Calcitonin gene-related peptide elevates calcium and polarizes membrane potential in MG-63 cells by both cAMP-independent and -dependent mechanisms

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Burns, Douglas M., Lisa Stehno-Bittel, and Tomoyuki Kawase. Calcitonin gene-related peptide elevates calcium and polarizes membrane potential in MG-63 cells by both cAMP-independent and -dependent mechanisms. Am J Physiol Cell Physiol 287: C457–C467, 2004; 10.1152/ajpcell.00274.2003.—Published data suggest that the neuropeptide calcitonin gene-related peptide (CGRP) can stimulate osteoblastic bone formation; however, interest has focused on activation of cAMP-dependent signaling pathways in osteogenic cells without full consideration of the importance of cAMP-independent signaling. We have now examined the effects of CGRP on intracellular Ca2+ concentration ([Ca2+]i) and membrane potential (Em) in preosteoblastic human MG-63 cells by single-cell fluorescent confocal analysis using fluo 4-AM-fura red-AM and bis(1,3-dibarbituric acid)-trimethine oxanol [DiBAC4(3)] bis-oxonol assays. CGRP produced a two-stage change in [Ca2+]i: a rapid transient peak and a secondary sustained increase. Both responses were dose dependent with an EC50 of ~0.30 nM, and the maximal effect (initially ~3-fold over basal levels) was observed at 20 nM. The initial phase was sensitive to inhibition of Ca2+ mobilization with thapsigargin, whereas the secondary phase was eliminated only by blocking transmembrane Ca2+ influx with verapamil or inhibiting cAMP-dependent signaling with the Rp isomer of adenosine 3’,5’-cyclic monophosphorothioate (Rp-cAMPS). These data suggest that CGRP initially stimulates Ca2+ discharge from intracellular stores by a cAMP-independent mechanism and subsequently stimulates Ca2+ influx through L-type voltage-dependent Ca2+ channels by a cAMP-dependent mechanism. In addition, CGRP dose-dependently polarized cellular Em, with maximal effect at 20 nM and an EC50 of 0.30 nM. This effect was attenuated with charybdotoxin (~20%) or glyburide (glibenclamide; ~80%), suggesting that Em hyperpolarization is induced by both Ca2+-activated and ATP-sensitive K+ channels. Thus CGRP signals strongly by both cAMP-dependent and cAMP-independent signaling pathways in preosteoblastic human MG-63 cells.

osteoblastic cells; calcium; membrane potential; potassium channels; adenosine 3’,5’-cyclic monophosphate

OVER THE PAST 15 YEARS evidence has accumulated indicating that peripheral peptidergic neurons can influence cells within target tissues through localized release of neuropeptides (2, 14–17, 23, 27, 41). One important peripheral neuroeffector that may play a role in bone metabolism is α-calcitonin gene-related peptide (CGRP), a 37-residue peptide produced in specific neurons by alternative splicing of the primary transcript from the calcitonin gene (10).

Numerous publications provide in vivo demonstration that CGRP innervation is associated with bone formation (and similar mineralizing processes), particularly during development, growth, or repair (for detailed review, see Refs. 16 and 17). In vitro studies have demonstrated that CGRP stimulates the osteoblastic differentiation of bone marrow mesenchymal stromal cells (32, 33) and acts directly on osteoblastic cells to modulate phenotypic functions (7, 29, 37). These and similar data suggested that neuroactive CGRP plays a role in stimulating or maintaining bone formation in skeletal tissues. In further support of the bone anabolic action of CGRP, Vignery and coworkers demonstrated that CGRP protects against ovariectomy-induced bone loss (36) and that targeted transgenic expression of CGRP in mouse osteoblasts increases bone density 27–40% at skeletal sites by stimulating cancellous bone formation (1).

Despite data suggesting that CGRP can stimulate bone formation, its mechanism of action in osteoblast-related cells is not well understood. Historically, attention was drawn to CGRP’s pronounced stimulation of cAMP formation in immature osteoblast-related cells isolated from bone and bone linings from several species (28), but this cAMP response varies considerably in different osteoblastic cell types (Refs. 3, 19, and 28; Burns and Kawase, unpublished observations). In addition, we (18, 20, 21) and others (11, 12, 38, 39) have recently reported that CGRP can stimulate cAMP-independent intracellular signaling pathways in several osteoblastic cell types. We thought it significant that mature osteoblast-like cells respond to CGRP by increasing intracellular Ca2+ ([Ca2+]i) and/or altering membrane potential (Em) while yielding small increases (if any) in cAMP production (18, 20, 21). Similarly, Drissi et al. (11, 12) demonstrated that CGRP increases [Ca2+]i, but not cAMP, in human OHS-4 osteoblast-like cells. Recently, Villa et al. (38, 39) demonstrated that CGRP’s 2.2-fold stimulation of proliferation in primary cultures of human osteoblasts is cAMP independent. Collectively, these data suggest that cAMP may not represent the primary mode of CGRP signaling in mature osteoblastic cells.

