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$^{8-37}$h-CGRP antagonizes actions of amylin on carbohydrate metabolism in vitro and in vivo

Ming-Wei Wang, Andrew A. Young, Timothy J. Rink and Garth J.S. Cooper

Physiology Department, Amylin Corporation, 9373 Towne Centre Drive, San Diego, California 92121, USA

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$^{8-37}$hCGRP is a potent antagonist at calcitonin gene-related peptide (CGRP) liver membrane receptors, it also blocks vasodilator actions of CGRP and the related peptide, amylin, in intact rats We now report that $^{8-37}$ hCGRP antagonizes amylin inhibition of insulin-stimulated labelled glucose uptake into isolated rat soleus muscle, and inhibits amylin-evoked elevation of plasma lactate and glucose in fasted anaesthetized rats The different relative potencies of the 3 ligands (amylin CGRP, $^{8-37}$hCGRP) on the liver receptor and on the soleus muscle point to distinct CGRP receptors in liver membranes and amylin receptors on muscle cells

Amylin, Calcitonin gene-related peptide, Rat soleus muscle, Glucose, Lactate, Plasma calcium

1 INTRODUCTION

Amylin is a 37-amino acid protein which is the major constituent of the islet amyloid found in type 2 diabetic patients [1] Amylin is expressed primarily in pancreatic β-cells and is co-secreted from these cells along with insulin, following stimulation by nutrients such as glucose and arginine [2]. Amylin exerts powerful effects on glucose metabolism in isolated skeletal muscle, and in intact animals. In isolated rat soleus muscle, amylin was found to inhibit insulin-stimulated incorporation of labelled glucose into glycogen with an EC_{50} near 1 0 nM [3] Among subsequently reported effects of amylin on skeletal muscle in vitro is a decrease in glucose uptake [4], an increase in lactate production [3], a decrease in total glycogen [5] and activation of phosphorylase-*a*, the rate limiting enzyme for glycogen breakdown [6,7]

Calcitonin gene-related peptide (CGRP) is a widely distributed peripheral and central neurotransmitter Amylin and CGRP are structurally related, α-hCGRP and β-hCGRP have respectively 43 and 46% amino acid sequence identity with human amylin as shown in Fig 1 All known forms of amylin and CGRP share 2 post-translational modifications essential for full biological activity, namely a Cys^2–Cys^7 disulfide bond and an amidated C-terminal carboxyl group [8] Amylin and CGRP also appear to be functionally related They both inhibit insulin-stimulated incorporation of labelled glucose into glycogen with comparable potency [3] CGRP is a potent vasodilator [9], and amylin also has vasodilator activity [10–12]

In vivo, amylin is reported to reduce insulin-stimulated glucose clearance, and partly to reverse insulin inhibition of hepatic glucose output in animals during hyperinsulinemic euglycaemic clamps [13–15] Infusions of CGRP are reported to exert similar effects [13,16]. Both amylin [17,18] and CGRP [19,20] have a hypocalcemic action through the inhibition of bone resorption We have found that intravenous injections of amylin [12] and CGRP evoke a similar spectrum of responses, but with different relative potencies, CGRP is approximately 65 times more potent as a hypotensive agent than is amylin and evokes more prolonged falls in blood pressure, CGRP is slightly less potent than amylin in evoking increments in lactate and glucose, but is slightly more potent in promoting decrements in calcium (M-W Wang et al., in preparation).

CGRP and amylin may evoke their phsyiologic responses via a family of membrane receptors at which they act with different relative potencies The CGRP

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Position          5    10   15   20   25   30   35
                  |    |    |    |    |    |    |
Human amylin      KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY-NH2
Rat amylin        KCNTATCATQRLANFLVRSSNNLGPVLPPTNVGSNTY-NH2
α-hCGRP           ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF-NH2
β-hCGRP           ACNTATCVTHRLAGLLSRSGGMVKSNFVPTNVGSKAF-NH2
8-37hCGRP               VTHRLAGLLSRSGGVVKNNFVPTNVGSKAF-NH2
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Fig 1 Amino acid sequences Sequences of human amylin, rat amylin, α-human calcitonin gene-related peptide (CGRP), β-human CGRP, and $^{8-37}$hCGRP For all except the last, there is an intramolecular disulfide linkage between Cys^2 and Cys^7

