ M. However, if insulin (10nM) was added during exposure of the cells to glucagon, the sensitivity of this uncoupling process to blockade by PIA was dramatically increased (Fig. 1). Under these conditions an EC₅₀ of 4.0 $\pm$ 2.1 nM for PIA was observed. Insulin itself did not affect the uncoupling process.

Fig. 1. Effect of N^6 -(phenylisopropyl)adenosine in preventing glucagon both blocking insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase and causing the desensitization of adenylate cyclase in intact hepatocytes

In studies on the activation of the cyclic AMP phosphodiesterase (■), hepatocytes, incubated at 37°C, were challenged with various concentrations of PIA for 1 min before the addition of glucagon (10 nM). After 6 min, insulin (10 nM) was added and the incubation continued for a further 5 min before the cells were harvested and analysed on Percoll gradients. For studies on the effects of PIA on glucagon desensitization a similar protocol was followed, except that in some sets of experiments insulin was absent (○), whereas in other sets (●) it was present. These data represent typical experiments.

Adenosine (10 μ M) also decreased the degree of 'uncoupling' of (glucagon + GTP)-stimulated adenylate cyclase activity in membranes from glucagon (10 nM)-treated cells by $54 \pm 9\%$ ($n = 3$; S.D. from separate experiments with different cell preparations). 2-Deoxyadenosine (10 μ M) was completely ineffective in decreasing (<8% decrease) the degree of uncoupling elicited by glucagon treatment of hepatocytes.

As shown in Fig. 2, PIA, at concentrations (100 nM) sufficient to elicit inhibitory responses in various cells (Cooper, 1982), but not to block desensitization in the absence of insulin (Fig. 1), exerted no effect whatsoever on the magnitude of the rise in intracellular cyclic AMP concentration that was elicited by glucagon (10 nM). This was also apparent when the cyclic AMP phosphodiesterase inhibitor Ro-20-1724 was present in the incubation mixture (see also Heyworth *et al.*, 1984). Similarly, in broken membranes PIA (100 nM) caused no apparent change in activity (<7%) of adenylate cyclase assayed in either the presence or the absence of glucagon, even with GTP concentrations as high as 100 μ M. [In such instances our assays of adenylate cyclase activity contained Ro-20-1724 (1 mM) rather than theophylline to block degradation of cyclic AMP.]

Fig. 2. Glucagon's elevation of intracellular cyclic AMP concentrations in hepatocytes is unaffected by the presence of N^6 -(phenylisopropyl)adenosine

Hepatocytes were challenged with glucagon (10 nM) for various periods of time before their intracellular concentration of cyclic AMP was determined (●). The change in the intracellular concentration of cyclic AMP was also determined when 100 nM-PIA was added 1 min before challenge with glucagon (○). Results of a typical experiment are shown.

Discussion

Insulin activates the peripheral-plasma-membrane cyclic AMP phosphodiesterase both in intact hepatocytes (Heyworth *et al.*, 1983b; Houslay *et al.*, 1983a,b) and in purified plasma membranes (Marchmont & Houslay, 1980b; Heyworth *et al.*, 1983a; Houslay *et al.*, 1983a,b). However, if hepatocytes were exposed first to glucagon, insulin failed to elicit any activation of the plasma-membrane cyclic AMP phosphodiesterase in intact hepatocytes (Table 1; Heyworth *et al.*, 1983b). Such an action of glucagon in blocking an effect of insulin has also been observed in adipocytes (Green, 1983b; Cushman *et al.*, 1983). In these instances glucagon was shown to inhibit insulin's stimulation of glucose transport. However, for this inhibitory effect to be observed, the adipocytes had first to be incubated with adenosine deaminase in order to remove the extracellular adenosine that accumulates in the medium

of these cells. Similarly, when PIA was added to adipocytes previously depleted of endogenous adenosine, glucagon failed to exert any inhibitory effect on insulin-stimulated glucose transport (Green, 1983a). Isoprenaline exerted similar effects to those of glucagon under these various conditions (Green, 1983a; Simpson *et al.*, 1983; Souness *et al.*, 1983). However, the effects of β -adrenergic agents on glucose transport appear to be extremely complex, involving both cyclic AMP-dependent and -independent mechanisms (see, e.g., Pessin *et al.*, 1983; Kashiwagi *et al.*, 1983).

We find here that analogous interactions between either adenosine or its non-metabolizable analogue, PIA, and glucagon and insulin can be observed. In this instance they were both shown to prevent glucagon from blocking insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase (Table 1; Fig. 1).

Adenosine presumably exerted its effects here through an R-site adenosine receptor rather than a P-site receptor. The evidence suggesting that this is the case is as follows. Firstly, 2-deoxyadenosine, a specific agonist for P-site receptors (see, e.g., Fain & Malbon, 1979; Londos *et al.*, 1983), did not mimic the action of adenosine in this system. Secondly, PIA, which is known (Fain & Malbon, 1979; Londos *et al.*, 1983) to interact specifically with the R-site receptor, exerted a potent effect on this system (Table 1; Fig. 1). Thirdly, the effect of PIA was blocked by the methylxanthine, theophylline (Table 1), which interacts with R-site, and not P-site, adenosine receptors (Fain & Malbon, 1979; Londos *et al.*, 1983). We therefore believe that the action of adenosine in relieving glucagon's block on insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase is mediated by an R-site receptor. Indeed, as PIA mimics the action of adenosine in blocking the inhibitory effect of glucagon on insulin's stimulation of the glucose-transport system in adipocytes, then presumably this effect is also exerted via an R-site receptor. This would appear to extend the similarities between these two systems.

