Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 461-467

Suppression of Insulin-Stimulated Glucose Transport in L6 Myocytes by Calcitonin Gene-Related Peptide

D. Kreutter, S. J. Orena and K. M. Andrews

Department of Metabolic Diseases, Pfizer Central Research, Groton, CT 06340

Received August 31, 1989

The binding of calcitonin gene-related peptide (CGRP) to L6 myocytes, the coupling of this receptor to adenylyl cyclase and the resultant effects on insulin-stimulated 2-deoxyglucose uptake were examined. L6 cells express specific binding sites for CGRP. Binding of human [^{125}I]CGRP was inhibited by rat CGRP with an IC_{50} of approximately 10^{-9} M. Synthetic human calcitonin at concentrations up to 10^{-6} M had no effect on the binding of CGRP, suggesting that L6 cells express CGRP receptors, rather than calcitonin receptors which are also capable of binding CGRP. The CGRP receptor appeared to be coupled to adenylyl cyclase. Concentrations of CGRP greater than $3x10^{-9}$ M increased the cellular content of cAMP. At $3x10^{-8}$ M, CGRP increased cAMP 500-fold. CGRP at 10^{-10} M and above suppressed the stimulation of 2-deoxyglucose uptake by insulin. Acute incubation of L6 cells with insulin stimulated 2-deoxyglucose uptake 1.6-fold, which was inhibited up to 70% by CGRP. Our results demonstrate that the specific binding of CGRP to L6 cells causes large increases in the cellular content of cAMP and inhibition of insulin-stimulated 2-deoxyglucose uptake, but the differences in the dose-response curves suggest that the suppression of insulin action by CGRP cannot be solely explained by the increase in cAMP.

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide that arises from alternative splicing of the calcitonin gene (1). CGRP stimulates adenylyl cyclase in skeletal muscle, resulting in increased concentrations of cAMP (2-4). The peptide also induces insulin resistance in isolated soleus muscle preparations (5,6). Similar effects on insulin action have been observed using ß-adrenergic agonists, which are also coupled to the cAMP messenger system. In isolated soleus muscle, isoprenaline inhibits insulin-stimulated glycogen synthesis (7). Stimulation of ß-adrenergic receptors also inhibits insulin action in isolated fat cells and myocytes (8-10). In these model systems, ß-adrenergic agonists inhibit insulin-stimulated glucose transport by both cAMP-dependent and independent mechanisms. In addition, agents such as

0006-291X/89 $1.50

forskolin and isobutylmethylxanthine that increase cAMP levels by stimulation of adenylyl cyclase and inhibition of phosphodiesterase, respectively, suppress insulin action by cAMP-independent mechanisms (8,11). The aim of the present study was to determine whether the inhibitory effects of CGRP on insulin responsiveness in isolated muscle could be demonstrated in L6 myocytes, and whether the effects on insulin action in these cells are mediated by the cAMP messenger system.

METHODS

L6 cells were obtained from American Type Culture Collection. Synthetic, rat CGRP and synthetic human calcitonin were purchased from Sigma Chemical Co. The radioimmunoassay kit for cAMP measurements and [1-^{14}C]2-deoxyglucose were purchased from New England Nuclear. Human (2-[^{125}I]-iodohistidyl^{10}) CGRP was obtained from Amersham.

L6 cells were grown in 6-well plates in alpha-MEM containing 2% fetal bovine serum. Cells were used for experiments after fusion into myotubes. Cells were depleted of serum for 5 hours in alpha-MEM. During the final forty minutes of this period, insulin and CGRP were added. At the end of 5 hours, the cell monolayer was washed with Dulbecco's phosphate buffered saline (D-PBS). Uptake of 0.1 mM [1-^{14}C]2-deoxyglucose in D-PBS was measured over 10 minutes at room temperature. Deoxyglucose uptake was linear for at least 30 minutes under these conditions. Uptake was terminated by aspirating the buffer and quickly washing the monolayer three times with ice-cold D-PBS. Cells were dissolved in 0.1 N NaOH and an aliquot was counted in a liquid scintillation counter.

