Cross-talk between glucagon- and adenosine-mediated signalling systems in rat hepatocytes: effects on cyclic AMP-phosphodiesterase activity

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The effect of adenosine analogues on glucagon-stimulated cyclic AMP accumulation in rat hepatocytes was explored. *N*⁶-Cyclopentyladenosine (CPA), 5'-*N*-ethylcarboxamidoadenosine and *N*⁶-(*R*-phenylisopropyl)adenosine inhibited in a dose-dependent manner the cyclic AMP accumulation induced by glucagon. This effect seems to be mediated through A₁ adenosine receptors. Pertussis toxin completely abolished the effect of CPA on glucagon-stimulated cyclic AMP accumulation in whole cells which suggested that a pertussis-toxin-sensitive G-protein was involved. On the other hand, this action of adenosine analogues on glucagon-induced cyclic AMP accumulation was reverted by the selective low-*K*_m cyclic AMP-phosphodiesterase inhibitor Ro 20-1724. Analysis of cyclic AMP-phosphodiesterase activity in

purified hepatocyte plasma membranes showed that glucagon in the presence of GTP inhibited basal PDE activity by 45% and that CPA reverted this inhibition in dose-dependent manner. In membranes derived from pertussis-toxin-treated rats, we observed no inhibition of cyclic AMP-phosphodiesterase activity by glucagon in the absence or presence of CPA. Our results indicate that in hepatocyte plasma membranes, stimulation of adenylate cyclase activity and inhibition of a low-*K*_m cyclic AMP phosphodiesterase activity are co-ordinately regulated by glucagon, and that A₁ adenosine receptors can inhibit glucagon-stimulated cyclic AMP accumulation by blocking glucagon's effect on phosphodiesterase activity.

INTRODUCTION

Cyclic AMP has been identified as the intracellular mediator of the action of a large number of hormones, neurotransmitters and autacoids. Its concentration within cells depends on the rate of its formation, catalysed by adenylate cyclase, and also on the rate of its degradation to AMP, by specific cyclic nucleotide phosphodiesterases (PDEs).

In rat liver, glucagon interacts with specific cell-surface receptors that activate adenylate cyclase through G_s [1]. Additionally, a glucagon fragment is responsible for inhibition of the liver calcium pump, also via G_s [2]. In contrast, adenosine receptors have been divided into A₁, A_{2a}, A_{2b} and A₃ subtypes according to the order of potency of adenosine analogues in radioligand-binding studies and functional measurements [3,4], and more recently, through molecular cloning (see [5,6] and references therein). In rat liver, the existence of A₂ adenosine receptors, coupled to adenylate cyclase in an activatory fashion via G_s, has been known for some time [3,7,8]. More recently, the presence of A₁ adenosine receptors, which inhibit adenylate cyclase activity, in liver has been suggested [9,10]. Hormone-like effects of adenosine and inosine in hepatocytes have also been observed [11,12].

There are many isoforms of cyclic nucleotide phosphodiesterase that have been divided into seven classes on the basis of substrate specificity and regulation of their activity (see [13–16] and references therein). A large number of studies have examined the regulation of low-*K*_m PDE activities because of the possible importance they might have in the regulation of cellular cyclic AMP levels [13–16]. Several reports have documented increased low-*K*_m cyclic AMP-PDE activity in response to hormones in various cell types [17–20], but in most cases the molecular mechanisms have remained unclear. One of the best characterized examples of these effects is the glucagon-induced inhibition of a

low-*K*_m cyclic AMP-PDE in particulate fractions of frog heart ventricle via a pertussis-toxin-sensitive G-protein [21].