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From among an assortment of osteoblast-like cell lines, clonal osteosarcoma-derived human MG-63 cells have been described as a model for early-stage immature preosteoblasts because of the nature of their response to factors like 1,25(OH)2-vitamin D3 and their regulated temporal development of bone-type alkaline phosphatase activity (4, 9). We very recently found (22) that MG-63 cells prominently express the type-1 CGRP receptor [calcitonin receptor-like receptor (CRLR)/receptor activity-modifying protein (RAMP)]1 and respond to CGRP with 35-fold increases in cAMP formation and rapid phosphorylation or dephosphorylation of several types of mitogen-activated protein kinase. Similar strong cAMP signaling has also been reported for immature lining and preosteoblastic cells (3, 19, 28, 34), and this correlation suggests that cAMP and cAMP-dependent pathways could be the primary mode of CGRP signaling in immature osteogenic cells.

Thus it was of considerable interest to determine whether CGRP also induces changes in [Ca2+]int or cellular E\textsubscript{m} in preosteoblastic human MG-63 cells. To this end, we have employed a sensitive, single whole cell fluorescent confocal analysis using a fluo 4-AM-Fura red-AM ratiometric assay to assay [Ca2+]\textsubscript{int} and similarly modified our bis(1,3-dibarbbituric acid)-trimethine oxanol [DiBAC\textsubscript{4}(3)] bis-oxonol assay to monitor E\textsubscript{m}. Expecting to find modest or negligible effects of CGRP on these parameters, we have instead demonstrated robust effects that exceed in complexity and magnitude those we had previously observed in a more mature model of osteogenic cells (18, 20, 21). In addition, we also report unexpected differences between our present results and our previous findings, including CGRP’s activation of cAMP-dependent, verapamil-sensitive membrane Ca\textsuperscript{2+} channels to produce a long-sustained Ca\textsuperscript{2+} increase in MG-63 cells.

Although cAMP-independent mechanisms figure into induction of the initial Ca\textsuperscript{2+} transient and polarization of E\textsubscript{m} as previously observed in UMR106 cells, the situation is substantially more complex in preosteoblastic MG-63 cells and could conceivably involve additional CGRP receptor subtypes.

**MATERIALS AND METHODS**

**Materials.** Human CGRP (Advanced Chemtech, Louisville, KY) was dissolved in 0.01% trifluoroacetic acid to a stock concentration of 100 \( \mu \)M and stored in 50-\( \mu \) aliquots at \(-40^\circ\)C; necessary dilutions were made immediately before use. Charybdoxatin (Sigma-Aldrich, St. Louis, MO) and adenosine-3',5'-cyclic monophosphorothioate, Rp isomer, triethylammonium salt (Rp-cAMPS; Calbiochem, La Jolla, CA) were dissolved in distilled water to concentrations of 500 \( \mu \)M and 5 mM, respectively. Glyburide (glibenclamide; Sigma-Aldrich) and charybdoxatin were dissolved in DMSO to 2 and 20 mM, respectively. Fluo 4-AM and fura red-AM were dissolved in DMSO to 1 and 4 mM, respectively. DiBAC\textsubscript{4}(3) (Molecular Probes, Eugene, OR) was dissolved in ethanol to 20 mM. Thapsigargin (Sigma-Aldrich) was dissolved in DMSO to a stock concentration of 10 mM.

**Cells and cell cultures.** Low-passage MG-63 osteogenic human osteosarcoma cells were obtained from the American Type Culture Collection (Manassas, VA) and plated, grown, and maintained in DMEM supplemented with 1 mM L-glutamine, standard penicillin-streptomycin, and 2% fetal bovine serum (FBS; JRH Biosciences, Mission, KS). Cells were normally plated at a density of \( 1 \times 10^4 \) cells/cm\(^2\) and cultivated in humidified 5% CO\textsubscript{2}-95% air at 37°C. Cultures were never allowed to become confluent. For the assays described below, cells were grown on sterile 1-mm-thick, 23-mm-diameter circular coverslips. All assays were performed in physiological saline solution (PSS; in mM: 130 NaCl, 5 KCl, 1.5 CaCl\textsubscript{2}, 1 glucose, 10 HEPES, pH 7.30).

