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Selective antagonism of calcitonin-induced osteoclastic quiescence (Q effect) by human calcitonin gene-related peptide-(Val^{8}Phe^{37})

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Exposure of isolated rat osteoclasts to calcitonin (CT) leads to an abrupt cessation of cell motility (Q effect) followed by cell retraction (R effect). We have previously shown that these effects are mediated by two G proteins that appear to activate separate post-receptor pathways. The present study demonstrates that the Q but not the R effect of CT (0.006 μM) is abolished in the presence of human calcitonin gene-related peptide (CGRP)-(Val^{8}Phe^{37}) (0.5 μM), a fragment analogue of human CGRP. This selective antagonism suggests that the Q effect could result from an action of CT upon a site that is distinct from that producing the R effect. The former site ('amylin site') also appears to interact with related peptides, amylin and CGRP, whilst the latter site ('CT site') specifically interacts with CT.

Osteoclasts have a high density of calcitonin (CT)-binding sites (1, 2). Their activation by CT leads to the generation of cyclic adenosine monophosphate (cAMP) (1, 2) and an elevation of intracellular calcium ($[Ca^{++}]_i$) (3, 4). These signals mediate respectively, the two qualitatively and kinetically distinct inhibitory responses, namely quiescence (Q effect) and retraction (R effect) (3). The two second messenger pathways also appear be linked proximally to different G-proteins (3). Our studies have shown that molecules, that are structurally homologous to CT, including amylin and calcitonin gene-related peptide (CGRP), produce the Q effect of CT, whilst failing to mimic its R effect (5). This observation prompted us to investigate whether the observed intracellular and functional dichotomy of the action of CT on the osteoclast was the result of activation of separate binding sites. We found that pretreatment of osteoclasts with a fragment of CGRP, human CGRP-(Val^{8}Phe^{37}), led to the abolition of the cAMP-dependent Q effects of both amylin and CT, whilst the R effect of CT remained unaffected.

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MATERIALS AND METHODS

Materials: Asu-(Cys^1-Cys^7)-eel calcitonin (eCT) (Elcatonin), human CGRP (hCGRP) and human amylin were gifts from Drs. F. Avaldi (ISF, Milan, Italy), J. Pless (Sandoz, Basle, Switzerland) and T.J. Rink (Amylin Corporation, San Diego, USA). The peptides were dissolved in de-ionized water containing acetic acid (0.001%, v/v; "Aristar" grade; BDH, Dorset, U.K.) and bovine serum albumin (0.01%, w/v; BSA; Sigma, Dorset, U.K.; "recrystallized"), then lyophilized and stored at -70°C. All other chemicals were purchased from either Sigma or BDH. Indo 1-AM was purchased from Molecular Probes (Eugene, U.S.A.). Tissue culture media and heat-inactivated foetal calf serum (FCS) were purchased from Flow Laboratories, UK, Ltd. (Rickmansworth, Herts., U.K.). Osteoclasts were isolated in Medium 199 buffered with *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulphonic acid (HEPES) and supplemented with FCS (10% v/v), benzyl penicillin (Glaxo, Middlesex, U.K.; 100 IU/l) and streptomycin (Glaxo; 100 mg /l) (referred to as Medium 199).

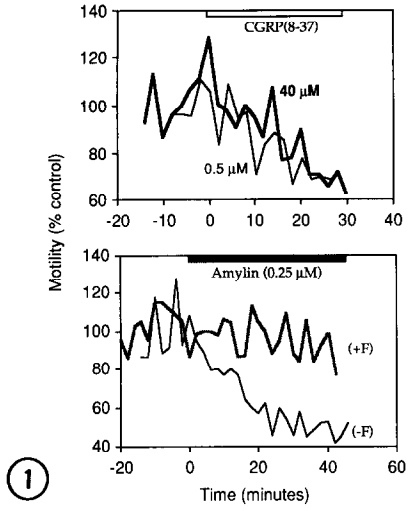
Osteoclast isolation and culture (6): The femora and tibiae from neonatal rats were used for isolation of osteoclasts. The adherent soft tissues were removed and the bones cut across their epiphyses in HEPES-buffered Medium 199. Osteoclasts were mechanically disaggregated by curetting the bones of each rat with a scalpel blade into 1 ml medium and agitating the suspension with a pipette. Larger fragments were allowed to settle for 10 s, before the supernatant was dropped onto plastic petri dishes (35 mm; Cellcult; Sterilin). The cells were then incubated at 37°C for 20 min to allow sedimentation and attachment of osteoclasts. This was followed by two gentle washes at 30 min intervals in order to obtain a functionally pure population of osteoclasts.