It is well established that, in rat liver, insulin can antagonize the ability of glucagon to increase the intracellular concentration of cyclic AMP [22,23], both by increasing peripheral plasma membrane PDE activity and by exerting a small inhibitory effect on adenylate cyclase activity [24]. Interestingly, activation of PDE by insulin can be blocked by pretreating hepatocytes with glucagon, an effect that can be relieved by the presence of adenosine [25]. A 'dense-vesicle' PDE activity has also been reported that can be stimulated by glucagon and by insulin in intact hepatocytes [26]. In contrast, several studies have shown that adenosine or its analogue, *N*⁶-(*R*-phenylisopropyl)adenosine (R-PIA), inhibits glucagon-stimulated cyclic AMP accumulation both in intact and cultured hepatocytes [9,27]. The aim of the present study was to characterize the process involved in the inhibition of glucagon-stimulated cyclic AMP accumulation by adenosine in rat hepatocytes. We provide evidence that glucagon increases cyclic AMP levels in these cells by two mechanisms: activation of adenylate cyclase and inhibition of a membrane-bound low-*K*_m cyclic AMP-PDE activity. We also show that adenosine analogues reduce glucagon-mediated cyclic AMP accumulation by interfering with glucagon-mediated inhibition of PDE activity.

EXPERIMENTAL

Materials

Nucleotides, phosphocreatine, creatine kinase and 5'-nucleotidase (from *Crotalus adamanteus* venom) were from Sigma Chemical Co. *N*⁶-Cyclopentyladenosine, 5'-*N*-ethylcarboxamidoadenosine, CGS-21680 and Ro 20-1724 were from RBI. *N*⁶-(*R*-Phenylisopropyl)adenosine was from Boehringer

Abbreviations used: CPA, *N*⁶-cyclopentyladenosine; NECA, 5'-*N*-ethylcarboxamidoadenosine; PDE, phosphodiesterase; R-PIA, *N*⁶-(*R*-phenylisopropyl)adenosine.

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Mannheim. [^3H]Cyclic AMP (36.1 Ci/mmol), [α - ^{32}P]ATP (32 Ci/mmol) and [adenylate- ^{32}P]NAD $^+$ (800 Ci/mmol) were purchased from DuPont-New England Nuclear.

Experiments with isolated cells

Male Wistar rats (approx. 200–225 g) fed *ad libitum* were used. Hepatocytes were isolated by the method of Berry and Friend [28] and incubated in Krebs–Ringer bicarbonate buffer supplemented with 10 mM glucose. Cyclic AMP accumulation was determined in cells plus medium by the method of Brown et al. [29]. All determinations were performed in cells stimulated, for 2 min, with the agents indicated.

Experiments with membrane preparations

Membranes were isolated from hepatocytes by a modification [30] of the method of Loten and Redshaw-Loten [31] and used immediately. Protein was quantified by the method of Lowry et al. [32], using BSA as standard.

Treatment with pertussis toxin and ADP-ribosylation

Pertussis toxin was purified by the method of Sekura et al. [33] from pertussis vaccine concentrates generously provided by the Institute of Hygiene (Mexico). Animals were injected with pertussis toxin (40 μg per 100 g intraperitoneally) 3 days before the experiment was performed. *In vitro*, pertussis-toxin-catalysed ADP-ribosylation was done as described [34].

Low- K_m cyclic AMP-PDE assay

PDE activity in rat hepatocyte membranes was determined by a modification of the two-step procedure of Rutten et al. [35]. Briefly, the assay medium contained: 33 mM Tris, pH 7.5, 5 mM MgCl_2 , 0.1 μM cyclic AMP (containing approx. 50 000 c.p.m. [^3H]cyclic AMP), 0.02% BSA, 0.1 mg/ml trypsin inhibitor and 0.1 μM CaCl_2 in a final volume of 0.1 ml. Incubation was initiated by the addition of 30 μg of protein, continued for 5 min at 37 °C and was ended by 2 min ebullition. Tubes were then cooled to 4 °C and a second incubation step was run for 30 min at 30 °C, initiated by the addition of 5 μg of 5'-nucleotidase per tube to catalyse the conversion of the [^3H]AMP formed during the first reaction to [^3H]adenosine. Tubes were then cooled to 4 °C and diluted to 1.5 ml with ice-cold 33 mM Tris buffer, pH 8.5. Assay mixtures were applied to columns filled with 3 ml of Dowex AG1-X2 (Bio-Rad, Cl $^-$ form, 200–400 mesh, converted to OH $^-$ form according to the manufacturer's instructions) equilibrated in 33 mM Tris buffer, pH 8.5. The columns were washed with 2.5 ml of 33 mM Tris buffer, pH 8.5; the PDE reaction products were eluted with 5 ml of 0.1 M NaHCO_3 and the radioactivity in the eluates was counted. Blank values were estimated in assays boiled immediately after the addition of membrane protein and were subtracted from all data points. PDE activity was linear with respect to time and to protein concentration under the conditions employed.

