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α-Calcitonin gene related peptide (α-CGRP) mediated lipid mobilization in 3T3-L1 adipocytes

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ABSTRACT

The neuropeptide, α-calcitonin gene-related peptide (α-CGRP), is expressed from sensory nerves that innervate fat. However, how α-CGRP may act in adipose tissue is unclear. Using 3T3-L1 adipocytes we observed that rat α-CGRP (rα-CGRP) evoked either a biphasic or monophasic reduction in intracellular free fatty acid (FFA) content. cAMP production was always monophasic and occurred when FFA responses were absent. Taken together with the observed potencies, these findings suggest that adipose tissue is a physiological target for α-CGRP. However, uncoupling of the FFA and CGRP-signaling responses with increasing passage number limits 3T3-L1 adipocytes as a suitable cellular model.

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino-acid neuropeptide peptide and a member of the calcitonin peptide family, which includes six members: calcitonin, amylin, adrenomedullin (AM), adrenomedullin-2 and two distinct forms of CGRP, α-CGRP and β-CGRP [16]. Although α-CGRP is the major form [29], there is nevertheless overlapping expression of both isoforms throughout the central and peripheral nervous systems, including motor and sensory neurons [1,13]. CGRP expression has also been observed in (i) human adipose tissue where its expression can be regulated by hormones such as glucose-dependent insulinotropic peptide [24,33], (ii) rat sensory neurons innervating both brown and white adipose tissues [8,31], and (iii) activated macrophages [21]. It is likely that the physiological actions of CGRP are primarily mediated through sensory nerve termini where it is released. These localized concentrations are difficult to measure but may be significantly higher than circulating concentrations [15,25], which typically range between 5 and 50 pM [7,27,34,38] and fall below levels reported to evoke biological effects [7,27].

The 'CGRP receptor' is formed by the combination of the calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1) [26]. This receptor displays greater affinity for CGRP than AM [2,3]. CGRP also displays equipotent activity to amylin at the amylin subtype 1 (AMY_1) receptor which is formed by the combination of the calcitonin receptor (CTR) and RAMP1 [3,14]. A wide range of biological roles have been reported for CGRP, including pain perception [28], vasodilation [4] and carbohydrate metabolism [19,20]. The identity of the receptor involved in each activity of CGRP is not known in all cases. However, the principal signaling molecule elevated by CGRP is cAMP [35].

Several in vivo and in vitro studies have highlighted a possible role for CGRP in lipid metabolism. For example, α-CGRP knockout mice are protected against diet-induced obesity, showing an overall reduction in adiposity [36]. Triglyceride content in isolated soleus muscle from Wistar rats is also lowered acutely following incubation with exogenous α-CGRP or following infusion of α-CGRP in whole animals [7]. α-CGRP also stimulates lipolysis, as indicated

Abbreviations: α-CGRP, αcalcitonin gene-related peptide; rα-CGRP, rat αcalcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; FFA, free fatty acid; AM, adrenomedullin; rAM, rat adrenomedullin; IP_3, Inositol tri phosphate.

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by increased glycerol release, in both cultured mesenchymal stem cell derived adipocytes [22] and 3T3-L1 adipocytes [5]. However, the physiological significance of CGRP's actions in adipocytes is still unknown as is the role of CGRP-expressing sensory nerve populations that innervate adipose depots [30]. There is only limited information available on potencies and underlying signaling mechanisms involved and it is currently unclear whether CGRP does indeed directly regulate adipose lipid stores.

3T3-L1 fibroblasts are a cell line originally cloned from mouse embryo fibroblasts, which can be chemically induced to undergo differentiation into 3T3-L1 adipocytes. Once differentiated, these mature adipocytes are characterized by elevated fatty acid synthase activity and large intracellular lipid droplets that are visible under a light microscope [32]. Therefore, 3T3-L1 adipocytes could be useful as a model system for studying the underlying mechanisms involved in the lipid-modulating effects of α-CGRP in adipose tissue. We therefore investigated the utility of differentiated 3T3-L1 adipocytes as a model system to dissect the receptor pharmacology and signaling pathways involved in the actions of α-CGRP.