Correspondence address M-W Wang, Physiology Department, Amylin Corporation, 9373 Towne Centre Drive, San Diego California 92121, USA Fax (1) (619) 5522212

fragment, $^{8-37}$ hCGRP, which displaces labelled CGRP from liver membranes with an apparent affinity of 260 pM, has proved to be a competitive antagonist in inhibiting CGRP activation of adenylate cyclase [21] In vivo, $^{8-37}$CGRP reversibly inhibited the vasodilator action of CGRP [22], and has been shown to inhibit the vasodilator action of amylin [11,12] In the present experiments, we report the action of $^{8-37}$hCGRP to block amylin responses in isolated rat soleus muscle, and to block the hyperlactaemic and hyperglycaemic responses to a 25 5 nmol intravenous bolus of amylin in fasted anaesthetized rats

2. MATERIALS AND METHODS

2 1 *Materials*

2 1 1 *Animals* For in vivo experiments, Harlan Sprague-Dawley rats (332±9 g, males, age 93±5 days) were used They were housed at 22 7±0 8°C in a 12 12 hour light dark cycle (experiments being performed during the light cycle) and fed and watered ad libitum (Diet LM-485, Teklad, Madison, WI) Animals were fasted for 20±0 5 h before experiments For isolated soleus muscle assays, rats weighed 200–250 g and were fasted for 4 h before use

2 1 2 *Chemicals* Soluble insulin, (Humulin-R, 100 U/ml) was purchased from Eli Lilly and Co , Indianapolis, IN The conversion factor between activity units, U, and molar units for insulin used in the present study was 1 μU/ml=7 1 pM Human $^{8-37}$CGRP (Lot #ZH201) was from Bachem (Torrance, CA) Chemical identity and purity of this peptide was determined as being around 98% by amino acid analysis, gas phase protein sequencing, and FAB mass spectrometry

Since activities of synthesized amylin vary [23], the activity of rat amylin [24] used in this study (lot #ZG485, Bachem) was first determined using the soleus muscle-based assay [3,25], the measured EC_{50} was 6 7±1 5 nM Stock solutions of rat amylin and $^{8-37}$hCGRP were prepared fresh daily in 150 mM NaCl Concentrations of amylin and $^{8-37}$ hCGRP in protein-free stock solutions were verified using quantitative amino acid analysis as previously described [26]

[U-^{14}C]-glucose (12 6 GBq/mmol) was purchased from New England Nuclear (Wilmington, DE) All other reagents were of analytical grade or better

2 2 *In vitro methods*

Isolation and incubation of stripped rat soleus muscles in the presence of various concentrations of insulin, amylin and $^{8-37}$hCGRP, and determination of rates of radioglucose incorporation into glycogen, were performed according to previously described methods [3,25] Muscles were pre-incubated in Erlenmeyer flasks containing 10 ml Krebs-Ringer bicarbonate buffer at 37°C with the following composition (in mM) NaCl, 118 5, $NaHCO_3$, 25, KCl, 5 94, $CaCl_2$, 2 54, KH_2PO_4, 1 19, $MgSO_4$, 1 19, D-glucose, 5 5, pH 7 40 Flasks were gassed continuously with O_2 CO_2 (95 5 v/v) After preincubation of muscles in this medium for 30 min at 37°C in an oscillating water bath muscle strips were transferred to similar vials containing the same medium with added [U-^{14}C]glucose (at 0 5 μCi/ml), human insulin (7 1 nM), rat amylin (100 nM) and increasing concentrations of $^{8-37}$ hCGRP (0, 1 10, 100, 1000, 1×10^4 3×10^4, 1×10^5 nM) (Fig 3) Muscles were incubated for a further 60 min, then blotted and [U-^{14}C]glucose incorporation into glycogen was measured Four muscle strips were incubated at each treatment condition, and each experiment was repeated 3 times

2 3 *In vivo methods*

2 3 1 *Surgery and instrumentation* Anaesthesia was induced in 18-h fasted rats using 5% halothane which was then maintained at 2% during surgery and at 0 8 1% during subsequent metabolic recordings Tracheotomy and cannulation of right femoral artery and vein were performed and core temperature controlled with a thermoregulator (Model 73A, YSI, Yellow Springs, OH) which switched a heated operating table