RESULTS AND DISCUSSION

As mentioned above, there is evidence that adenosine and its A_1 -selective analogue, R-PIA, can inhibit glucagon-stimulated cyclic AMP accumulation in hepatocytes [9,10,27]. The precise mechanism of this action is incompletely understood. To investigate this phenomenon further, we first examined its pharmacological specificity. As shown in Figure 1, the adenosine analogues tested inhibited 1 μM glucagon-stimulated cyclic AMP accumulation in

Figure 1 Effect of adenosine analogues on glucagon-stimulated cyclic AMP accumulation

Freshly isolated rat hepatocytes were incubated for 2 min at 37 °C with 1 μM glucagon in the absence or presence of different concentrations of CPA (▲), CGS-21680 (●), R-PIA (△) or NECA (○) and cyclic AMP was determined as described in the Experimental section. The cyclic AMP concentration was 0.68 ± 0.2 and 3.64 ± 0.4 pmol per mg wet cell weight in the absence of any agent or with 1 μM glucagon, respectively. Results are expressed as a percentage of the effect of glucagon alone. Plotted are the means and vertical lines represent the S.E.M. for eight experiments using different cell preparations.

a dose-dependent manner. CPA and R-PIA decreased cyclic AMP accumulation to a greater extent than NECA and CGS-21680; CPA was consistently more potent than R-PIA (IC_{50} 30 pM and 10 nM, respectively) (Figure 1). These data suggest that the effect is mediated through A_1 adenosine receptors. Recently, Nagy and DeSilva [9] reported a very similar IC_{50} for R-PIA (19 nM) in cultured hepatocytes, and also found that this effect can be blocked by the A_1 -specific antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPDX).

In most systems studied, A_1 adenosine receptors are coupled in an inhibitory fashion to adenylate cyclase through G_i [5,6]. Therefore we next examined the role of G_i in the inhibition of glucagon-stimulated cyclic AMP accumulation by adenosine in intact cells. Bacterial toxins have proved to be useful tools for the identification of distinct G-proteins. In liver, pertussis toxin ADP-ribosylates the α subunits of G_{i2} and G_{i3} , blocking their actions [1,33]. The results in Figure 2 show that the potent inhibitory effect of CPA on glucagon-stimulated cyclic AMP accumulation was abolished by pertussis toxin. The effectiveness of the toxin treatment was monitored by examining ADP-ribosylation of substrates in membranes derived from control and toxin-treated rats. As can be seen in the inset of Figure 2, the 40–41 kDa proteins in membranes from intoxicated cells were no longer ADP-ribosylated by pertussis toxin *in vitro*, indicating that these substrates were already ADP-ribosylated *in vivo* (>98%) under the conditions employed. Thus the data clearly indicate that a pertussis-toxin-sensitive G-protein, probably a form of G_i , was involved in the inhibition of glucagon-stimulated cyclic AMP accumulation induced by activation of A_1 adenosine receptors.

All the previous experiments were performed in the absence of

Figure 2 Effect of pertussis toxin on the inhibitory action of CPA on glucagon-stimulated cyclic AMP production

Freshly isolated hepatocytes from control (○) or pertussis-toxin-treated rats (●) were incubated with $1 \mu\text{M}$ glucagon in the absence or presence of different concentrations of CPA for 2 min at 37°C ; cyclic AMP was determined as described. Cyclic AMP accumulations (in pmol per mg wet cell weight) in hepatocytes from control rats were: basal 0.53 ± 0.10 ; $1 \mu\text{M}$ glucagon 3.77 ± 0.40 ; and in cells from pertussis-toxin-treated rats: basal 0.20 ± 0.10 ; $1 \mu\text{M}$ glucagon 1.50 ± 0.40 . Results are means $\pm$ S.E.M. for eight experiments with different cell preparations. The inset shows a representative autoradiogram of pertussis-toxin-catalysed ADP-ribosylation of hepatocyte membranes derived from the control (lane 1) or pertussis-toxin-treated rats (lane 2).