For measurements of cAMP content, cells were serum deprived for 5 hours and then incubated with CGRP for 15 minutes at 37°C. Media was aspirated and cells dissolved in 10 mM HCl. After neutralization of pH with NaOH, an aliquot was used for RIA.

For binding experiments, cells were serum deprived for 5 hours. The monolayer was washed 3 times with D-PBS, and [I^{125}]CGRP plus various concentrations of CGRP in 120 mM NaCl/ 4.7 mM KCL/ 5 mM $MgCl_2$/ 1 mM EGTA/ 5 mg/ml fatty acid free bovine serum albumin/ 1 mg/ml bacitracin/ 10 mM HEPES, pH 7.4 were added. After 16 hours at 4°C, the buffer was aspirated and the monolayer was washed 3 times with ice-cold D-PBS. Cells were solubilized in 0.1 N NaOH and an aliquot was counted for I^{125} in a liquid scintillation counter.

RESULTS

The binding of [I^{125}]CGRP to L6 cells is shown in Figure 1. The specific binding of ligand represented 80% of the total binding. Unlabeled CGRP

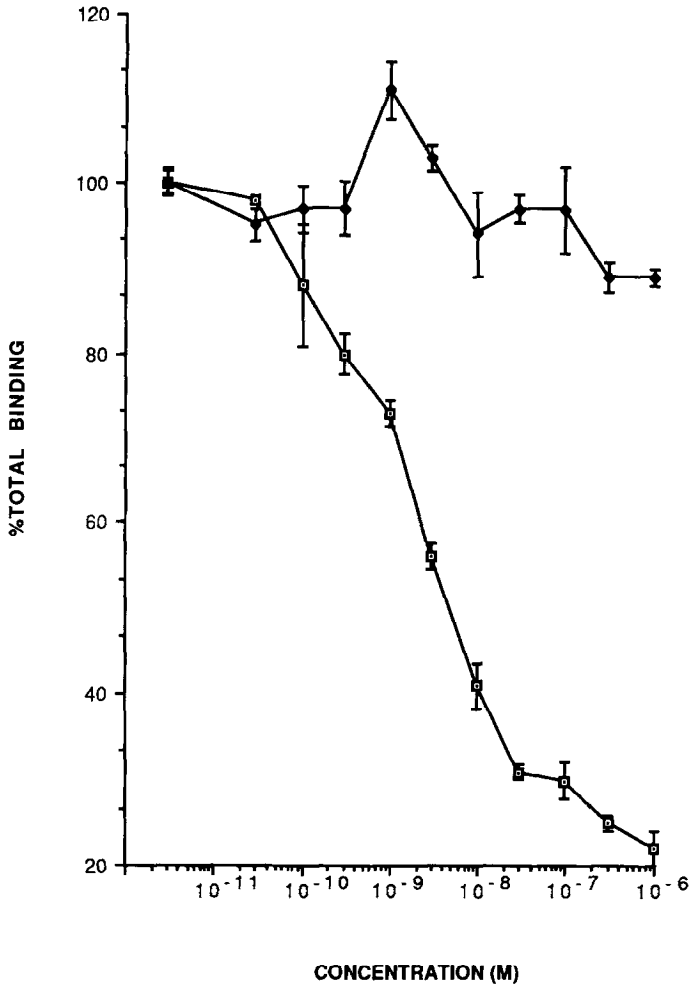

Figure 1. Binding of [^{125}I]CGRP to L6 cells.
Cells were depleted of serum for 5 hours in alpha-MEM, followed by incubation with 100,000 DPM of human (2-[^{125}I]-iodohistidyl^{10})CGRP plus various concentrations of unlabelled, rat CGRP (⊡—⊡) or human calcitonin (♦—♦) for 16 hours at 4°C. The cell monolayer was washed, dissolved in 0.1 N NaOH and an aliquot was counted in a liquid scintillation counter. Results are the mean ± S.E. of triplicate determinations in duplicate experiments.