The femoral arterial line was connected to a pressure transducer (Spectramed P23XL transducer, Model 13-4615-58 amplifier, Gould, Cleveland, OH) and perfused with heparinized saline (2 U/ml) at 3 0 ml/h The femoral venous line was used for acute (bolus) injections, and $^{8-37}$hCGRP was added to this infusate for chronic administration

Signals for arterial pressure were sampled and stored with 12-bit precision at 20 Hz using a computerized data acquisition system (DT2801A A/D converters, DataTranslation, Marlboro, MA, AST Premium 386 computer, AST Research, Irvine, CA Labtech Notebook software, Laboratory Technologies Corp , Wilmington, MA)

2 3 2 *Treatment groups* There were three treatment groups

(1) Amylin bolus (n=7) after an initial 2-h infusion, animals received 100 μl (i v) saline containing 25 5 nmol freshly dissolved rat amylin.
(2) $^{8-37}$hCGRP Primed continuous infusion with amylin bolus (n=3) animals were injected at t=−30 min with a 160 nmol bolus of $^{8-37}$hCGRP, followed by a continuous infusion of this peptide at 1 6 μmol/h for 2 h, then at 320 nmol/h for a further hour, the total $^{8-37}$hCGRP delivered being 3 7 μmol/rat (11 1 μmol/kg) At t=0 min, animals received 100 μl (i v) saline containing 25 5 nmol fresh rat amylin as in the group above
(3) Saline controls (n=7), instead of fresh amylin, rats were injected with 100 μl of saline vehicle

2 4 *Chemical methods and data analysis*

Arterial samples were drawn 0 5, 0 25 and 0 h before, and 0 5, 1, 1 5, 2, 3, and 4 h after bolus injection Samples were collected into heparinized capillaries and separated plasma analyed for glucose, lactate and total calcium Glucose and lactate were analyzed by immobilized enzyme chemistries (glucose oxidase L-lactate oxidase, Analyzer model 2300-STAT, YSI, Yellow Springs, OH) Calcium was measured using a dye-binding assay (*o*-Cresolphthalein complexone, Sigma procedure #587, Sigma Chemical Co , St Louis, MO)

Statistical analysis was performed using the non-paired two-tailed Student's t-test (pooled variances method) contained in the SYSTAT system [27], with levels of significance as stated Results are reported as means ±SEM Sigmoid dose–response analyses from which EC_{50} values were derived, used a least-squares iterative routine to fit a 4-parameter logistic function [28]

3 RESULTS AND DISCUSSION

3.1 *Effects in skeletal muscle*

Figure 2 shows that 100 μM $^{8-37}$hCGRP could completely abrogate the ability of 100 nM rat amylin to suppress insulin-stimulated incorporation of labelled glucose into glycogen in isolated rat soleus muscle This result is most readily attributed to blockade of amylin action at the cell surface, presumably at amylin receptors since both amylin and $^{8-37}$hCGRP are large molecules and therefore unlikely to permeate cell membranes The design of the experiment makes it unlikely that $^{8-37}$hCGRP was acting in some non-specific manner, because it restored a complex metabolic response to insulin Applied in the absence of amylin, 100 μM $^{8-37}$hCGRP did not change insulin-stimulated glucose incorporation into glycogen, indicating that $^{8-37}$hCGRP has no amylin-agonist activity in this assay

Figure 3 shows the dose-response curve for $^{8-37}$hCGRP in the soleus muscle preparation. In the presence of 100 nM rat amylin, the IC_{50} is calculated to