**Measurement of [Ca\textsuperscript{2+}]\textsubscript{int}.** The data reported here were obtained with detailed single-cell measurements of [Ca\textsuperscript{2+}]\textsubscript{int} using the dual-dye ratiometric method of Lipp and Niggli (25, 26) and laser-scanning fluorescent confocal microscopy. In this protocol, subconfluent densities of MG-63 cells grown on circular 1-mm-thick coverslips were loaded for 30 min with 1 \( \mu \)M fluo 4-AM and 4 \( \mu \)M fura red-AM in standard DMEM supplemented with 1% FBS. Slides were rinsed twice with PSS + 0.2% FBS, mounted individually to a 23-mm-diameter Affluor slide chamber (Molecular Probes), immersed in 1 ml of PSS + 0.2% FBS, and then examined at 22°C on the stage of an inverted Nikon Eclipse T-300 microscope equipped for fluorescent dark-field imaging and coupled directly to a Bio-Rad RadiancePlus 2000 triple-laser computer-controlled scanning confocal apparatus (Bio-Rad Laboratories, Hercules, CA). Experimental treatments were introduced by withdrawing 400 \( \mu \)l of the chamber bath volume, into which was mixed the desired treatment (peptide or reagent). This solution was carefully introduced back into the slide-holding chamber with careful mixing, thus avoiding exposure of cells to unusually high levels of vehicle or treatment reagents.

To estimate [Ca\textsuperscript{2+}]\textsubscript{int}, dye-loaded cells were excited with the 488-nm line of an argon laser running at approximately <1.5 mW. The standard confocal pinhole setting for this assay was 3.2 mm, and when emissions were collected with the >40 or >60 objectives the “optical slice” was typically 0.6 \( \mu \)m thick. Fluor 4 emission at 520 ± 15 nm strongly increases as [Ca\textsuperscript{2+}] increases, whereas fura red emission at 620 ± 40 nm decreases as [Ca\textsuperscript{2+}] increases. These two emissions from individual mouse-delineated cellular cross sections were collected in separate channels. General cellular fluorescence was found to be so low as to be negligible in these confocal assays, but backgrounds were still collected in each assay.

Time course profiles were initially collected as raw data (total pixels within a computer-designated cellular cross section for each designated emission), and a background time course (equivalent areas without cells, or identical cells without dye) was collected and stored for each assay and subtracted from each time course emission. Ratiometric of these two fluorescent emissions provides a fluorescent report of [Ca\textsuperscript{2+}] levels that is independent of dye-loading patterns and similar problems. Evaluating this ratio vs. a [Ca\textsuperscript{2+}] standard curve prepared in cell homogenates and read on a coverslip with the confocal microscope under identical conditions (pinhole, laser power, objective, emission settings) permitted accurate estimation of [Ca\textsuperscript{2+}]\textsubscript{int}. These values generally agreed with values obtained through identical assays conducted instead with fura 2 and a UV laser-equipped Zeiss confocal microscope (Burns and Stehno-Bittel, unpublished data) and are presented as a quantitative estimation of [Ca\textsuperscript{2+}]\textsubscript{int}.

Although these assays require additional steps in data processing, the mean population analysis of a field of single cells yields a result that is identical, albeit with better signal-to-noise ratio, to what is measured in a cuvette assay (data not shown). These [Ca\textsuperscript{2+}]\textsubscript{int} assays were most reliable when conducted in PSS + 0.2% FBS, because most cells displayed periodic 10–20% fluctuation in [Ca\textsuperscript{2+}]\textsubscript{int} when studied in DMEM + 0.2% FBS (possibly because of the presence of the ionotropic glutamate receptor channel).

**Measurement of E\textsubscript{m}.** The basic method for measuring changes in cellular E\textsubscript{m} was similar to what we have previously reported (18) and is a modification of Civitelli’s original protocol (8). Subconfluent MG-63 cells grown on coverslips were rinsed with PSS containing 0.2% FBS and then incubated for 40 min in the same medium supplemented with 0.5 \( \mu \)M levels of the bis-oxonol dye DiBAC\textsubscript{4}(3). As cells polarize, the 500- to 600-nm fluorescent emission from DiBAC\textsubscript{4}(3) decreases (5, 8), and pilot experiments conducted in a
quartz cuvette with a Shimadzu RC-5391-PC spectrofluorometer confirmed this phenomenon.