displaced bound [I^{125}]CGRP with an IC_{50} of approximately 1 nM. Calcitonin at concentrations up to 1 μM had no effect on the binding of CGRP. Receptor occupancy appears to be coupled to adenylyl cyclase. As shown in Figure 2, CGRP increased the concentration of intracellular cAMP in a dose dependent manner. The ED_{50} for increasing cAMP agreed well with the IC_{50} for inhibition of [I^{125}]CGRP binding. Concentrations of CGRP greater than $3x10^{-9}$ M were required to increase cAMP. The maximal

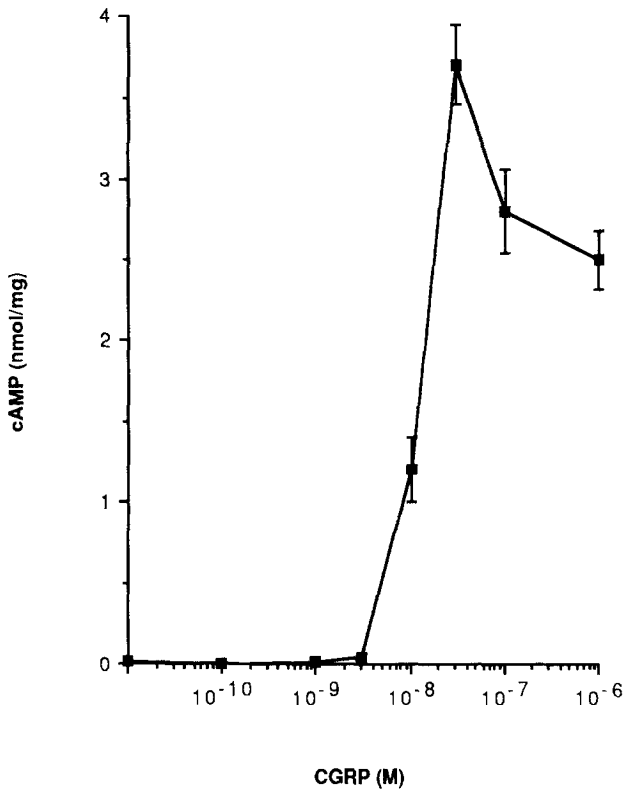

Figure 2. Increase in intracellular cAMP by CGRP.
Cells were depleted of serum for 5 hours in alpha-MEM, followed by incubation with CGRP for 15 minutes at 37°C. The cell monolayer was washed and dissolved in 10 mM HCl. An aliquot was neutralized and used for the determination of cAMP by RIA. Results are the mean ± S.E. of triplicate determinations in duplicate experiments.

effect of CGRP was observed at $3x10^{-8}$ M. At this concentration, CGRP increased the cellular content of cAMP 500-fold from a basal value of 6.9 pmol/mg protein to 3.7 nmol/mg protein. Higher concentrations of CGRP resulted in somewhat lower concentrations of cAMP, although the levels were still greatly elevated over basal. At 10^{-7} M and 10^{-6} M peptide, the cellular content of cAMP was 2.8 nmol/mg protein and 2.5 nmol/mg protein, respectively.

Acute incubation (40 minutes) of L6 cells with 1 μM insulin stimulated 2-deoxyglucose uptake 1.6-fold (Fig. 3). Simultaneous addition of CGRP suppressed the stimulatory effect of insulin. As shown in Figure 3, concentrations of CGRP as low as 10^{-10} M significantly inhibited insulin action. The maximum inhibition induced by CGRP was approximately 70%, and was observed at 10^{-8} M peptide. At 10^{-7} M CGRP the suppression of insulin action was partially reversed.