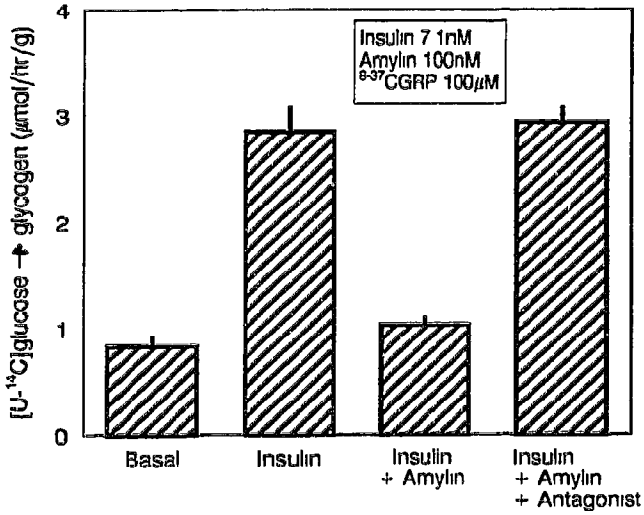

Fig 2 In vitro agonist and antagonist activity Isolated soleus muscles were pre-incubated for 30 min in Krebs–Ringer bicarbonate buffer containing no added hormones (basal), 7 1 nM insulin, 7 1 nM insulin + 100 nM amylin, and 7 1 nM insulin + 100 nM amylin + 100 μM $^{8-37}$hCGRP Net incorporation of [U-^{14}C]glucose added to the medium for the next hour was measured in extracted glycogen and expressed as the calculated rate of glucosyl units transferred onto glycogen/h/g wet muscle n=12 muscle strips, means ± SEM

be 5 9 μM with a standard error of ±0 13 log units Schild analysis [29] of the shifts invoked by $^{8-37}$hCGRP (0 1, 1, 10, 30, 100 μM) in the amylin dose–response for suppression of insulin-stimulated glucose incorporation into soleus muscle glycogen (data not shown) returned a pA_2 of 7 1, corresponding to a K_B of 80 nM That is, $^{8-37}$hCGRP has an apparent K_B for skeletal muscle amylin receptors about 300 times greater than the value of 260 pM reported for displacement of CGRP from membranes [21] Thus, as previously reported in the liver membrane assay, $^{8-37}$hCGRP has a higher affinity than does amylin while in the soleus muscle assay amylin has a higher affinity than does $^{8-37}$hCGRP. These results further indicate that amylin (and CGRP) effects on skeletal muscle are mediated via receptors distinct from those in liver membranes. Collectively, the data in the present study combined with previous reports suggest the following order of potency for three ligands considered here, for the liver receptor, CGRP>$^{8-37}$hCGRP>amylin, and for the soleus muscle receptor, amylin≥CGRP≫$^{8-37}$hCGRP

3.2 *Effects in anaesthetized rats*

Figure 4 shows that intravenous infusion of $^{8-37}$hCGRP into anaesthetized rats suppressed the increase in plasma lactate and glucose normally evoked by an intravenous bolus of 25 5 nmol rat amylin The transient fall in blood pressure normally evoked by this dose of amylin was also blocked, confirming previous findings [11] Inhibition of the vasodilation is unlikely to explain the inhibition of the lactaemic and glycaemic response to amylin since these responses also occurred following subcutaneous amylin injection when there was no change in blood pressure $^{8-37}$hCGRP itself evoked significant changes in neither plasma lactate and glucose, nor in blood pressure at blocking doses Interestingly, infusion of $^{8-37}$hCGRP appeared not to inhibit

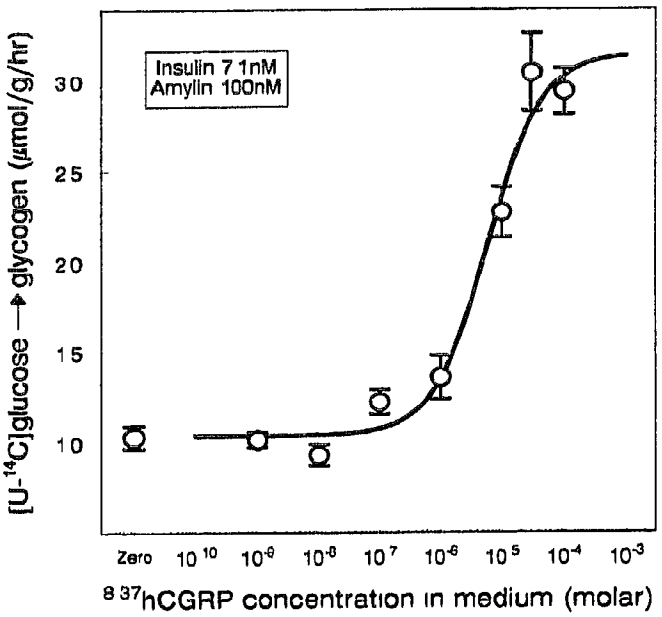

Fig 3 In vitro dose-response In vitro doseresponse for the antagonism by $^{8-37}$hCGRP of the effect of 100 nM amylin to inhibit insulin-stimulated [U-^{14}C]glucose incorporation in glycogen in isolated soleus muscle The estimated EC_{50} was 5 92 μM ±0 13 log units