The data reported here were generated by single whole cell assay of $E_m$ changes in MG-63 cells plated onto circular 1-mm coverslips with laser-scanning confocal fluorescent microscopy (as above). Excitation was with the 488-nm line of the argon laser (running at 1.5 mW), and the 500- to 620-nm fluorescent emission (620-nm dichroic mirror + 500-nm LP emission filter) was collected. Bis-oxonol dye is not as bright as fluo 4, so a 3- to 3.5-mm pinhole was used, yielding an effective optical slice of $\approx 0.8$ μm when the $\times 40$ or $\times 60$ objectives were used. These data were collected to permit estimation of relative $E_m$ values vs. that of an initial 5-min baseline (see below). Between 8 and 20 cells were studied in each microscopic field. As before, data were collected and stored as time course profiles.

Data analysis. For both assays the specific cellular fluorescence (defined as the total number of bright pixels/cell cross section − background intensity pixel data) was imported into MicroSoft Excel. Accurate $[Ca^{2+}]_{int}$ values were obtained as described above. For $E_m$ time courses, each data string from a cell was normalized vs. the initial average baseline (average initial fluorescent pixel count/cell over 5 min) to adjust for differences in the area of cellular footprint. It has so far proven impossible to quantitate the DiBAC$_{4}(3)$ emission against a standard curve to produce quantitative estimation of $E_m$ values; thus all $E_m$ data are presented as fluorescence intensity of emission as a fraction of the initial value, much as we did previously (18).

Pooling of single-cell recordings and aggregate analysis. It was important to determine the overall response within a given microscopic field, even if individual cells were heterogeneous in their time course response. Thus the “mean population value” for each time point was determined by averaging the aggregate value from each of the cells being examined in a particular experiment, and this mean value ± SE is plotted for each time point. In this manner, rather complex responses could be analyzed for general parameters and overall properties. It is of interest that the aggregate response of a number of imaged cells (generally when $n > 6$ cells) strongly resembles the data obtained from analyzing hundreds of adherent cells in cuvette-sized assays in the spectrofluorometer. Whenever the magnitude of a response with one treatment was evaluated against the magnitude obtained with a different treatment, the area under the curve (AUC) for the corresponding time interval from each result was estimated and directly compared.

Statistical analysis. Time course profiles that are plots of the mean population value determined by averaging the same time point from the individual profiles recorded in 8–22 individual cells display means ± SE. For every case, the standard deviation is $<5\%$ of the mean value shown. When necessary, mean population values are compared point by point at areas of interest against the baseline mean population profile using a simple two-tailed Student’s t-test to determine whether the values are different with a statistical significance of $P < 0.05$ or better. With regard to multiple comparisons and a single set of controls, comparisons were by one-way ANOVA with group comparisons made by Tukey’s test. Again, $P < 0.05$ was accepted as significant.

RESULTS

Imaging and estimation of CGRP-increased $[Ca^{2+}]_{int}$ in single attached MG-63 cells. The dual-dye method pioneered by Lipp and Niggli (25, 26) for laser-scanning confocal fluorescent microscopy was adapted to use with adherent MG-63 cells for estimation of $[Ca^{2+}]_{int}$. Microscopic fields containing 11–23 individual cells could be analyzed with an effective optical slice of 0.6 μm (or less). Rapid brief sampling with very low background provided extremely good sensitivity and avoided significant photobleaching of the dyes.

Figure 1 demonstrates CGRP-induced increases in $[Ca^{2+}]_{int}$ in $>90\%$ of MG-63 cells within 45 s of application of 10 nM human CGRP (Fig. 1B vs. A). Additionally, $>80\%$ of responsive cells increased $[Ca^{2+}]_{int}$ in nuclear and/or perinuclear regions as part of this response. Neither salmon nor human calcitonin at doses of 1–100 nM had any significant effect on $[Ca^{2+}]_{int}$, and no effects were observed with equal volumes of 0.01% trifluoroacetic acid or 0.1% DMSO (as vehicle controls). Baseline recordings of vehicle-stimulated individual cells revealed only minor 1–2% fluctuations in fluo-4-to-fura red ratio, and simultaneous imaging of fluo 4 demonstrated little falloff in signal intensity over 10–20 min of laser scanning.

To simplify these studies, entire whole cells were measured in our assays. Ratiometric measurement permitted quantitation of results through utilization of a standard $[Ca^{2+}]_{int}$ curve constructed in cell homogenates prepared from subconfluent basal cultures. Figure 1C presents one representative collection of multiple $[Ca^{2+}]_{int}$ recordings from a single experiment. Although both response time and magnitude of response differed significantly between individual cells, there was an apparent overall biphasic pattern of response.