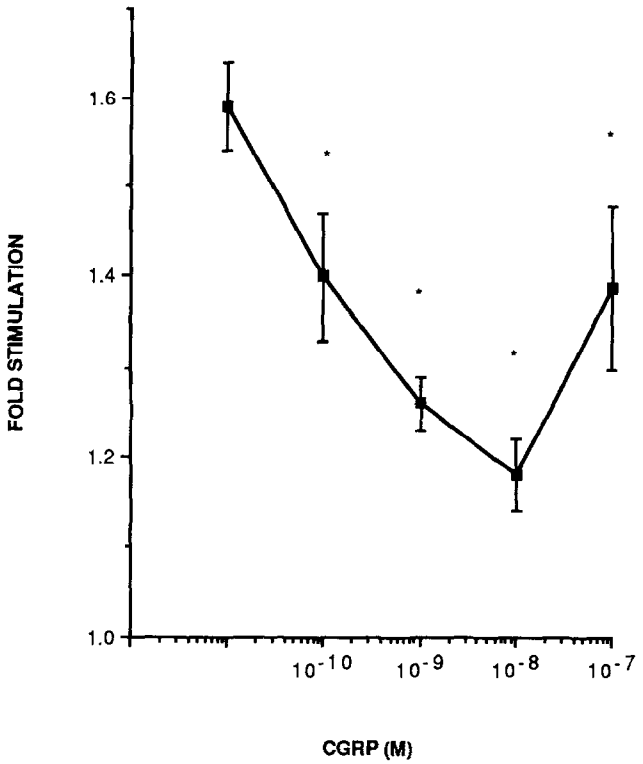

Figure 3. Induction of insulin resistance by CGRP.
Cells were depleted of serum for 5 hours in alpha-MEM. During the final forty minutes of this period, 10^{-6} M insulin and the indicated concentrations of CGRP were added. At the end of 5 hours, the cell monolayer was washed, and uptake of 0.1 mM [1-^{14}C]2-deoxyglucose was initiated. After 10 minutes at room temperature, the monolayer was washed, dissolved in 0.1 N NaOH and an aliquot was counted in a liquid scintillation counter. Results are the mean ± S.E. of triplicate determinations in five experiments. * indicates $p < .05$

DISCUSSION

To our knowledge, the results of the present study are the first demonstration that CGRP interacts specifically with adenylyl cyclase-coupled receptors on L6 myocytes. Labelled peptide was displaced by rat CGRP with an IC_{50} of 10^{-9} M. The apparent affinity of this receptor agrees well with that observed in rat liver membranes where the Kd is ~$3x10^{-10}$ M (12, unpublished observations), and gastric smooth muscle cells where the Kd is $3x10^{-9}$ M (13). The high affinity of the receptor for CGRP and the lack of competition by calcitonin suggest that CGRP interacts with L6 cells via specific CGRP receptors, rather than by binding to calcitonin receptors.

Concentrations of CGRP greater than $3x10^{-9}$ M increased the intracellular content of cAMP. At $3x10^{-8}$ M, CGRP caused a 500-fold increase in the concentration of cAMP. This effect is consistent with observations in mouse soleus muscle cells (3), cultured chick myocytes (2) and isolated mouse diaphragm (4), although the magnitude of the effect in L6 cells is greater. The effect of $3x10^{-8}$ M CGRP in L6 cells is comparable to the effect of 10^{-7} M forskolin (7ß-deacetyl-7ß-(-N-methylpiperazino) -butyryl forskolin) in these cells (unpublished observations).

We have observed that incubation of L6 cells with CGRP suppresses the effect of insulin on the uptake of 2-deoxyglucose. Acute incubation with insulin stimulates 2-deoxyglucose uptake 1.6-fold, similar to the effect observed by Klip et al. with these cells (11). CGRP between 10^{-10} and 10^{-7} M suppresses this stimulation up to 70%. These results are consistent with the reports that CGRP inhibits insulin-stimulated glycogen synthesis in isolated rat soleus muscle (5,6). However, the effects that we observe occur at lower concentrations of peptide, and the inhibition is of greater magnitude. Our results suggest that CGRP exerts its effect on insulin action at the level of glucose entry into the cell.