Materials and methods

Cell culture

Three distinct batches of 3T3-L1 cells were used in these studies. Batch #1 was kindly provided by Dr. Yu Wang, (University of Auckland, New Zealand) [37]. Batches #2 and #3 were obtained from the American Tissue Culture Collection (ATCC). All cells were grown and maintained in a humidified incubator at 37 °C and 5% CO_2. 3T3-L1 fibroblasts were maintained as subconfluent cultures in high glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen; New Zealand) supplemented with 10% fetal bovine serum (FBS, Invitrogen, New Zealand). 3T3-L1 fibroblasts were grown to confluence in 6-well plates (for lipid assays) or 24-well plates (cAMP assay). Differentiation of 3T3-L1 fibroblasts was induced by incubation with 0.25 μM dexamethasone (Sigma–Aldrich), 0.5 mM isobutylmethylxanthine (IBMX, Sigma–Aldrich) and 10 μg/mL insulin (Actrapid; Novo Nordisk) for 48 h. This was followed by incubation with 10 μg/mL insulin for 48 h. Cells were maintained in DMEM with 10% FBS for 96 h to allow differentiation, which was monitored using light microscopy [37]. We refer to these differentiated cells as 3T3-L1 adipocytes.

Determination of triglyceride and FFA content in 3T3-L1 fibroblasts and adipocytes

3T3-L1 fibroblasts or differentiated adipocytes were serum-deprived for 30 min in 900 μL DMEM supplemented with 0.1% bovine serum albumin (BSA). All peptides were made up initially as 1 mM stocks in distilled H_2O and stored as aliquots at −30 °C. 100 μL of 10 times the required concentration of either rat α-CGRP (Bachem, Bubendorf, Switzerland) or rat AM (Bachem, Bubendorf, Switzerland) constituted in DMEM supplemented with 0.1% BSA was then added to the wells and plates incubated for 60 min. Lipid extraction was then performed immediately. Media was aspirated from the wells and the cells washed with 1 mL of PBS. Cells were scraped with a cell scraper to detach them from the plate surface and 0.5 mL of PBS was added to each well and the cells transferred to a 15 mL glass tube (on ice). Wells were washed a further three times with 0.5 mL of methanol and the cells then transferred to the same 15 mL glass tube on ice. Chloroform (3 ml) was added to the 15 mL glass tube (on ice) giving a 1:2 ratio of methanol:chloroform. The glass tubes were vortexed and incubated for overnight at 4 °C while shaking at 200 rpm. 0.6% NaCl (2 mL) was added to each glass tube, vortexed and centrifuged at 2000 rpm for 10 min at 4 °C. The lower organic phase was extracted into scintillation vials and the chloroform evaporated off under oxygen-free nitrogen gas. The dried extract was re-dissolved in 500 μL of absolute ethanol and vortexed thoroughly. Samples were stored at 4 °C for a maximum of 24 h prior to analysis for lipid content. Analyses of FFA and triglyceride content were performed as per the manufacturer's instructions, by an accredited technician (SMF) on a Synchron CX5CE (Beckham Coulter, Inc., USA).

Determination of cAMP content in 3T3-L1 fibroblasts and adipocytes

cAMP measurements were performed as previously described [2], modified for use in 24-well plates. 3T3-L1 fibroblasts or adipocytes in 24-well plates were serum-starved for 30 min with 450 μL serum free media containing 0.5 mM IBMX and 0.1% BSA. 50 μL of serum free media containing 0.5 mM IBMX, 0.1% BSA and 10 times the appropriate hormone concentration or forskolin (Tocris) was added to each well and the plates incubated at 37 °C for 15 min. Media was aspirated and cAMP extracted by the addition of 500 μL of ice-cold absolute ethanol. Plates were incubated in a freezer for at least 5 min and then analyzed immediately or stored in a freezer until analysis.

For cAMP analysis, ethanol was evaporated and cell extracts resuspended in 125 μL cAMP assay buffer (5 mM EDTA, 20 mM HEPES, pH 7.5). Extracts (100 μL) were transferred to 1.5 mL microfuge tubes, followed by 100 μL ^{3}H-cAMP and 200 μL protein kinase A solution. The 1.5 mL microfuge tubes were gently mixed by inversion and then incubated at 4–8 °C for between 2 and 24 h. Activated charcoal (100 μL) was added to the microfuge tubes which were mixed and then centrifuged at 3000 rpm (Eppendorf 5402 centrifuge) for 5 min at 4–8 °C. Supernatant (200 μL) was removed to a 4 mL scintillation vial and 2 mL Starscint scintillation fluid (Packard) was added to each vial. The samples were vortexed and counted in a microbeta Trilux (Perkin–Elmer). Corrected counts per minute were converted to a percentage of the maximal (forskolin) response which was expressed as 100%, samples that were treated with buffer only were expressed as 0%.