Averaged time course changes in CGRP-increased $[Ca^{2+}]_{int}$ in single attached MG-63 cells. As suggested by multiple single-cell profiles, $>90\%$ of MG-63 cells responded to 10 nM CGRP with an obvious two-phase change in $[Ca^{2+}]_{int}$. Overall similarities were analyzed in more detail by collecting 11–22 single-cell profiles in an individual experiment and determining the mean population value at each time point ± SD. Three different examples of summed aggregate data for MG-63 cells stimulated with 10 nM human CGRP are presented in Fig. 2 (A and B demonstrate two separate experiments containing 16 or 22 cells, respectively). As summarized in Fig. 2C, analysis of 270 single adherent MG-63 cells in 15 additional experiments demonstrated essentially the same biphasic time course.

The average initial $[Ca^{2+}]_{int}$ response peaked approximately threefold over basal $[Ca^{2+}]_{int}$ by 40–50 s after application of peptide (average peak response vs. baseline at 50 s was significant with $P < 0.001$). After declining over ~180 s, a second phase of increased $[Ca^{2+}]_{int}$ began that peaked approximately twofold over baseline $[Ca^{2+}]_{int}$ after another 100–130 s (the second phase average response was significant vs. baseline $[Ca^{2+}]_{int}$ at 300 s with $P < 0.001$). This second increase in $[Ca^{2+}]_{int}$ was sustained for at least another 150 s.

Averaged dose-response relationship in CGRP-increased $[Ca^{2+}]_{int}$ in single attached MG-63 cells. As shown in Fig. 3, the area under the initial peak of CGRP-stimulated $[Ca^{2+}]_{int}$ was calculated and plotted against CGRP dose to determine dose response. A mean population value profile was determined for each concentration of CGRP (as before), and a number of experiments with 0.01–80 nM human CGRP demonstrated a dose-response curve. Maximal effects were obtained by ~20 nM, and both 40 and 80 nM CGRP produced substantially less of an increase than 20 nM. Increases were statistically significant at $P < 0.05$ (or better) for doses of 0.01 nM CGRP and greater. The apparent $EC_{50}$ was ~0.25 nM CGRP. The secondary phase $[Ca^{2+}]_{int}$ response was also ana-
lyzed separately and displayed a very similar EC$_{50}$ (data not shown).

Characterization of individual phases of [Ca$^{2+}$]$_{int}$ increase. When cells were pretreated with thapsigargin (10 µM; 20 min), the initial CGRP-induced Ca$^{2+}$ transient was inhibited by ~75% at 50 s of CGRP application ($P < 0.001$), whereas the secondary delayed phase was not significantly influenced (Fig. 4A). Pretreatment with verapamil (2 µM; 20 min) completely eliminated the second phase while having only minor effects on the initial phase (Fig. 4B). Similarly, when cells were pretreated with Rp-cAMPS, a potent inhibitor of cAMP-dependent protein kinase (30 µM; 20 min), the initial phase was inhibited 27% (without statistical significance) whereas the second phase was eliminated (Fig. 4C). When cells were preincubated for 20 min with Ca$^{2+}$-free PSS + 0.2% FBS supplemented with 1 mM EGTA, the initial phase was only slightly attenuated but the second phase was eliminated (data not shown). As shown in Fig. 5, when cells were stimulated with CGRP in the presence of CGRP(8–37) (2 µM), a known antagonist of the type 1 CGRP receptor (41), the initial phase was attenuated by ~55% ($P < 0.01$ vs. CGRP alone) whereas the second phase was nearly eliminated ($P < 0.001$ vs. CGRP alone).

Imaging and estimation of $E_m$ in single adherent CGRP-stimulated MG-63 cells. The vasodilatory effects of CGRP are accounted for by hyperpolarization of cellular $E_m$ (30). In our previous studies (18, 21), we demonstrated that CGRP can induce membrane hyperpolarization in osteoblastic UMR106 and RCOB-3 rat cells, both of which give only small cAMP responses when stimulated with CGRP. Thus it was of great interest to determine whether CGRP affects $E_m$ in MG-63 cells. Using DiBAC$_4$(3) bis-oxonol dye and confocal microscopy, we next monitored the relative cellular $E_m$ of single adherent MG-63 cells treated with 10 nM human CGRP (Fig. 6). More than 90% of CGRP-stimulated MG-63 cells responded within 50 s and lost ~20% of their bis-oxonol fluorescence over 300 s (Fig. 6, B vs. A). Because bis-oxonol fluorescent emission decreases in parallel with increased negative polarity of cellular $E_m$, these large changes represent significant hyperpolarization of the membrane potential. Unfortunately, it was not...
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Fig. 2. Mean population value [Ca\textsuperscript{2+}] response time courses from 2 individual experiments and 15 additional independent experiments demonstrate a common pattern of response. Shown are 3 mean population value [Ca\textsuperscript{2+}] response time courses for MG-63 cells treated with 10 nM human CGRP. Data for these plots were derived by calculating the mean ± SE at each time point (sampled every 7 s) from the [Ca\textsuperscript{2+}] measured in each of the individual cells followed. A and B: these data were obtained from 2 separate experiments measuring 15 or 22 individual cells, respectively. Cellular heterogeneity in these responses was similar to that demonstrated in Fig. 1C, but a common 2-phase pattern is evident in each experiment. C: the mean population value from 15 experiments in which 270 individual cells were measured demonstrates an essentially identical 2-part Ca\textsuperscript{2+} response pattern.

