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CORRELATION OF β_3-ADRENOCEPTOR-INDUCED ACTIVATION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE WITH ACTIVATION OF LIPOLYSIS IN RAT WHITE ADIPOCYTES

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Abstract—The lipolytic action of the β_3-adrenoceptor-selective agonist 4-[2-[(2-hydroxy-2-(3-chlorophenyl)ethyl)-amino]propyl]-phenoxyacetic acid (BRL 37344) was compared to that of isoprenaline in adipocytes derived from rat white adipose tissue. Concentration–response curves for activation of lipolysis by each agonist correlated well with the dose–response curves for activation of cAMP-dependent protein kinase (A-Kinase). Addition of propranolol at a concentration (0.1 μM) sufficient to block β_1- and β_2-adrenoceptors did not affect the stimulation of either parameter by BRL 37344 or isoprenaline, indicating that lipolysis was predominantly dependent on β_3-adrenoceptor stimulation. Blockade of β_3-adrenoceptors by 3 μM propranolol antagonized both A-Kinase activation and glycerol release. Activation of lipolysis by BRL 37344 was blocked by treatment of the cells with *N*-[2-*p*-(bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide (H89) a potent and selective isoquinolinesulphonamide inhibitor of A-Kinase activity. Taken together, these results indicate that lipolysis in rat white adipocytes is primarily controlled by β_3-adrenoceptors, and that cyclic AMP generation alone is responsible for activation of lipolysis in this tissue.

It is now generally accepted that lipolysis in rat adipocytes is mediated by β_3-adrenoceptors [1] rather than by β_1 or β_2-adrenoceptors. This classification is due to the demonstration of three "atypical" properties. First, in functional studies, both rat brown and white adipocyte β-adrenoceptors have been shown to possess a low affinity for both selective [2] and non-selective [3] β_1- and β_2-antagonists. Secondly, lipolysis can be stimulated by certain β_1/β_2-antagonists exemplified by CGP 12177 [4]. Thirdly, a novel series of β_3-adrenoceptor agonists, including 4-[2-[(2-hydroxy-2-(3-chlorophenyl)ethyl)-amino]propyl]-phenoxyacetic acid (BRL 37344‡) (Fig. 1) show enhanced potency and selectivity for lipolysis compared to isoprenaline [5, 6]. The pharmacological evidence for the existence of a distinct β_3-adrenoceptor has been strengthened by cloning and expression studies from human [7], rat [8] and mouse [9] genomic libraries. These cloned β_3-adrenoceptors possess essentially the same pharmacological profile as the physiological receptors, with some small changes, attributed to species differences, between the human and rat cloned β_3-adrenoceptors [8, 10]. However, the primary sequence homology of each of the cloned β_3-adrenoceptors is at least 90%, significantly different from the extent of homology of each with the β_1- or β_2-adrenoceptors (about 60%).

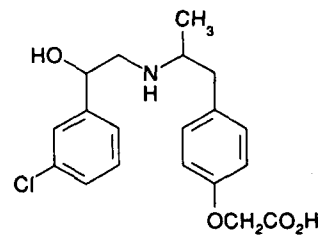

Fig. 1. Structure of BRL 37344.

In rat white adipocytes, receptor binding studies with a variety of ligands demonstrate only β_1 adrenoceptors [11], while functional studies suggest that the β-adrenoceptors coupled to stimulation of lipolysis are predominantly of the β_3-subtype [12]. This apparent contradiction is thought to be due to the inability of β-antagonist ligands to bind to β_3-adrenoceptors when used in the nanomolar concentration range, with non-specific binding effects

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‡ Abbreviations: A-Kinase, cAMP-dependent protein kinase; PIA, N^6-[*R*-(−)-1-methyl-2-phenethyl]adenosine; H89, *N*-[2-*p*-(bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide; BRL 37344, 4-[2-[(2-hydroxy-2-(3-chlorophenyl)ethyl)-amino]propyl]-phenoxyacetic acid; PMSF, phenylmethylsulphonylfluoride; IBMX, 3-isobutyl-1-methylxanthine; DMSO, dimethylsulphoxide; TCA, trichloroacetic acid; EGTA, ethylene glycol-bis(β-aminoethylether)*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; EC_{50}, concentration of the drug eliciting a half-maximal response; BSA, bovine serum albumin.