Statistics

Data analysis and curve fitting was performed using GraphPad Prism (version 4.03, GraphPad Software Inc., San Diego, CA). pEC_{50} values were determined by fitting a four-parameter logistic equation to the cAMP, FFA and TG concentration-response data. When the Hill slope was not significantly different (*f*-test) from one for agonist potency curves the curves were re-fitted with a Hill slope constrained to one and a pEC_{50} obtained. For the FFA concentration-response data both one-site and two-site curves were fitted to the data and compared. Two-site curves are presented when this fitted the data better. Statistical significance was achieved at $p < 0.05$ and determined using unpaired two-tailed *t*-tests or one-way ANOVA followed by post hoc Bonferroi tests as appropriate. All data represent the mean ± SEM of two wells (triglyceride and FFA) or four wells (cAMP).

Results

3T3-L1 fibroblasts were studied first to investigate whether they were intrinsically responsive to α-CGRP. cAMP concentration-response curves were conducted prior to commencing studies in differentiated 3T3-L1 adipocytes. In a representative experiment, rα-CGRP elicited a concentration-dependent increase in intracellular cAMP content with a pEC_{50} of 9.6 (Fig. 1A). Triglyceride content was below the limit of detection and unable to be measured. Despite elevating cAMP, rα-CGRP at a concentration of 100 nM had

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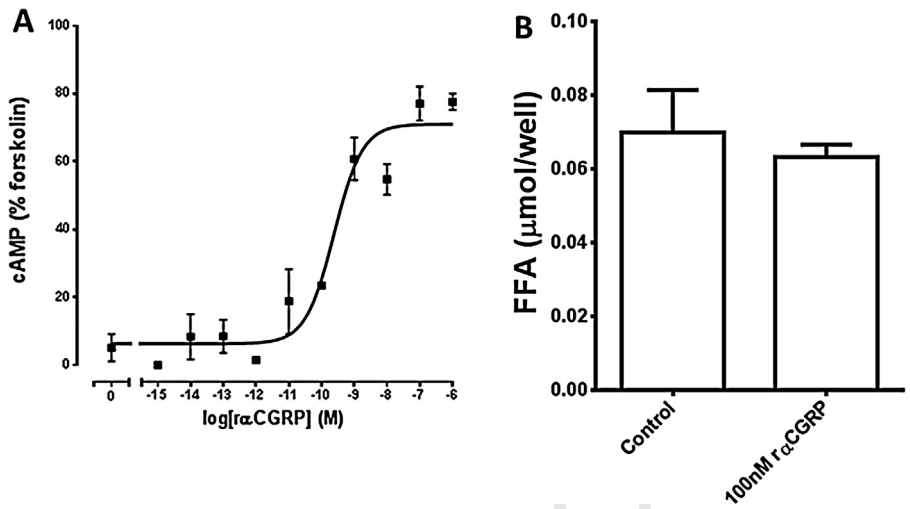

Fig. 1. Effect of rα-CGRP on cAMP production and lipid content in3T3-L1 fibroblasts (Batch #1). A representative experiment showing the effect of (A) a range of rat α-CGRP concentrations on cAMP production and (B) no significant effect of 100 nM rα-CGRP on FFA content. Data are representative of three (A) or two (B) independent experiments. Values are expressed as Mean ± SEM ($n = 4$ wells cAMP, $n = 2$ wells FFA).

no measureable effects on FFA content, which had a detection limit of 0.02 μmol/well (Fig. 1B).

Experiments were then conducted on the same batch of 3T3-L1 adipocytes that were differentiated at passage 4 and which contained measurable triglyceride content. Incubation with either 100 nM rα-CGRP ($p < 0.05$), 100 nM rAM ($p < 0.05$) or 1 pM rα-CGRP ($p < 0.01$), but not 1 pM AM produced a significant decrease in intracellular FFA (Fig. 2A). We measured both extracellular FFA and glycerol release in the medium in response to CGRP. However, their absolute concentrations were too close to the limit of detection in this experimental system to accurately determine whether corresponding increases of FFA and glycerol occurred in the medium. This decrease in intracellular FFA content was not associated with a significant change in triglyceride content (Fig. 2B). In parallel experiments, both 100 nM rα-CGRP and 100 nM rAM elicited significant increases in cAMP content. Interestingly, despite stimulating a significant decrease in FFA content, 1 pM rα-CGRP did not increase cAMP production (Fig. 2C). Similar results were observed at passage 5 (data not shown).