Quantitative analysis of cell motility (3): The petri dishes were then placed in the incubation chamber of an inverted phase-contrast microscope (Diaphot, Nikon UK, Ltd.) linked via a charge coupled device (CCD) camera to a 480 hour-mode time-lapse video recorder (Mitsubishi, Tokyo, Japan). The composite video signal was fed into a 256-grey level imaging system (Sight Systems, Newbury, Berks., U.K.) centered on a 60-Mb dual hard disc IBM microcomputer. The capture of digitised grey images into the computer memory at 2-min intervals was followed by redigitisation of the cell boundry using a mouse (Microsoft, New York, U.S.A.), an edge detection facility and a binary overlay. A tracing of each cell was then retrieved sequentially, overlaid on the previous outline and analysed using a software package programmed to measure the area within each tracing together with area change due to cell movement (shape area). Absolute cell area (A) was expressed as a percentage of control cell area. Motility was expressed as the change of shape area (ΔA) per unit cell spread area. Frame-shift errors were determined by assessing the position of two or more reference points (etch marks) selected arbitarily on the substrate.

RESULTS

Human CGRP-(Val^{8}Phe^{37}) is a weak Q-effect agonist: Exposure of isolated osteoclasts to human CGRP-(Val^{8}Phe^{37}) (0.5 and 40 μM) caused weak inhibitory effects on cell motility (Figure 1, top panel), without producing a significant R-effect (not shown). Motility was reduced by approximately 30% and there was a significant regression of response over time at both concentrations (0.5 μM: $r = 0.83$, $p < 0.001$; 40 μM: $r = 0.5$, $p < 0.05$).

Human CGRP-(Val^{8}Phe^{37}) abolishes the Q-effect of amylin: Figure 1 (bottom panel) reveals the effect of 0.25 μM-[amylin] on osteoclast motility with or without preincubation for 45 min, with 40 μM-[hCGRP-(Val^{8}Phe^{37})]. In the absence of the fragment, amylin produced a classical Q-effect. There was a significant resgression of response over time ($r = 0.89$; $p < 0.001$, half time = 18 min, slope = -1.5). However, in the presence of an approximately 100-fold greater molar concentration of the fragment, osteoclasts failed to respond to amylin ($r = 0.33$; $0.1 > p > 0.05$, slope = - 0.4).



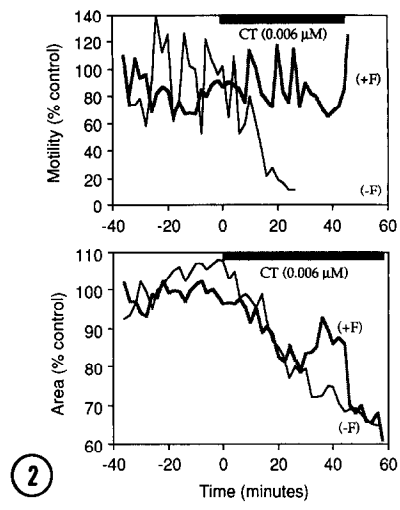


Fig. 1.
Top panel: Motility traces of isolated rat osteoclasts showing the inhibitory effect of human calcitonin gene-related peptide-(Val^{8}Phe^{37}) (0.5 and 40 µM) [CGRP(8-37)].
Bottom panel: Motility traces of isolated rat osteoclasts showing the effect of amylin (0.25 µM) with (+F) or without (-F) pretreatment with human calcitonin gene-related peptide-(Val^{8}Phe^{37}) (40 µM). The results are expressed as a percentage of control motility (change of shape area per unit cell spread area) (see Materials and Methods).

Fig. 2.
Motility (top panel) and spread area (bottom panel) traces of isolated rat osteoclasts showing the effect of calcitonin (CT) (0.006 µM) with (+F) or without (-F) pretreatment with human calcitonin gene-related peptide-(Val^{8}Phe^{37}) (0.5 µM). The results are expressed as a percentage of control motility (change of shape area per unit cell spread area) or cell spread area (A) (see Materials and Methods).

Human CGRP-(Val^{8}Phe^{37}) abolishes the Q effect but not the R effect of CT: Figure 2 shows the effect of 0.006 µM-[CT] on osteoclast motility with or without preincubation for 50 min, with an approximately 100-fold higher concentration (0.5 µM) of the fragment, hCGRP-(Val^{8}Phe^{37}). In the absence of the fragment, CT produced both Q and R effects. There was a significant regression of motility and cell spread area over time (Q effect: $r = 0.89$, $p < 0.001$, half time = 8 min, slope = -2.9; R effect: $r = 0.96$, $p < 0.001$, half time = 25 min). In the presence of the fragment, the Q effect of CT was abolished ($r = 0.105$, $p = 0.62$, slope = + 0.3), whilst the R-effect was unaffected ($r = 0.85$, $p < 0.001$, half time = 31 min).

