Vol. 167, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1990 Pages 183-188

Intracellular calcium in the control of osteoclast function

I. Voltage-insensitivity and lack of effects of nifedipine, BAYK8644 and diltiazem

Harish K. Datta, Iain MacIntyre and Mone Zaidi

Cell Biology Unit, Royal Postgraduate Medical School,

London W12 ONN, UK

Received January 3, 1990

We have recently demonstrated that in the rat osteoclast, a rise in the ambient calcium concentration induces a rapid elevation of cytosolic calcium, and that this phenomenon is accompanied by a complete inhibition of osteoclastic bone resorption. Here, we have attempted to characterise the electrophysiological nature of the putative 'calcium-activated' calcium channel. We have established that calcium influx into the osteoclast that occurs on exposure to elevated extracellular calcium is independent of membrane voltage and is insensitive to modulation by organic calcium channel modulators, namely nifedipine, BAYK8644 and diltiazem. The latter compounds were also unable to block the reduction of cell spread area and the inhibition of bone resorption produced in response to elevated extracellular calcium levels.

During the process of bone resorption the osteoclast generates and is consequently exposed to very high levels of ionised calcium (1). We have recently shown that the free ionised calcium concentration at the osteoclast resorptive site can directly regulate osteoclastic resorption by modulating the levels of intracellular calcium (2). A moderate elevation of the ambient calcium concentration induces a rapid elevation of cytosolic calcium (2 - 4): the calcium signal is inhibitory to osteoclast function (2, 3, 5) and is accompanied by cell detachment (6) and retraction (5) as well as the inhibition of secretory activity (2, 7). The mechanism of 'calcium-activated' intracellular calcium elevation (2) is presently unclear, but it is generally agreed that this involves the movement of calcium ions across the osteoclast membrane (2, 3). This is because this response is mimicked by ionomycin and blocked by ionic nickel (2). In the present study we have attempted to further characterise the nature of calcium entry in terms of voltage-dependence and sensitivity to dihydropyridines (nifedipine, BAYK8644) and diltiazem, a benzodiazepine.

MATERIALS AND METHODS

Osteoclast isolation and culture (8): Newborn Wistar rats were killed by cervical dislocation and their femora and tibiae removed. The bones were freed of adherent soft tissues and cut across their epiphyses in HEPES-buffered Medium 199 supplemented with heat-inactivated fetal calf serum (FCS; 10% v/v; Gibco, UK), benzyl penicillin (Glaxo, Middlesex, UK; 100 μIU/ml) and streptomycin (Glaxo; 100 μg/ml) (SP). 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 seconds,

0006-291X/90 $1.50

before the supernatant was dropped onto appropriate substrate (bone slices, glass coverslips or plastic petri dishes). (All experiments with nifedipineand BAYK 8644 were performed in the dark).

Measurement of $[Ca^{++}]_i$ in isolated osteoclasts (2, 9): The solutions were made up as follows: Balanced salt solution (BSS): 140 mM-NaCl, 5 mM-KCl, 2.5 mM-$CaCl_2$, 2 mM-$MgCl_2$, 10 mM-Na_2HPO_4, 5 mM-$NaHCO_3$, 10 mM glucose, 1 g/l bovine serum albumin (BSA) and 10 mM HEPES, and buffered to pH 7.4. The K^+-substituted solutions were prepared by replacing Na^+ by K^+ to the required molarity. The cells, settled on a coverslip (20 mm diameter, 0 grade thickness), were incubated with 1 µg/ml indo 1-AM in BSS without BSA or serum, for 10 minutes. Dye-loaded osteoclasts were transferred to a perspex bath (1 ml) mounted on a heated stage on the microspectrofluorimeter. Solutions were pre-heated (37°C) and passed through the chamber at a rate of 1.8 ml/min. The dual-emission microspectrofluorimeter is constructed from a Nikon Diaphot inverted microscope, fitted with epifluorescence. The dye-loaded cells are excited at 340 nm and the fluorescent signal directed to the sideport to which is attached a variable aperture, a shutter and a beam splitter containing a dichroic mirror (455 nm). Transmitted and reflected light through the dichroic mirror is filtered at 470-490 nm and 405 nm respectively and the intensities recorded by separate photomultiplier tubes (PM28B, EMI). Single photon currents in each tube were converted to TTL pulses and counted by a dual photon counter (Newcastle Photometric Systems, Newcastle-upon-Tyne, UK). Photon counts/s in each channel were recorded in a BBC microcomputer, the ratio of intensities (405 nm/495 nm) calculated and displayed.