depleting the signal at higher concentrations [12]. However, the demonstration of β_1-adrenoceptors from binding studies has recently been supported by functional studies that demonstrate a small, subordinate role for β_1-adrenoceptors in the regulation of rat white adipocyte lipolysis [2]. In these studies, the β-antagonist, CGP 20712A, caused a consistent rightward shift in isoprenaline-stimulated (non-selective) lipolysis, but did not affect the lipolytic response to BRL 37344 (a β_3-selective agonist), indicating a β_1-component in isoprenaline action. Comparison of adenylyl cyclase activation in rat adipocyte membranes by isoprenaline and BRL 37344 yielded a similar result with respect to CGP 20712A shifts [13]. BRL 37344 was found to be a partial agonist compared to isoprenaline in adenylyl cyclase studies, leading Hollenga and co-workers [2, 13] to conclude that the β_3-adrenoceptor contributes some 62% of the cyclic AMP produced, the rest being accounted for by β_1-adrenoceptor stimulation. These workers also observed that EC_{50} values (concentration of the drug eliciting a half-maximal response) for activation of adenylyl cyclase were similar for isoprenaline and BRL 37344; and that concentrations of either agonist stimulating adenylyl cyclase by approximately 10% were sufficient to activate lipolysis maximally, indicating that in rat white adipocytes significant compartmentalization of cyclic AMP occurs. They also observed that, while dose–response relationships of adenylyl cyclase activation for isoprenaline and BRL 37344 were similar, BRL 37344 was 10-fold more potent than isoprenaline in stimulating lipolysis.

These results imply that cyclic AMP in rat white adipocytes is functionally compartmentalized, with β_3- and to a lesser extent β_1-adrenoceptors efficiently targeted to cyclic AMP generation in a region of the cell where it can activate the cAMP-dependent protein kinase (A-Kinase) cascade resulting in triglyceride lipase activation. Alternatively, such results could be interpreted as BRL 37344 stimulating lipolysis by cyclic AMP-independent, as well as cyclic AMP-dependent mechanisms [13]. In the present studies we have evaluated these alternate hypotheses by measuring adenylyl cyclase, A-Kinase and glycerol production in rat white adipocytes stimulated by isoprenaline and BRL 37344. In addition we have selectively blocked β_1- and β_3-adrenoceptors with propranolol (0.1 and 3.0 μM) to evaluate the relative contribution of β_1- and β_3-adrenoceptors to the control of lipolysis.

MATERIALS AND METHODS

Materials. Nucleotides (ATP, NAD, cAMP, phosphocreatine, N^6-[*R*-(−)-1-methyl-2-phenethyl]-adenosine (PIA), adenosine), enzymes (collagenase type A, glycerol dehydrogenase, adenosine deaminase, creatine kinase) and bovine serum albumin (BSA) (Cohn fraction V) were obtained from Boehringer Mannheim (Lewes, U.K.). *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) (free acid) and leupeptin were from Calbiochem/Novabiochem (Nottingham, U.K.). [γ^{32}P]ATP (6000 Ci/mmol) was from Du Pont (NEN) Ltd, (Stevenage, U.K.) and [α^{32}P]ATP (>400 Ci/mmol) and [2,8-^{3}H]cAMP were from Amersham International Plc (Aylesbury, U.K.). Most other biochemicals including DL-propranolol hydrochloride and (−)isoprenaline (+)bitartrate were obtained from the Sigma Chemical Co. (Poole, U.K.). All other chemicals were of analytical reagent grade. Phosphocellulose paper (P81) was obtained from Whatman Ltd (Maidstone, U.K.). Malantide, a peptide substrate for A-Kinase, whose sequence (RTKRSGSVYEPLKI) corresponds to that of the site phosphorylated by A-Kinase on the β-subunit of phosphorylase kinase [14], was obtained from Ocean Biologicals (Edmonds, WA, U.S.A.). The peptide was dissolved in water and the concentration determined by absorbance (ε_{275} = 1380 M/cm) [15]. H89 (*N*-[2-*p*-(bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide), was synthesized as described [16, 17]. The compound was dissolved in dimethylsulphoxide (DMSO) at a concentration of 10 mM.