Further experiments were conducted to quantify the potency of rα-CGRP. Concentration-response curves were constructed to examine the effects of α-CGRP on FFA and triglyceride content. α-CGRP potently stimulated a biphasic reduction in intracellular FFA content in 3T3-L1 adipocytes differentiated from fibroblasts at passage 5 (pEC_{50}: 12.4 and 8.8, Fig. 2D) again without any significant change in triglyceride content (Fig. 2E). However, when these experiments were repeated with 3T3-L1 adipocytes at passage 8 in parallel with cAMP assays, the rα-CGRP-stimulated reduction of intracellular FFA was no longer evident (Fig. 2F). Triglyceride content was unchanged (Fig. 2G) but a robust concentration-dependent cAMP response (Fig. 2H) was still evident (pEC_{50}: 10.3) showing that an intrinsic signaling response was maintained. Similar results were observed at passage 7 (data not shown).

These findings suggested that the metabolic effects of rα-CGRP on FFA content in 3T3-L1 adipocytes were passage-dependent. To confirm this possibility, a new batch of 3T3-L1 fibroblasts were obtained from ATCC (batch #2) and the experiments repeated (Fig. 3). 3T3-L1 adipocytes differentiated at passage 3 recapitulated the rα-CGRP-stimulated reduction in intracellular FFA (Fig. 3A) with no accompanied change in triglyceride content (Fig. 3B). However, the potency of this response (pEC_{50}: 10.7) was now intermediate compared to the high (pEC_{50}: 12.4) and low (pEC_{50}: 8.79) potency responses obtained for the previous batch of 3T3-L1 adipocytes (Fig. 2D). cAMP production was robustly increased in a concentration-dependent manner (Fig. 3C) with a potency (pEC_{50}: 10.8) similar to that observed in the previous batch (pEC_{50}: 10.3) (Fig. 2I).

Similar results were obtained at passage 4 for both FFA reduction (pEC_{50}: 10.4) and cAMP production (pEC_{50}: 10.0). However, at increased passage number (passage 5), the rα-CGRP-stimulated reduction of intracellular FFA was again lost (Fig. 3D) with no significant change in triglyceride content (Fig. 3E). A cAMP response (pEC_{50}: 10.1) was sustained (Fig. 3F). Similarly, at passage 6 rα-CGRP did not affect intracellular FFA content (data not shown). cAMP analysis was not conducted. A third batch of 3T3-L1 adipocytes obtained from ATCC (batch #3) rα-CGRP did not modulate intracellular FFA at passage 3 or 4 (data not shown).

Discussion

Although CGRP has reported effects on lipid metabolism in adipose tissue, whether these represent physiological actions are unknown. We therefore assessed differentiated 3T3-L1 adipocytes as a model system to dissect the receptor pharmacology and signaling pathways involved. 3T3-L1 adipocytes are a well characterized model and have been shown previously to respond to CGRP [5]. They have also been shown to express CLR, a CGRP receptor component [23].

For differentiated 3T3-L1 adipocytes, cAMP production evoked by rα-CGRP was reproducible across multiple batches and passages of cells with similar potencies. In fact, cAMP responses in 3T3-L1 adipocytes were approximately 5-fold more potent than in 3T3-L1 fibroblasts (pEC_{50}: 10.5 ± 0.1, $n = 3$ combined experiments vs. 9.80 ± 0.2, $n = 3$ combined experiments; $p < 0.05$) suggesting enhanced expression, coupling or activity of a CGRP-responsive receptor. At lower passage number, there was a biphasic reduction in FFA content by rα-CGRP with potency responses in the low picomolar and nanomolar ranges. These potencies are similar to a similar biphasic response reported for isolated rat soleus muscle where rα-CGRP increased FFA content in parallel with a decrease in triglyceride content [7]. By contrast, the decrease in FFA content in 3T3-L1 adipocytes evoked by rα-CGRP was not accompanied by significant changes in triglyceride content. Although this may be due to the sensitivity of triglyceride measurement, inspection of the data suggests mobilization of intracellular FFA by rα-CGRP, possibly through cellular export or further metabolism.