DISCUSSION

Osteoclasts are the physiological target cells for the hormone, CT. A number of studies have characterised the binding of CT to its receptors on the osteoclast surface (for example: 1, 2) and have convincingly demonstrated elevations in cAMP levels in response to CT, both in bone cultures (1) and cell populations (2). Using biophysical techniques that have allowed us to quantitate osteoclast motility to micrometric precision (3), we have resolved the acute effects of CT into two distinct

components, the Q effect and the R effect (3). We have suggested that these qualitatively and kinetically distinct effects of CT are mediated by separate G proteins activating separate post-receptor pathways: the Q effect is cAMP-dependent, whilst the R effect is $[Ca^{++}]_i$-dependent (3). In addition, we have demonstrated that the structurally homologous peptides, amylin and CGRP (20% homology, 7), selectively mimic the Q, but not the R effect of CT, even at a 100-fold higher molar concentration (5).

In the present study, a 29 amino acid fragment of human CGRP, hCGRP-($Val^{8}Phe^{37}$), has been used to antagonise the action of the peptides on the osteoclast. This fragment has previously been shown to antagonise the action of CGRP (and CT) on cAMP-linked receptors in rat liver cells (8, 9). However, the fragment did not inhibit adenylate cyclase activity in CT-receptor positive $LLCPK_1$ kidney cells in response to CT; instead it was weakly agonistic at a concentration of 0.1 μM and above (8, 9). In contrast, we found that despite having a weak agonistic effect, the fragment completely abolished the Q effect of amylin on the osteoclast. Whilst these findings, in themselves, potentially indicated the presence of a different receptor site mediating the action of amylin on the osteoclast, this was confirmed by using the fragment in studies with CT. We found that pretreatment of osteoclasts with hCGRP-($Val^{8}Phe^{37}$) (0.5 μM) completely abolished the Q effect of CT (0.006 μM), whereas the cells continued to retract.

Thus, it is possible that CT acts upon two receptor sites on the osteoclast. One of these, the 'amylin site', can bind amylin, CGRP and CT and produce the Q-effect by a cAMP-dependent pathway, whilst the other, the 'CT-specific site', selectively binds CT resulting in cell retraction. However, in the light of our pharmacological studies at the single cell level, we cannot rule out the possibility that distinct sites present on a single receptor complex are being activated to produce distinct functional effects (Figure 3).

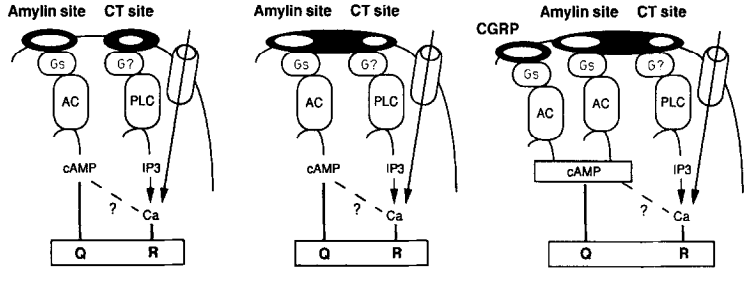


Fig. 3.
Hypothetical relationships between the functional responses of the calcitonin gene peptides and their second messenger pathways and possible receptor configurations.
Abbreviations: CT - calcitonin; CGRP - calcitonin gene-related peptide; Q - quiescence; R - retraction; IP_3 - inositol trisphosphate; cAMP - cyclic adenosine monophosphate; AC - adenylate cyclase; PLC - phospholipase C; Gs - stimulatory G protein; G? - uncharacterised G protein.

Our results are consistent with the diversity of receptor subtypes reported for the peptides encoded by the CT gene family (10). There are at least two classes of CT/CGRP receptors in the nervous system; one of these is highly specific for salmon CT and does not seem to be linked to adenylate cyclase (11, 12). However, vascular receptors for CGRP (13, 14), receptors for amylin in the muscle and pancreas (15, 16), as well as ectopically-expressed CT receptors (7) exert effects via a cAMP-dependent system and appear to cross-react with related peptides. Indeed, it may be possible to classify these receptors once the cloning data is amassed. Finally, our studies documenting the antagonism of the action of amylin by hCGRP-(Val^{8}Phe^{37}) may have widespread implications in further studies on the biology of this novel peptide and its potential physiological role in carbohydrate metabolism.

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