any phosphodiesterase inhibitor. Interestingly, we observed that 3-isobutyl-1-methylxanthine blocks the effect of the adenosine analogues on glucagon-stimulated cyclic AMP accumulation in isolated hepatocytes (results not shown). This xanthine derivative is a promiscuous agent that antagonizes adenosine action at receptor level; it is also a potent inhibitor of PDE. Therefore, to avoid ambiguity, we tested the action of Ro 20-1724, a well-known PDE inhibitor that does not interact with adenosine receptors [36,37]. Interestingly, Ro 20-1724 blocked in a dose-dependent manner the inhibition induced by the adenosine analogues CPA and R-PIA (Figure 3). Taken together, our results suggest that not only a pertussis-toxin-sensitive G-protein but also a PDE could be involved in the cross-talk between glucagon and adenosine receptors to regulate cyclic AMP concentration.

Cyclic nucleotide phosphodiesterases constitute a multigene family of enzymes with distinct characteristics. The cyclic AMP-specific (type IV) PDEs are important regulators of intracellular signalling and are identified by their high affinity and selectivity for cyclic AMP and specific inhibition by antidepressants like rolipram and Ro 20-1724 [13].

We measured PDE activity in purified hepatocyte plasma membranes from control and pertussis-toxin-treated rats. In membranes from control animals, basal PDE activity was not

Figure 3 Effect of Ro 20-1724 on the cyclic AMP accumulation induced by glucagon

Hepatocytes were incubated with $1 \mu\text{M}$ glucagon plus $0.1 \mu\text{M}$ CPA (▲), R-PIA (△) or CGS-21680 (●) in the absence or presence of different concentrations of Ro 20-1724. Results are presented as a percentage of the effect of glucagon alone. Basal cyclic AMP accumulation was as in Figure 1 and did not change significantly in the presence of Ro 20-1724. Cyclic AMP accumulations (in pmol per mg wet cell weight) in the presence of $1 \mu\text{M}$ glucagon and the indicated concentrations of Ro 20-1724 were as follows: $0 \mu\text{M}$, 4.10 ± 0.20 ; $1 \mu\text{M}$, 6.70 ± 0.50 ; $10 \mu\text{M}$, 7.3 ± 0.60 ; $100 \mu\text{M}$, 8.10 ± 0.40 . Results are the means $\pm$ S.E.M. for triplicate determinations of nine experiments with different membrane preparations. Other indications as in Figure 1.

Figure 4 Effect of glucagon on PDE activity in purified plasma membranes

Membranes were incubated in the absence of any agent (basal 100%), with $100 \mu\text{M}$ GTP (○), or with $100 \mu\text{M}$ GTP and different concentrations of glucagon. Basal PDE activity was 11.5 ± 0.9 pmol per mg protein per min. Each point represents the mean $\pm$ S.E.M. for eight experiments performed in triplicate with different membrane preparations.

affected in the presence of $100 \mu\text{M}$ GTP ($99 \pm 5\%$ of basal activity). Interestingly, in the presence of GTP ($100 \mu\text{M}$) glucagon induced a dose-dependent decrease of PDE activity (Figure 4).

Figure 5 Effect of glucagon and CPA on PDE activity in purified plasma membranes

PDE assays were performed as described in the Experimental section with hepatocyte plasma membranes derived from (a) control or (b) pertussis-toxin-treated rats. Membranes were incubated in the absence of any agent (basal 100%), with 100 μ M GTP ($\circ$), with 1 μ M glucagon plus 100 μ M GTP in the absence ($\bullet$) or presence of different concentrations ($\blacktriangle$) of CPA. Basal PDE activities were (in pmol per mg protein per min): 12.08 ± 0.60 [membranes from control animals, (a)] and 23.20 ± 1.90 [membranes from pertussis-toxin-treated rats, (b)]. Each point represents the mean $\pm$ S.E.M. for four experiments performed in triplicate with different membrane preparations.