Preparation of adipocytes. Adipocytes were prepared from the epididymal fat pads of Sprague–Dawley rats (180–200 g, fed *ad lib.*, supplied by OLAC Ltd, U.K.) by the method of Rodbell [18], as modified by Londos and co-workers [19, 20]. Briefly, the fat pads were minced and incubated with collagenase (6 mg/g tissue, 3 mL/g tissue) for 1 hr at 37° in a shaking water bath gassed with 95% O_2/5% CO_2 in modified Krebs buffer (118 mM NaCl, 4.7 mM KCl, 10 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$ and 30 mM HEPES, pH 7.4), supplemented with 4% (w/v) BSA and 200 nM adenosine. The cells were washed three times by flotation, then incubated in the same buffer supplemented with 4% (w/v) BSA, 100 nM PIA, and 0.5 U/mL adenosine deaminase. Adipocytes were incubated in a final volume of 1 mL in a shaking water bath at 37°. Exact conditions and details of incubation additions are given in the legends to the figures.

Assay of glycerol release and A-Kinase activation. Following incubation, an aliquot (500 μL) of the infranatant was removed and added to 100 μL of 10% (w/v) trichloroacetic acid (TCA). Glycerol content was assessed using a fluorimetric assay as described [21]. The adipocytes were resuspended by gentle agitation, and an aliquot (200 μL) frozen in liquid N_2. Subsequently, the cells were thawed in 2.5 vol. of 10 mM NaH_2PO_4, 10 mM EDTA, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.2 M NaCl (pH 6.8), homogenized by vigorous vortex-mixing, and the infranatant obtained after centrifugation (12,000 *g*, 4 min, 4°) used in the A-Kinase assay.

A-Kinase activity-ratio assay. Measurement of the degree of activation of A-Kinase (activity-ratio assay) was carried out as described [22]. Cell extract (10 μL) was added to 50 μL of assay buffer consisting of 70 mM $NaHPO_4$ [(pH 6.8), 14 mM $MgCl_2$, 1.4 mM ethylene glycol-bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.014% (v/v) Tween-20], ±28 μM cAMP at 4°. The tubes were equilibrated for 1 min at 30°, and the kinase reaction initiated by addition of 2.1 mM [γ^{32}P]ATP (10 μL, 0.6–1.0 μCi). The reaction was terminated after 10 min by addition of 10 μL 1 M HCl. Subsequently, 50 μL of each sample were spotted onto 2 × 2 cm phosphocellulose paper squares (P81), and the

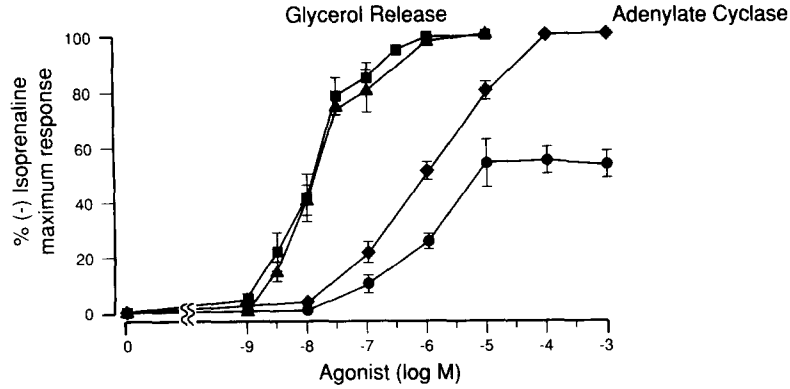

Fig. 2. Concentration–response curves for the stimulation of glycerol release (■, ▲) from adipocyte preparations (N = 5–7), and for the stimulation of adenylyl cyclase (◆, ●) in adipocyte membrane preparations (N = 4) elicited by isoprenaline (■, ◆) and BRL 37344 (▲, ●). Data are represented as the percentage of the isoprenaline maximal response. Errors are SEM. Absolute rates of adenylyl cyclase activity were: basal 142.8 ± 35.8 pmol cAMP/mg/min; isoprenaline maximum, 698.8 ± 104.1 pmol cAMP/mg/min. Absolute values of glycerol release were: basal 46.5 ± 9.5 nmol/mg dry weight cells/min; isoprenaline 979 ± 218 nmol/mg cells/min.