possible to quantify the signal from this dye, but DiBAC\textsubscript{4}(3) emission has been reported to decline ~25% with a 100-mV shift in $E_m$ (5). Parallel experiments in which vehicle (0.01% trifluoroacetic acid) alone was added demonstrated that no more than a 2–3% decline in DiBAC\textsubscript{4}(3) fluorescent emission normally occurs over 900 s of low-level laser illumination.

Figure 6C presents multiple single-cell profiles from an experiment using 11 cells stimulated with 10 nM CGRP. These data are typical of >15 independent experiments. As observed for CGRP-induced changes in [Ca\textsuperscript{2+}]$_{int}$, considerable heterogeneity was evident between individual single-cell traces, and yet there was an evident pattern to the response. The $E_m$ of some cells were observed to oscillate significantly during the experiment, consistent with our observation that ~50% of attached MG-63 cells periodically displayed oscillations in $E_m$ both before and after application of CGRP.

Averaged time course and dose-response relationship of CGRP-induced $E_m$ hyperpolarization in adherent MG-63 cells. When all single-cell records from independent experiments (with 7–20 cells) were normalized vs. original baseline emission intensities over 5 min (to correct for cellular “footprint”—see MATERIALS AND METHODS) and used to calculate mean population values for each time point, the resulting plot demonstrated the general similarity in the averaged response of the cell population (Fig. 7). Data from an initial experiment (Fig. 7A) and summary data from 15 individual experiments examining a total of 180 cells (Fig. 7B) in which MG-63 cells were stimulated with 20 nM human CGRP proved to be very similar. The vast majority of cells responded by 50 s to 20 nM human CGRP with a steep initial drop in bis-oxonol fluorescence, a partial reversal, and then a second steep drop to yield maximal reduction in fluorescence after 300 s. Hyperpolarization of $E_m$ was sustained for at least 600 s.

As with the [Ca\textsuperscript{2+}]$_{int}$ assay, multiple doses of human CGRP were used to construct a dose-response curve (net decrease in bis-oxonol emission at the 300 s point plotted against peptide concentration). Just as observed for stimulation of [Ca\textsuperscript{2+}]$_{int}$, maximal effect was obtained with 20 nM CGRP and less of an effect was obtained with 40–80 nM. The EC$_{50}$ was ~0.30 nM (data not shown).

Characterization of CGRP-induced $E_m$ hyperpolarization. A number of multiecell experiments were performed using the maximally effective dose of CGRP (20 nM) with standard concentrations of specific or selective inhibitors to determine the involvement of different pathways or membrane channels in CGRP-induced hyperpolarization of $E_m$ (Fig. 8). Rp-cAMPS (30 μM; 20-min pretreatment) produced an 8% reduction in effect 300 s after treatment that was not statistically significant; however, it did significantly delay the onset of $E_m$ polarization, possibly by eliminating the initial phase of polarization. A selective inhibitor of Ca\textsuperscript{2+}-activated K\textsuperscript{+} (K$_{Ca}$) channels, charybdotoxin (5 μg/ml; 5-min pretreatment), attenuated CGRP-induced reduction in bis-oxonol emission by 20% (with

Fig. 3. Dose-response curve for CGRP-stimulated increases in [Ca\textsuperscript{2+}]$_{int}$. The area under the initial transient peak (between 5 and 280 s of stimulation) was quantified and plotted against the dose of human (h)CGRP administered. A dose-response curve was generated, but less of an effect was obtained with 40 and 80 nM than was obtained at 20 nM. The EC$_{50}$ is estimated as ~0.30 nM. Each point and vertical bar represent the mean ± SE of data obtained in 4–6 independent experiments.
Polarization and produced a general 55% inhibition of the overall effect at 300 s after treatment \( (P < 0.01 \, \text{vs. CGRP alone}) \); the same compound produced a 75% inhibition of the cAMP formation induced by 20 nM CGRP (22). As shown above (Fig. 5C), CGRP(8–37) also inhibited the initial \( \text{Ca}^{2+} \) peak while eliminating the cAMP-dependent secondary-phase \( \text{Ca}^{2+} \) increase. However, available experimental data do not confirm the presence of a second type of CGRP receptor.