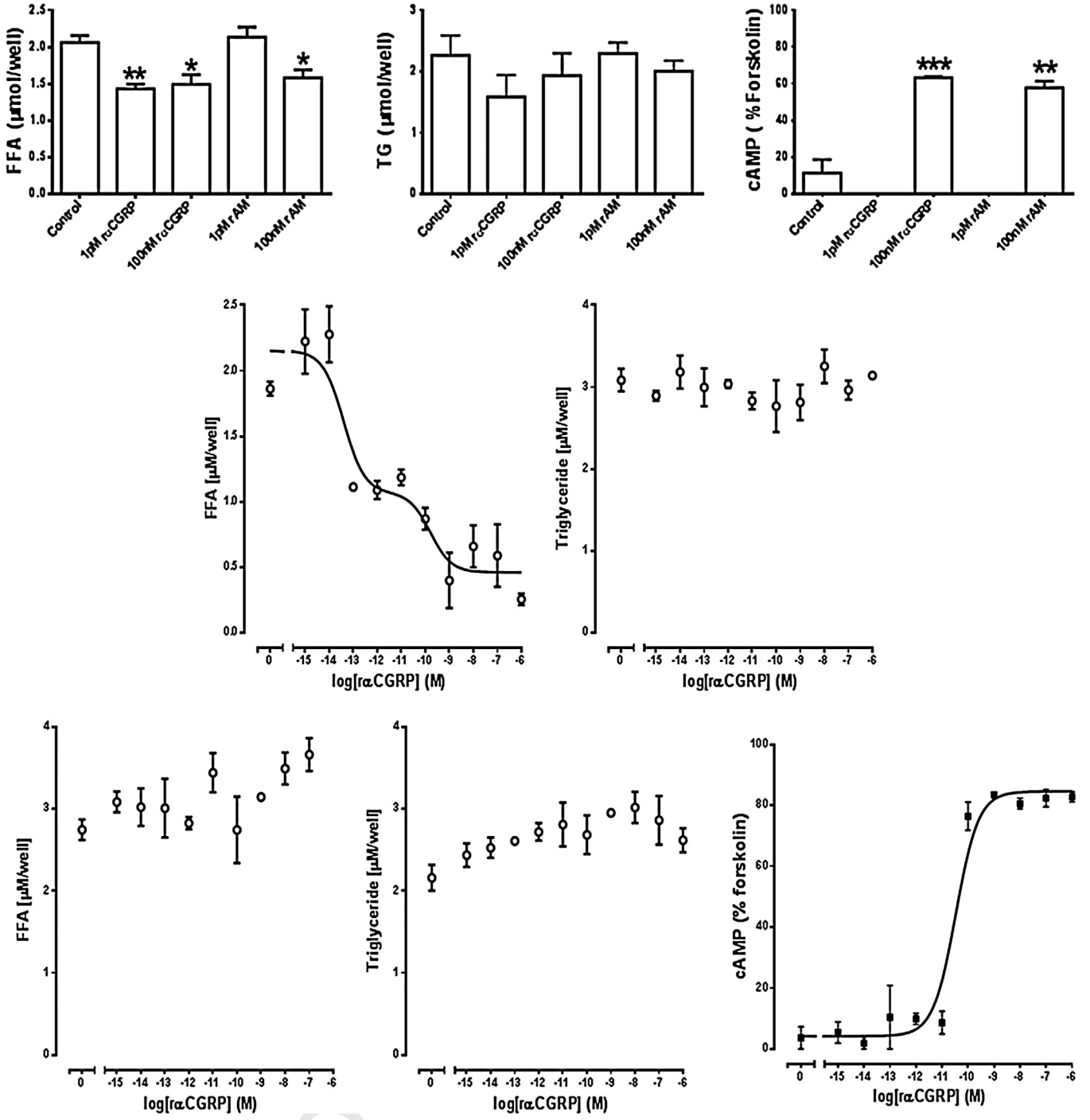

Fig. 2. Effect of rα-CGRP and rAM on cAMP production and lipid content in 3T3-L1 adipocytes (Batch #1). 3T3-L1 adipocytes at passage 4 were incubated with either rat α-CGRP or rat AM and (A) FFA, (B) triglyceride or (C) cAMP content measured. 3T3-L1 adipocytes at passage 5 (D–E) or 8 (F–H) were incubated with a range of rat α-CGRP concentrations and FFA, triglyceride or cAMP content measured. Values are expressed as Mean ± SEM ($n = 4$ wells cAMP, $n = 2$ wells FFA and triglyceride) of data from a single experiment.