papers given four washes of 5–10 min each in 0.5% (v/v) tetraphosphoric acid, 38 mM H_3PO_4 [23], and dried by rinsing in acetone. Radioactivity was estimated by Cerenkov counting in water. Blanks were obtained by acidifying the samples before addition of [γ^{32}P]ATP, and were subtracted from all values. The activity ratio was obtained by dividing the radioactivity (dpm) obtained in the absence of exogenous cAMP by that obtained in its presence. The incorporation of ^{32}P into malantide was linear for at least 20 min under the incubation conditions used. Values for activity ratios obtained in rat white adipocytes were: control incubations 0.06 ± 0.012 (N = 10 preparations), rising to 0.32 ± 0.075 (N = 4 preparations) upon challenging with isoprenaline and 0.32 ± 0.083 with BRL 37344 (N = 5 preparations).

Preparation of adipocyte membranes. Isolated adipocytes were suspended in 30 mL of hypotonic lysing buffer [1 mM Tris–HCl, pH 7.4, 1 mM EDTA, containing 100 μM benzamidine, 100 μM phenylmethylsulphonylfluoride (PMSF) and 1 μg/mL leupeptin]/g of original tissue at ambient temperature, and hand-homogenized in a Potter-Elvehjem homogenizer (10 strokes). The resulting homogenate was then centrifuged at 15,000 *g* for 5 min at 4°, the fat cake removed, and the supernatant centrifuged at 15,000 *g* for 15 min at 4°. The pellet was resuspended by hand-homogenization as before (five strokes) and centrifuged at 15,000 *g* for 15 min at 4°. The resulting pellet containing crude membranes was resuspended in 10 mM Tris–HCl, pH 7.4, containing 1 mM EDTA, to a final concentration of 1–2 mg protein/mL, and frozen in liquid N_2 for subsequent adenylyl cyclase assay [24].

Adenylyl cyclase assay. Adenylyl cyclase was assayed at 30° in triplicate [25] by addition of plasma membranes (60–120 μg protein) to an incubation mixture (final vol. 100 μL) containing [α^{32}P]ATP (1.0 mM, 0.5 μCi), 10 mM theophylline, 20 mM phosphocreatine, creatine kinase (240 U/mL), 5 mM $MgSO_4$, 1 μM GTP and 25 mM Tris–HCl, pH 7.4. The cyclic AMP formed over 20 min was separated from ATP by the method of Salomon *et al.* [26].

Other methods. Protein content was determined by the modified Lowry method of Peterson [27], using BSA as a standard. Results are expressed as the mean ± SEM. Significance was assessed using the unpaired Student's *t*-test.

RESULTS AND DISCUSSION

Concentration–response curves for the stimulation of adenylyl cyclase and glycerol release elicited by isoprenaline and BRL 37344 are shown in Fig. 2. Activation of adenylyl cyclase by isoprenaline displayed an EC_{50} of 1×10^{-6} M, similar to that found for BRL 37344 (1.5×10^{-6} M). However, BRL 37344 was only a partial agonist in activation of adenylyl cyclase, showing an intrinsic activity of 0.55. These results agree with those reported recently by Hollenga *et al.* [13], who also showed, using the selective β_1-adrenoceptor-antagonist, CGP 20712A, that the response elicited by BRL 37344 is solely due to activation of β_3-adrenoceptors, while that elicited by isoprenaline is due to activation of both β_1- and β_3-adrenoceptor populations.

In contrast to the above, both agents were full agonists with respect to stimulation of lipolysis as measured by glycerol efflux (Fig. 2). Half-maximal stimulation by BRL 37344 occurred at 11 nM, and that evoked by isoprenaline at 10 nM. These data differ to those of Hollenga *et al.* [13] who showed that BRL 37344 was 10-fold more potent than isoprenaline at stimulating lipolysis in isolated white adipocytes derived from Wistar rats. We are presently unable to explain these potency differences except to note the difference in rat strain between this work and the work of Hollenga *et al.* [13].