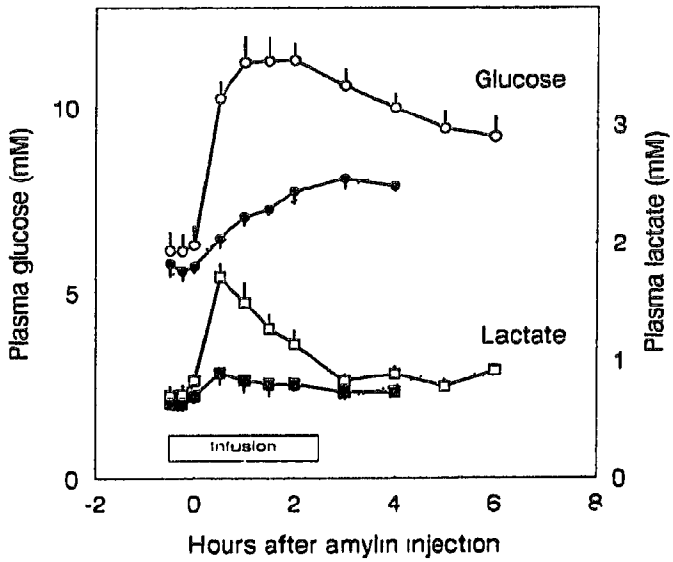

Fig 4 In vivo amylin agonist and antagonist responses Amylin-induced changes in plasma glucose ( ) and lactate (□) without (n=7) and with (●, ■, n=3) a primed-continuous $^{8-37}$hCGRP infusion The response to saline alone (, n=7) is also shown Symbols represent the means ± SEM

the calcium lowering effect of rat amylin (data not shown). This result is consistent with an amylin influence on calcium homeostasis via actions on osteoclasts [17,18] through receptors distinct from those mediating amylin actions in skeletal muscle and from those mediating vasodilator effects

It is interesting that a simple modification to CGRP, the removal of 7 N-terminal amino acids with the Cys^2–Cys^7 disulfide bond, converts a full agonist into a relatively potent antagonist with no apparent agonist action at the receptor subtypes examined in the current experiments Apparently, the C-terminal 30 amino acids contain motifs that can bind tightly to the liver CGRP receptor and somewhat less tightly to the muscle amylin receptor Although $^{8-37}$hCGRP antagonizes the vascular effects of CGRP (and amylin), this antagonist can also inhibit amylin action at amylin doses that have no measurable vascular effects Therefore $^{8-37}$hCGRP should be a useful pharmacological tool for exploring amylin's biologic actions, and its role in metabolic regulation and disorders

3.3. *Conclusions*

These results demonstrate that $^{8-37}$hCGRP is an effective inhibitor of amylin action in isolated skeletal muscle. The data are consistent with $^{8-37}$hCGRP being a competitive amylin receptor antagonist The finding that this antagonist inhibits amylin-evoked elevation of plasma lactate and glucose, supports the proposal that amylin's metabolic effects in vivo reflect its action on skeletal muscle to promote glycogenolysis and release of lactate