The difference observed between the effects of rα-CGRP on skeletal muscle and adipose tissue may reflect fundamental differences in their metabolic roles as well as the compliment of lipid handling proteins present. The concentration of CGRP observed to reduce FFA in 3T3-L1 adipocytes was much lower than those typically reported in circulation [7,27,34]. However, at specific sites of action the concentration of CGRP may be tightly regulated. In order to assess the biological importance of this response further in vivo and ex vivo studies should be performed to determine the CGRP concentration and the potency at the site of action in adipose tissue. Changes to the expression of accessory proteins, including receptor-component protein (RCP) and RAMP1, may sensitize the system to CGRP [10,39].

Interestingly, the decrease in FFA content by 1 pM rα-CGRP was approximately 10- to 100-fold lower than that required to elevate cAMP. This observation points toward the existence of cAMP-independent signaling mechanisms and is consistent with similar discrepancies reported between cAMP signaling and α-CGRP-mediated effects on lipid metabolism [5,7]. Given the potential for CGRP receptors to couple several downstream signaling

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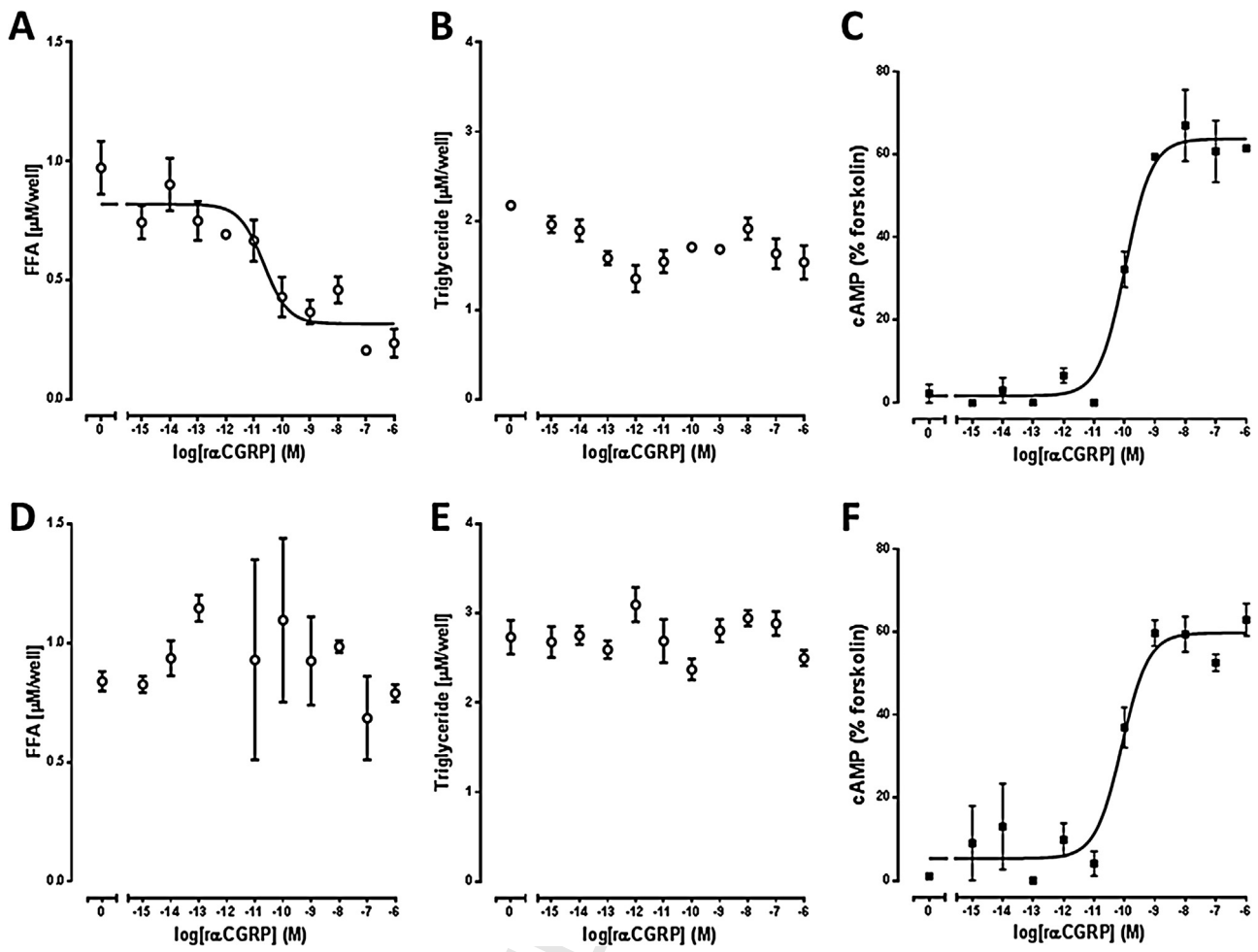