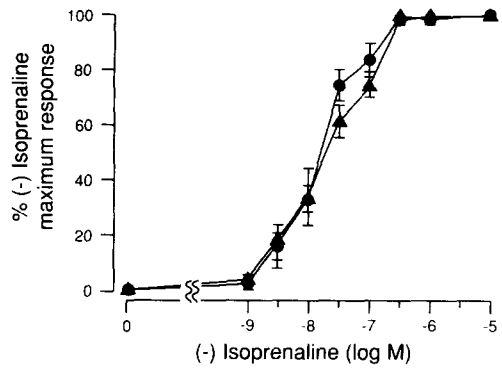

Fig. 3. Concentration–response curves for the stimulation of glycerol release (●) and A-Kinase activation (▲) by isoprenaline. Data are represented as the percentage of isoprenaline maximal response. Errors are SEM of four preparations, performed in triplicate. Values for activity ratios obtained in adipocytes were: control incubations 0.06 ± 0.012 rising to 0.32 ± 0.075 upon challenging with isoprenaline.

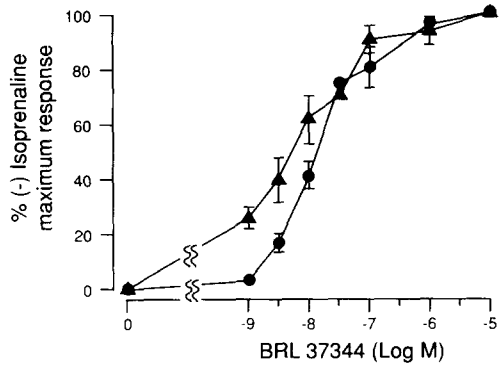

Fig. 4. Concentration–response curves for the stimulation of glycerol release (●), and for activation of A-Kinase (▲) by BRL 37344. Data are represented as the percentage of BRL 37344 maximum response. Errors are SEM of five preparations, performed in triplicate. Values for activity ratios obtained in adipocytes were: control incubations 0.06 ± 0.012 rising to 0.32 ± 0.083 upon challenging with BRL 37344.

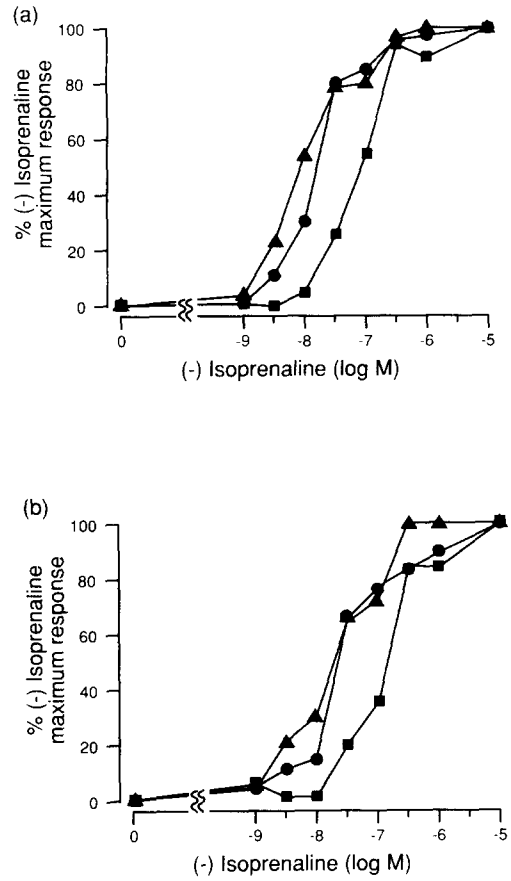

Fig. 5. Concentration–response curves for the stimulation of (a) glycerol release and (b) A-Kinase activation from adipocytes incubations (N = 3) elicited by isoprenaline in the absence (▲) or presence of 0.1 μM (●) or 3.0 μM (■) propranolol. Data are represented as the percentage maximal isoprenaline response, SEM were in all cases < 10% of the mean values, and are omitted for clarity.

The lipolytic responses shown in Fig. 2 were achieved by concentrations of either agonist that were insufficient to activate significantly adenylyl cyclase in crude membrane preparations derived from these cells. Furthermore, maximal lipolytic activity was achieved at concentrations which elicited only 25% and 50% stimulation of adenylyl cyclase by BRL 37344 and isoprenaline, respectively. Although total cellular cyclic AMP content was not measured in these studies, it has been shown that cyclic AMP content agrees with adenylyl cyclase using noradrenaline as an agonist [28]. Such observations suggest either that the β_3-adrenoceptor-mediated response is more efficiently targeted to activation of hormone-sensitive lipase, and ultimately glycerol release; or, that cAMP is not the obligatory second messenger transducing the lipolytic signal.