**DISCUSSION**

With careful perusal of published data, there should be little doubt that CGRP can act directly on osteoblastic cells to increase bone mass, but the literature also displays a wide variation in CGRP-induced signaling observed within different types of osteogenic cells. In fact, it is widely believed that mature osteoblastic cells downregulate CGRP receptors as they differentiate and no longer respond to CGRP—because strong stimulation of cAMP formation is not always observed in mature osteoblastic models. This view stems directly from the assumption that CGRP action in osteogenic cells is mediated predominantly by cAMP-dependent signaling pathway(s). Thus the actual mechanism of action behind CGRP’s stimulation of immature and mature osteogenic cells is a question central to the basic issue of whether CGRP plays a bone anabolic role.

In investigating this concept, we previously studied the effects of CGRP on \([\text{Ca}^{2+}]_{\text{int}}\) and \(E_{\text{m}}\) in mature osteoblastic rat UMR106 (18, 20, 21) and RCOB-3 (20) cells. CGRP did not elicit large increases in cAMP production (Kawase and Burns, unpublished observations), whereas it did invoke sizable \( \text{Ca}^{2+} \) transients and \(E_{\text{m}}\) hyperpolarization in UMR106 (18, 20, 21) and RCOB-3 cells (Ref. 20; \( \text{Ca}^{2+} \) data not published). This is consistent with a subsequent demonstration (6) that UMR106 cells do not seem to express the type 1 CGRP receptor (which...
generally couples tightly to adenylate cyclase activation). Thus these findings and demonstration of similar strong cAMP-independent CGRP signaling in other mature osteoblastic cell lines (11, 12) and primary human osteoblasts (38, 39) strongly suggested that cAMP-dependent signaling pathways are no longer the predominant mediators of CGRP action in mature osteoblastic cells (18). On the basis of previous data (3, 28, 34), the converse argument could also be made—that cAMP-dependent signaling pathways predominate in immature osteogenic cells.

We have now tested in preosteoblastic human MG-63 cells the working hypothesis that cAMP-dependent mechanisms would predominate without appreciable contribution from cAMP-independent signaling pathways. MG-63 cells were previously shown to strongly express mRNA for one of the components (CRLR) of a type 1 CGRP receptor (35). We recently demonstrated (22) the expression of both CRLR and RAMP1 proteins in these cells and determined that CGRP strongly stimulates cAMP formation. We could not detect expression of multiple CGRP receptors by analyzing saturation-binding kinetics with $^{125}$I-His$^{10}$-labeled human CGRP during the course of these studies (22). On the basis of these data, we assumed that only one receptor subtype was present and that it would predominantly signal through cAMP-dependent pathways. Thus we investigated $[\text{Ca}^{2+}]_{\text{int}}$ and $E_m$ values during CGRP treatment just to verify that no changes occurred. As shown in the present study, CGRP produces strong changes in both these parameters. The results reported here show both unexpected similarities to and surprising differences from our data from UMR106 cells, and, on evaluation, these data require that we discard or reformulate our simple hypothesis.

With regard to methodology, we have in the present study introduced and successfully adapted the dual-dye confocal fluorescence laser-scanning microscopy method of Lipp and Niggli (25, 26) to monitor CGRP-induced changes in $[\text{Ca}^{2+}]_{\text{int}}$ and $E_m$. To simplify these complex actions, we have only considered individual whole cells; future studies will need to examine apparent subcellular changes visualized by fluorescent imaging within...
CGRP was shown to induce a 2.3-fold initial peak in 
Ca²⁺ and mechanism of action. In human osteoblastic OSH-4 cells, cAMPS, indicating that this response is primarily produced by ATP-sensitive potassium channels are blocked (by a 5-min pretreatment with 500 μM glyburide; gray inverted triangles), cAMP signaling is blocked (20-min pretreatment with 10 μM Rp-cAMPS; open circles), and Ca²⁺-activated potassium channels are blocked (by 5-min pretreatment with 5 μM charybdotoxin, gray diamonds). For comparison, the nonchallenged response to CGRP is shown as filled circles. In all cases, the mean population value ± SE is plotted. Each experiment contained between 11 and 15 cells, and each cell was measured every 7 s. Each experiment was repeated 3–4 times.