Fig. 3. Effect of rα-CGRP on cAMP production and lipid content in3T3-L1 adipocytes (Batch #2). 3T3-L1 adipocytes at passage 4 (A–C) or 5 (D–F) were incubated with a range of rat α-CGRP concentrations and FFA, triglyceride and cAMP content measured. Values are expressed as Mean ± SEM ($n = 4$ wells cAMP, $n = 2$ wells FFA and triglyceride) of data from a single experiment.

mechanisms independent of cAMP, including Ca^{2+}/IP_3 accumulation, this potential signaling mechanism warrants further investigation [18,35].

Significant passage-dependent variation was observed in the ability of rα-CGRP to mobilize FFA in differentiated adipocytes despite reproducible and robust cAMP responses. These findings suggest that adipocytes express the machinery to modulate lipid content but that these properties are unstable and can become uncoupled from receptor signaling. A similarly unstable phenotype was reported previously in 3T3-L1 adipocytes where lipoprotein lipase expression in response to glucose-dependent insulinotropic peptide decreased with passage number [9]. It may be possible to take advantage of this passage-dependent effect using genomic or proteomic techniques to isolate the specific factors through which receptor activation by CGRP is coupled to lipid modulation. However, the variable CGRP-responsive phenotype of this cell type could make these experiments challenging.

One possibility for the loss of FFA response is a dysregulation in cAMP-independent signaling pathways. Evidence suggests that G_q coupled IP_3 accumulation is an important pathway activating lipolysis in adipocytes [6]. Interestingly, in 3T3-L1 fibroblasts, α-CGRP stimulated Ca^{2+} transients only in approximately 30% of the cell population [23], suggesting that α-CGRP mediated Ca^{2+}/IP_3 signaling is inherently variable in this cellular system. Although it is not known how this finding relates to differentiated 3T3-L1 adipocytes, it is possible that coupling to Ca^{2+}/IP_3 signaling pathways is not essential for differentiation or survival and could therefore be diminished through passaging.

AM also evokes lipolysis as measured as glycerol release in a cultured rat adipocyte model, consistent with downstream activation of cAMP and protein kinase A [17]. However, by comparison with the high potency response rα-CGRP in the low picomolar range, rAM only reduced FFA content and increased cAMP at the higher concentration of 100 nM. This observation is consistent with the known lower potency for rAM as compared to rα-CGRP for CGRP or amylin receptors [2,14]. Due to the unstable phenotype observed for the 3T3-L1 adipocytes, it was not feasible to undertake a complete characterization of the receptor and signaling pathways responsible for the lipid mobilizing effects of rα-CGRP and rAM.

In summary, rα-CGRP stimulated a decrease in intracellular FFA in 3T3-L1 adipocytes that appeared to be uncoupled from changes in cAMP. Taken together with the high potency of these metabolic effects on FFA content, these findings point toward adipose tissue as a physiological target site for CGRP-expressing sensory neurons that innervate fat. Inhibitor studies would be useful for dissecting out the signaling pathways involved. However, the transient nature of the FFA response and loss of the high potency response with increasing passage number precludes the use of 3T3-L1 adipocytes as a suitable cellular model. Primary cultured or stem cell derived adipocytes may provide useful alternatives [11,12]; however care should be taken to determine their robustness as a model system.

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