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REFERENCES

[1] Cooper, G J S , Willis, A C , Clark, A , Turner, R C , Sim, R B and Reid, K B (1987) Proc Natl Acad Sci USA 84, 8628–8632
[2] Ogawa, A , Harris, V , Mccorkle, S K , Unger, R H and Luskey, K L (1990) J Clin Invest 85 973–976
[3] Leighton, B and Cooper, G J S (1988) Nature 335, 632–635
[4] Kreutter, D , Orena, S J and Andrews, G C (1990) Diabetes 39, 121A
[5] Young, D A , Deems, R O , Deacon, R W , Mcintosh, R H and Foley, J E (1990) Am J Physiol 259, E457–E461
[6] Young, A A , Mott, D M Stone K and Cooper, G J S (1991) FEBS Lett 28 149–151
[7] Deems, R O , Deacon, R W and Young, D A (1991) Biochem Biophys Res Commun 174, 716–720
[8] Roberts, A , Leighton, B , Todd, J A , Cockburn, D , Schofield, P N , Sutton, R , Holt, S , Boyd, Y , Day, A J , Foot, E A , Willis, A C , Reid, K B M and Cooper, G J S (1989) Proc Natl Acad Sci USA 86, 9662–9666
[9] O'Halloran, D J and Bloom, S R (1991) Br Med J 302, 739–740
[10] Brain, S D , Wimalawansa, S , MacIntyre, T and Williams, T J (1990) Am J Pathol 136, 487–490
[11] Gardiner, S M , Compton, A M , Kemp, P A , Bennett, T , Bose, C , Foulkes, R and Hughes, B (1991) Diabetes (in press)
[12] Young, A A , Wang, M -W , Rink, T J and Cooper, G J S (1991) J Physiol (Proceedings) (in press)
[13] Molina, J M , Cooper, G J S , Leighton, B and Olefsky, J M (1990) Diabetes 39, 260–265
[14] Koopmans, S J , van Mansfeld, A D M , Jansz, H S , Krans, H M J , Radder, J K , Frolich, M , deBoer, S F , Kreutter, D K , Andrews, G C and Maassen, J A (1991) Diabetologia 34, 218–224
[15] Frontoni, S , Choi, S B , Banduch, D and Rossetti, L (1991) Diabetes 40, 568–573
[16] Choi, S B , Frontoni, S and Rossetti, L (1991) Am J Physiol 260, E321–E325
[17] Datta, H K Zaidi, M , Wimalawansa, S J , Ghatei, M A , Beacham, J L , Bloom, S R and MacIntyre, I (1989) Biochem Biophys Res Commun 162, 876–881
[18] Towhidul-Alam, A S M , Moonga, B S , Bevis, B J R , Huang, C L -H and Zaidi, M (1991) J Physiol (in press)
[19] Zaidi, M , Brain, S D , Tippins, J R , Di Marzo, V , Moonga, B S , Chambers, T J , Morris, H R and MacIntyre I (1990) Biochem J 269, 775–780
[20] Zaidi, M , Chambers, T J , Bevis, P J , Beacham, J L , Gaines Das, R E and MacIntyre, I (1988) Q J Exp Physiol 73, 471–485
[21] Chiba, T , Yamaguchi, A , Yamatani, T , Nakamura, A , Morishita, T , Inui, T , Fukase, M , Noda, T and Fujita, T (1989) Am J Physiol 256, E331–E335
[22] Gardiner, S M , Compton, A M , Kemp, P A , Bennett, T , Bose, C , Foulkes, R and Hughes, B (1990) Biochem Biophys Res Commun 171, 938–943
[23] Lehman-de Gaeta, L S , Willis, A C , Young, A A , Foot E A , Albrecht, E , Leighton, B , Rink, T J and Cooper, G J S (1991) Diabetes 40, 236A
[24] Leffert, J D , Newgard, C B , Okamoto, H , Milburn, J L and Luskey, K L (1989) Proc Natl Acad Sci USA 86, 3127–3130
[25] Young, A A , Gedulin, B , Wolfe-Lopez D , Greene, H E , Rink, T J and Cooper, G J S , Am J Physiol (submitted)
[26] Heinrikson, R L and Meredith, S C (1984) Anal Biochem 136, 65–74
[27] Wilkinson, L (1988) in SYSTAT The System for Statistics, Systat Inc , Evanston, IL
[28] DeLean, A , Munson, P J , Guardabasso, V and Rodbard, D (1988) in ALLFIT Simultaneous Fitting of Families of Sigmoidal Dose Response Curves Using the Four-Parameter Logistic Equation, National Institute of Child Health and Human Development, Bethesda, MD
[29] Schild, H O (1947) Br J Pharmacol 2, 189