It has been proposed that cAMP-dependent protein kinase may play a role in modulating the effect of insulin (14). The tyrosine kinase activity of the insulin receptor is suppressed by cAMP (15). However, agents that are well known to act through the cAMP messenger system, such as ß-adrenergic agonists, forskolin and isobutylmethylxanthine, affect insulin action by both cAMP-dependent and independent mechanisms (8-11). The effect of CGRP on insulin action in L6 cells does not appear to be solely mediated by cAMP. Concentrations of peptide that significantly suppress insulin-stimulated deoxyglucose uptake have no effect on intracellular cAMP concentration, suggesting a dissociation between the effects of peptide on deoxyglucose uptake and cAMP, consistent with the report that the effects of forskolin on cAMP and insulin-stimulated deoxyglucose uptake in L6 cells are separate phenomena (11). We observe an effect of CGRP on cAMP concentration only when the concentration of peptide is increased to 10^{-8} M. Thus, the effects on insulin action are observed at much lower concentrations than the effects on cellular cAMP content. However, it is interesting to note that the effects of CGRP on both cAMP levels and insulin action are biphasic, and that the maximal response of both parameters is observed between 10^{-8} and $3x10^{-8}$ M peptide, with higher concentrations resulting in less of a response. It is conceivable that the partial reversal of the insulin resistance at 10^{-7} M CGRP is related to the decrease in cAMP observed at this concentration of peptide, suggesting some involvement of the cAMP messenger system in the action of very high concentrations of CGRP.

In summary, we have reported that CGRP binds specifically to L6 cells and increases the cellular content of cAMP. However, CGRP suppresses the effect of insulin on these cells primarily through a cAMP-independent mechanism. Only extremely high concentrations of peptide may have a cAMP-mediated component of action. Our results suggest that the effect of CGRP on insulin action may be exerted at the level of glucose entry into the cell. The exact nature of this effect remains to be determined.

REFERENCES

1. Breimer, L.H., MacIntyre, I. and Zaidi, M. (1988) Biochem. J. 255, 377-390.

2. Laufer, R. and Changeux, J.-P. (1987) EMBO J. 6, 901-906.

3. Mulle, C., Benoit, P., Pinset, C., Roa, M. and Changeux, J.-P. (1988) Proc. Natl. Acad. U.S.A. 85, 5728-5732.

4. Takami, K., Hashimoto, K., Ushida, S., Tohyama, M. and Yoshida, H. (1986) Jpn. J. Pharmacol. 42, 345-350.

5. Leighton, B. and Cooper, G.J.S. (1988) Nature 335, 632-635.

6. Cooper, G.J.S., Leighton, B., Dimitriadis, G.D., Parry-Billings, M., Kowalchuk, J.M., Howland, K., Rothbard, J.B., Willis, A.C. and Reid, K.B.M. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 7763-7766.

7. Challiss, R.A.J., Lozeman, F.J., Leighton, B. and Newsholme, E.A. (1986) Biochem. J. 233, 377-381.

8. Kashiwagi, A., Hueckstead, T.P. and Foley, J.E. (1983) J. Biol. Chem. 258, 13685-13692.

9. Kuroda, M., Honnor, R.C., Cushman, S.W., Londos, C. and Simpson, I.A. (1987) J. Biol. Chem. 262, 245-253.

10. Shanahan, M.F., Edwards, B.M. and Ruoho, A.E. (1986) Biochim. Biophys. Acta 887, 121-129.

11. Klip, A., Ramal, T., Douen, A.G., Bilan, P.J. and Skorecki, K.L. (1988) Biochem. J. 255, 1023-1029.

12. Yamaguchi, A., Chiba, T., Okimura, Y., Yamatani, T., Morishita, T., Nakamura, A., Inui, T., Noda, T. and Fujita, T. (1988) Biochem. Biophys. Res. Commun. 152, 383-391.

13. Maton, P.N., Sutliff, V.E., Zhou, Z.C., Collins, S.M., Gardner, J.D. and Jensen, R.T. (1988) Am. J. Physiol. 254, G789-794.

14. Haring, H., Kirsch, D., Obermaier, B., Ermel, B. and Machicao, F. (1986) Biochem. J. 234, 59-66.

15. Tanti, J.-F., Gremeaux, T., Rochet, N., van Obberghen, E. and Le Marchand-Brustel, Y. (1987) Biochem. J. 245, 19-26.