In contrast, the second sustained phase of elevated [Ca²⁺]ₘₙ in MG-63 cells was not appreciably sensitive to thapsigargin but was inhibited by extracellular EGTA and verapamil. This phase was also inhibited by Rp-cAMPS and compound H-89 (data not shown) and eliminated by 2 μM CGRP (8–37). These data suggest that, in the second phase, activated CGRP receptors stimulate Ca²⁺ influx through a voltage-dependent Ca²⁺ channel and this process is initiated by cAMP and further integrated by cAMP signaling pathways. Similarly, in PTH-treated UMR106 cells, the slow secondary phase was demonstrated to be mediated by a cAMP-dependent, verapamil-sensitive membrane Ca²⁺ channel (44). In addition, the secondary phase observed in CGRP-treated OHS-4 cells, which is substantially smaller than that obtained here, was also shown to result from transmembrane influx as it was nifedipine sensitive; however, the secondary phase in OHS-4 cells is cAMP independent (11, 12).

CGRP-induced membrane hyperpolarization in MG-63 cells. Compared with Ca²⁺ mobilization, CGRP action on Eₘₚ has rarely been studied in osteoblastic cells at any stage of differentiation. In our previous study (18), we for the first time demonstrated with standard cuvette assays that CGRP induces Ca²⁺ release from intracellular stores in a cAMP-independent manner. However, because thapsigargin showed an incomplete elimination of this phase and both EGTA and verapamil produced a small inhibition, there may be a small contribution to the initial phase (perhaps 15–25% of the total effect) through very rapid transmembrane Ca²⁺ influx as has been shown for PTH (44).

The initial transient peak was inhibited 75% by thapsigargin but not substantially affected by extracellular EGTA or Rp-cAMPS, indicating that this response is primarily produced by
membrane hyperpolarization in adherent UMR106 cells. Judging from the dose-response relationship and the very small cAMP response elicited by CGRP in these cells, we suggested that cAMP-dependent signaling pathways were not involved in this action. In the present study of human preosteoblastic MG-63 cells using more sensitive protocols, we were surprised to find very similar time course profiles and dose-response relationships.

This action was further characterized with several specific pharmacological inhibitors. Rp-cAMPS delayed or suppressed the most initial transient phase of \( E_m \) polarization but failed to significantly inhibit the sustained phase. Conversely, glyburide almost completely blocked the second phase but not the initial phase, whereas charybdotoxin showed only slight effects on either phase. Therefore, these findings suggest that the initial phase is induced by a cAMP-dependent mechanism (possibly the rapid opening and closing of a Cl\(^{-}\) channel) and the large sustained major phase is produced by activating cAMP-independent \( K_{ATP} \) channels. However, our experiments do not rule out the possibility that either phase is also somewhat influenced by a \( Ca^{2+} \)-dependent mechanism.

**Hints of a second CGRP receptor subtype.** CGRP(8–37) has been described as a selective antagonist of type 1 CGRP receptors. CGRP(8–37) was able to eliminate certain effects (secondary phase of increased [Ca\(^{2+}\)]\(_{int} \), Fig. 5C) while only attenuating other effects by 55% [initial Ca\(^{2+}\) peak (Fig. 5C) and the bulk of cellular \( E_m \) polarization (Fig. 8)]. Although modest, the differences in level of inhibition between the two sets of effects did suggest that different receptors could be involved in this process. Unfortunately, careful analysis of the recent literature reveals several reports (40, 42) questioning the data described and discussed here is summarized in Fig. 9.

**Proposed explanation and scheme.** Our best interpretation of the data described and discussed here is summarized in Fig. 9, which indicates the different signaling pathways and effector molecules used by CGRP in MG-63 cells. The simplest explanation is that a type 1 CGRP receptor concomitantly couples to two or more different effector pathways in MG-63 cells. What would once have seemed an unlikely explanation is now supported by the literature, which indicates that transfected CGRP receptors can concomitantly signal by increasing both cAMP and \( Ca^{2+} \) (24, 31). Dual signaling has also been shown for the primary calcitonin receptor (31). Typically, changes in \( E_m \) have not been followed, and the exact mechanism(s) by which cellular \( E_m \) is modulated in osteogenic cells remains to be identified.

In conclusion, the present study provides data describing the activation of signaling pathways in an immature preosteoblastic human cell. Despite our initial working hypothesis, CGRP still induced strong ion-associated signaling by cAMP-independent mechanism(s). Although cAMP clearly plays a more important role in MG-63 cells than it does in UMR106 cells (18, 20, 21), cAMP-independent pathways are obviously important. Improvements on current assay protocols will undoubtedly enable more detailed study of CGRP-induced cellular changes.

These data further support the basic concept that bone is an important target tissue for the peripheral nervous system (13, 14, 16, 17, 27). Combined with our previous data (18, 20–22), these data provide a number of notable findings that collectively improve our understanding of CGRP-activated signaling pathways in osteoblastic cells.
GRANTS
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