The maximal inhibition was approx. 40 % of basal activity in the presence of 1 μ M glucagon, and EC_{50} was approx. 3 nM (Figure 4). CPA (10 μ M) in the presence of 100 μ M GTP did not modify basal PDE activity [105 ± 3 % of basal; mean $\pm$ S.E.M.; $n = 4$ (not shown)] but was able to reverse in a dose-dependent manner the inhibition of PDE induced by 1 μ M glucagon plus GTP (Figure 5a). In plasma membranes purified from pertussis-toxin-treated rats, there was almost no inhibition of PDE activity by glucagon plus GTP in the absence or presence of different concentrations of CPA. It should be mentioned, however, that basal PDE markedly increases in membranes from pertussis-toxin-treated rats (Figure 5b).

In hepatocytes, some PDEs have been previously reported to be activated by both glucagon and insulin [1,24–26]. These include the so-called ‘dense vesicle’ enzyme, which is a type III PDE (low K_m for cyclic AMP and for cGMP, milrinone-sensitive) [1,13] that is activated by glucagon through a mechanism involving its cyclic AMP-dependent phosphorylation [26]. Another key insulin-regulated enzyme is the so-called ‘peripheral plasma membrane’ PDE type IV, which is activated by tyrosyl phosphorylation stimulated by insulin [15] and whose activation can be blocked by glucagon [25,26]. These examples of PDE activation are the best studied so far in hepatocytes, but the mechanisms of the hormonal regulation of PDE activity are still not fully understood, probably owing to the existence of numerous PDE isozymes in these cells. Moreover, there has been no previous evidence of hormonal inhibition of PDE in liver, and the unique example of this kind of regulation was reported in frog heart ventricle [21]. In this respect, it is noteworthy that we obtained a 45 % inhibition of basal low- K_m cyclic AMP-PDE activity induced by glucagon, which is very similar to that reported by Brechler et al. [21] in a frog-ventricle particulate preparation.

Our results suggest that in liver cells, A_1 adenosine receptors modulate glucagon-stimulated cyclic AMP accumulation by blocking the inhibition of PDE activity induced by the peptide hormone. This A_1 adenosine effect seems to involve a pertussis-toxin-sensitive G-protein. In many cells, activation of A_1 receptors decreases cyclic AMP accumulation. This effect is frequently due to inhibition of adenylate cyclase [3–6]. In membranes obtained from liver cells we observed little effect of A_1 agonists on adenylate cyclase activity (results not shown). However, our data clearly showed a new action, i.e. blockade of the inhibition of PDE activity induced by glucagon. Our results also imply that activation of glucagon receptors increases cyclic AMP accumulation by both stimulating adenylate cyclase and inhibiting a specific cyclic AMP-PDE. It is clear that glucagon receptors activate adenylate cyclase through G_s ; however, the mechanism of their action on PDE activity is far from clear. Our results suggest that pertussis-toxin-sensitive G-proteins could be the molecular entities through which both receptor systems cross-talk, modulating PDE activity. Reconstitution and/or molecular biological approaches seem to be required to define precisely which molecules (G-protein subunits and PDE) mediate these effects.

A fascinating aspect of the present findings is the co-ordinated hormonal modulation of cyclic AMP synthesis and degradation. Glucagon receptors seem to be interacting with more than one G-protein. This is not surprising if we consider that, in liver cells, not all glucagon actions can be easily explained solely through increases in cyclic AMP levels; glucagon activates other signalling pathways such as that involving phosphoinositide turnover and calcium mobilization [38,39]. Initially, receptors were thought to interact exclusively with a single type of G-protein; however, it is now clear that a signal can be routed from one receptor to multiple G-proteins [40,41]. In this regard, our data suggest that glucagon receptors may interact with G-proteins other than G_s , and also that interplay of G-proteins in the regulation of PDE activity could participate in the cross-talk between different signalling pathways.

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