To gain further insight into this question, A-Kinase activity ratios were determined in the same adipocyte incubations as were assayed for glycerol release. Activation of A-Kinase closely paralleled glycerol efflux in response to isoprenaline (Fig. 3) and BRL 37344 (Fig. 4). EC_{50} values for activation of A-Kinase and lipolysis by BRL 37344 were 5×10^{-9} M and 1.1×10^{-8} M, respectively, and those elicited by isoprenaline were 1.8×10^{-8} M and 1.0×10^{-8} M, respectively; also, maximal activation of both lipolysis and A-Kinase was achieved by both agonists. Furthermore, stimulation of lipolysis and activation of A-Kinase are modulated in a similar manner after blockade by the β-adrenoceptor antagonist, propranolol. Addition of propranolol at a concentration (0.1 μM) which is sufficient to block β_1- and β_2-adrenoceptors [3] did not affect stimulation of lipolysis and activation of A-Kinase by BRL 37344 or isoprenaline (Figs 5 and 6), with respect to the

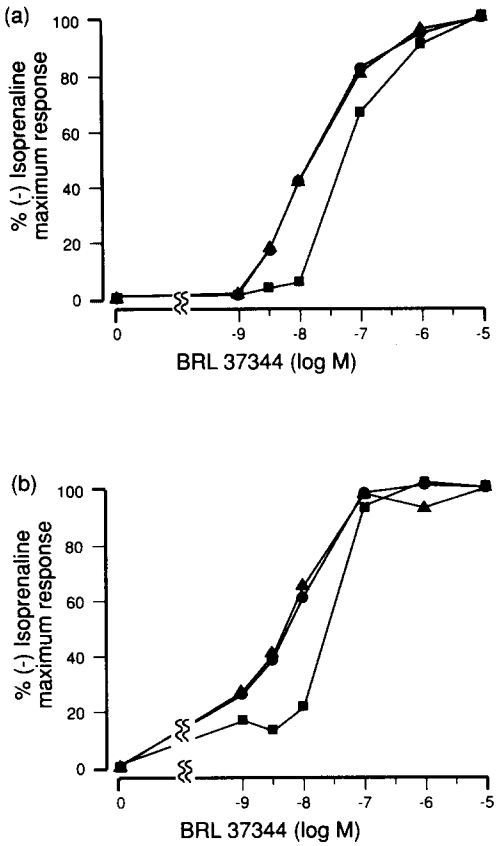

Fig. 6. Concentration–response curves for the stimulation of (a) glycerol release and (b) A-Kinase activation from adipocyte incubations (N = 3) elicited by BRL 37344 in the absence of (▲) or presence of 0.1 μM (●) or 3.0 μM (■) propranolol. Data are represented as the percentage maximal response to BRL 37344, SEM were in all cases < 10% of mean values, and are omitted for clarity.

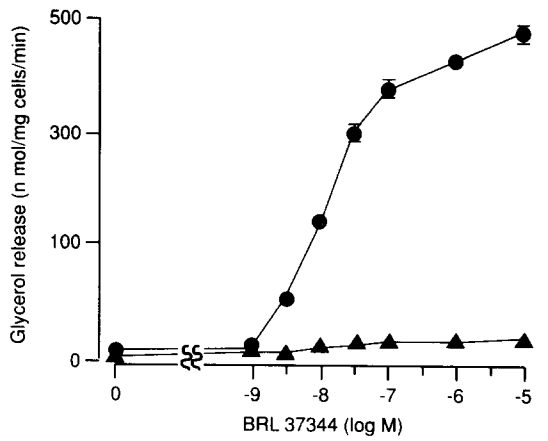

Fig. 7. Concentration–response curves for stimulation of lipolysis by BRL 37344 in adipocyte preparations which had (▲) or had not (●) been pretreated for 30 min with 100 μM H89, prior to the addition of agonist. Data are means ± SEM of three preparations, performed in triplicate.