R-site receptors expressing extremely high affinities for PIA, as is observed here, have usually been demonstrated either to inhibit or to activate adenylate cyclase activity. Here we confirm and extend, using intact-cell studies (Fig. 2), the observations by others (see Londos *et al.*, 1983) that no inhibitory R-site receptors are coupled to adenylate cyclase activity in the liver. Indeed, adenosine and PIA presumably exerted their effects here through a novel mechanism, as desensitization and the blockade of insulin's effect by glucagon are not cyclic AMP-mediated (Heyworth & Houslay, 1983a; Heyworth *et al.*, 1983b). One possibility is that these effects may be exerted through Ca^{2+}

mobilization (see, e.g., Fredholm, 1982; Endoh *et al.*, 1983).

Glucagon, as well as affecting insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase, also triggers the 'uncoupling' or desensitization of adenylate cyclase activity in hepatocytes by a process which is blocked by adenosine (Fig. 1). Such action also appeared to be mediated by an R-site receptor, as 2-deoxyadenosine was without effect on the process, and the ability of PIA to mimic adenosine was again blocked by theophylline. However, PIA appeared to prevent glucagon blocking insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase at considerably lower concentrations than those at which it was able to block glucagon desensitization (Fig. 1). This apparent anomaly was only resolved by our realization that the difference between the hepatocyte incubations in these two instances was simply the absence of insulin in the glucagon-desensitization experiments. For, although insulin did not affect glucagon desensitization itself, it increased the sensitivity of this event to PIA dramatically (Fig. 1). Under these conditions the sensitivity of both glucagon-mediated processes to PIA was very similar.

The observation that insulin can regulate the sensitivity of cellular events to adenosine is, however, not without a precedent, for Lambert & Jacquemin (1983) have demonstrated that insulin can effect a marked increase in the sensitivity of lipolysis in adipocytes to PIA. This suggests that there may well be post-receptor interactions between the insulin and adenosine effector systems.

The glucagon-mediated 'uncoupling' of adenylate cyclase has been suggested (Heyworth & Houslay, 1983a) to be due to a modification of N_s , the guanine nucleotide regulatory protein which mediates the action of stimulatory hormones on adenylate cyclase activity (Ross & Gilman, 1980). We have proposed that the effect of insulin on the liver plasma-membrane cyclic AMP phosphodiesterase and on adenylate cyclase is mediated by a specific species (N_{ins}) of guanine nucleotide regulatory protein (Heyworth *et al.*, 1983a,b; Heyworth & Houslay, 1983b; Houslay & Heyworth, 1983). This putative ' N_{ins} ' is seemingly activated by cholera toxin and is presumed to be inactivated by whatever process elicits glucagon's uncoupling of adenylate cyclase activity in hepatocytes (Heyworth *et al.*, 1983b). Our present observations that the effects of glucagon both in blocking insulin's activation of the plasma-membrane cyclic AMP phosphodiesterase and in 'uncoupling' adenylate cyclase from hormonal activation can be abolished by either adenosine or PIA reinforce our opinion that these two actions of glucagon are very

closely related and undoubtedly have a common origin. Even if the notion that this event took the form of a modification, eliciting the reversible inactivation, of both N_s and the putative ' N_{ins} ' involved in mediating certain of insulin's actions (Heyworth & Houslay, 1983a; Heyworth *et al.*, 1983b) is correct, we cannot ascertain precisely whether PIA either acted to prevent the purported modification effected by glucagon or actually aided the reversal of this process. However, the fact that insulin was seen to activate the plasma-membrane cyclic AMP phosphodiesterase when PIA was added after glucagon desensitization had been allowed to go to completion suggests that PIA might actually augment the reversal of the modification effected by glucagon (Table 1).

These investigations have uncovered a complex interplay between the functioning of adenosine, glucagon and insulin receptors. They may provide an insight into the molecular basis for the many observations (see, e.g., Schwabe *et al.*, 1975; Fain & Malbon, 1979; Green & Newsholme, 1979; Joost & Steinfelder, 1982; Souness *et al.*, 1983) where adenosine has been shown to potentiate certain of insulin's actions. Importantly, they draw analogy between the molecular mechanisms whereby insulin stimulates glucose transport in the adipocyte and activates the plasma-membrane cyclic AMP phosphodiesterase in hepatocytes. Indeed it has been suggested that insulin stimulates glucose transport through a mechanism involving at least two steps (Cushman *et al.*, 1983; Karnieli *et al.*, 1981). The first step is believed to be triggered by the internalization of the insulin receptor itself, involving a recruitment of inactive glucose carriers to the plasma membrane from an internal vesicle pool. The second step, which would involve the activation of these carriers, might then be that bearing analogy with the activation of the plasma-membrane cyclic AMP phosphodiesterase, involving a putative guanine nucleotide regulatory protein.

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