EC_{50} values. However, a shift in the threshold values of isoprenaline- (Fig. 5), but not BRL 37344- (Fig. 6) stimulated activation of lipolysis and A-Kinase was observed, indicating that a β_1-adrenergic component is present in isoprenaline action on rat adipocytes, in agreement with the observations of Hollenga *et al.* [13]. When the concentration of propranolol was increased to 3.0 μM, β_3-adrenergic responses were inhibited, with parallel shifts in glycerol release and activation of A-Kinase (Figs 5 and 6). Apparent pA_2 values obtained from the dose ratio shift at 3 μM propranolol are equivalent for the two parameters, both for BRL 37344 (A-Kinase, $pA_2 = 6.0$, glycerol efflux, $pA_2 = 6.10$) and for isoprenaline (A-Kinase, $pA_2 = 6.42$, glycerol efflux, $pA_2 = 6.40$). Therefore, the lipolytic response appears to be mediated predominantly through β_3-adrenergic receptors in rat white adipocytes.

Our results support and extend the observations of Hollenga and co-workers [13, 29] which led these workers to suggest either functional compartmentalization of the lipolytic cAMP pool, or the existence of a β_3-adrenoceptor-stimulated cAMP-independent signal transduction pathway activating lipolysis. To differentiate between these hypotheses we employed the novel, selective A-Kinase inhibitor, H89 [17]. This agent is a competitive inhibitor of ATP binding to the A-Kinase catalytic subunit. In *in vitro* studies performed on a number of purified kinases this compound was shown to inhibit A-Kinase with a K_i of 50 nM, 10-fold less than its K_i for inhibition of cGMP-dependent protein kinase, and at least 500–3000 times more potent than its inhibition of protein kinase C, myosin light-chain kinase, Ca^{2+}/calmodulin-dependent kinase II, and casein kinases I and II. In *in vivo* systems the observed potency of the inhibitor was much less, presumably reflecting poor cell uptake. However, selective inhibition of A-Kinase activity by pretreatment of pheochromocytoma PC12D cells with 30 μM H89 occurred without inhibition of the other kinase activities (as listed above) in the lysate [17]. We could find no effect of H89 upon lipolysis when adipocytes were pretreated with up to 10 μM of this compound (not shown). However Fig. 7 shows that pretreatment of adipocyte cell suspensions with 100 μM H89 completely inhibited lipolytic stimulation by all concentrations of BRL 37344. It proved impossible to assess the A-Kinase activation ratio, as no activity could be detected even in the presence of saturating (28 μM) concentrations of exogenous cAMP (not shown) presumably due to carry over of H89 in the extracts. This observation strongly implies an obligatory role for cAMP in mediating β_3-adrenoceptor stimulated lipolysis.

Discrepancies between cAMP concentration and lipolysis in response to β-agonists and other lipolytic agents have been interpreted as indicative of functional compartmentalization of the second messenger [13, 28–30]. However, as Londos and co-workers [20] have observed, it is difficult to directly relate linear rates of glycerol efflux to transient rises

in intracellular cAMP concentrations, due to concomitant activation of cAMP phosphodiesterases. While it is unlikely that a small, soluble molecule like cAMP could be restricted to a particular cytosolic compartment, some workers have recently reported preliminary characterization of a specific anchoring protein which interacts with the regulatory subunit of A-Kinase. This interaction serves to keep the holoenzyme located in specific subcellular compartments [31]. Such a molecular mechanism may underlie the compartmentalization of the lipolytic signal in rat adipocytes.

These observations demonstrate that the physiologically important lipolytic β-adrenoceptor on rat white adipocytes is the β_3-adrenoceptor and the lipolytic response is mediated by a functionally compartmentalized pool of cAMP or, more likely, protein kinase A. During the preparation of this manuscript other workers [32] demonstrated a direct relationship between agonist-stimulated lipolysis and A-Kinase activation. In the present study these conclusions are extended by the direct demonstration of the involvement of A-Kinase activation in lipolysis by the use of the selective A-Kinase inhibitor H89. Furthermore, the use of β-antagonists has further dissected the relative roles of β_1 and β_3-adrenoceptors in the control of lipolysis in rat adipose tissue.

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