Meaurement of cell spread area and motility (10, 11): Osteoclasts were settled in a tissue culture dish (35 mm; Cellcult; Sterilin) and were incubated at 37°C for 20 minutes to allow cell sedimentation and attachment. The wells were then washed with Medium 199 and 2 ml of the same medium was placed in each well. The dishes were placed in the incubation chamber of an inverted phase-contrast microscope (Diaphot, Nikon UK Ltd.) linked via a CCD camera to a 480 hour-mode time-lapse video recorder (Mitsubishi, Japan). The composite video signal was fed into a 256-grey level imaging system (Sight Systems, Newbury, Berks., UK). The timed capture of digitised grey images into the computer memory was followed by redigitisation of the cell boundry using a microsoft mouse (Microsoft), an edge detection facility and a binary overlay. A tracing of each cell was overlaid on the previous outline and analysed using a software package programmed to measure the area within each tracing and the area change due to cell movement (shape area). Total cell area (A) was expressed as a percentage of control cell area (± standard error of mean). Motility was expressed by a factor (change of shape area per unit cell area or $\Delta A/A$) (± standard error of mean).

Quantification of osteoclastic bone resorption (12, 13): Bone slices were prepared as previously described (12). Osteoclasts were dispersed on bone slices, and following incubation (37°C; 15 minutes) the slices were removed, washed gently in Minimal Essential Medium (MEM) containing 10% FCS and placed in separate wells. After further incubation (37°C, 10% humidified CO_2, 10 minutes), 100 µl of MEM/FCS containing the test substance was added. Finally, bone slices were incubated overnight (37°C, 10% humidified CO_2, 18 hours), and after fixation in glutaraldehyde the cells were stained with toluidine blue and examined by transmitted light microscopy. This enabled the assessment of multinucleate osteoclasts. Osteoclasts were counted on three slices for every variable. The slices were bleached by immersion in sodium hypochlorite solution (10%, v/v) for 30 minutes and dehydrated in aqueous ethanol (80%, v/v). Having been sputter-coated with gold, bone slices were randomised and examined in a computer-centered scanning electron mircoscope (Cambridge 360; Cambridge Instruments, Cambs., UK). The number of osteoclastic excavations, each defined by a continuous border, were counted. The area of the bone surface resorbed was calculated by tracing the outline of the concavities into an image processor (Sight Systems). The effect on the response, of treatment compared to control was assessed using Analysis of Variance.

RESULTS

Effect of high $[K^+]_e$ on osteoclast $[Ca^{++}]_i$, spread area, motility and bone resorption: Depolarisation of single isolated osteoclasts by exposure to K^+_e in Ca^{++}_e-containing or Ca^{++}_e-free medium for upto 5 minutes failed to elevate $[Ca^{++}]_i$ (Figure 1). Osteoclast size and motility was measured in response to depolarisation ($[K^+]_e$ - 20 mM and 80 mM) in different experiments conducted over 60 minutes. No reduction of cell spread area was observed. When computed and analysed using time-series analysis we found that total cell motility was unchanged. The individual components of motility, including pseudopodial

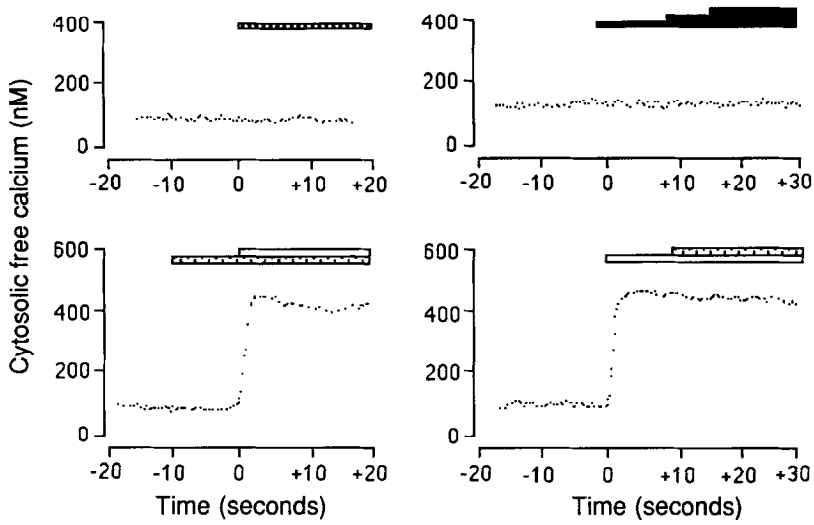

FIGURE 1

Top panels: The lack of effect of BAYK8644 (25 μM) (left panel) and of the stepwise elevation of $[K^+]_e$ (concentrations of 20, 40 and 60 mM) (right panel) on osteoclast $[Ca^{++}]_i$ at physiological $[Ca^{++}]_e$ (1 mM).

Bottom left panel: The effect of pretreatment with nifedipine (25 μM) (stippled bar) on the elevation of osteoclast $[Ca^{++}]_i$ in response to elevated $[Ca^{++}]_e$ (15 mM) (open bar).

Bottom right panel: The effect of treatment with nifedipine (25 μM) (stippled bar) on the plateau phase of elevated $[Ca^{++}]_i$ induced by the elevation of $[Ca^{++}]_e$ (15 mM) (open bar).

protrusion and retraction and cell movement were also not altered by depolarising concentrations of K^+_e. This was confirmed by observing time-lapse video recordings of individual cells. When cells were incubated at 20 and 40 mM $[K^+]_e$, there was no evidence of cytotoxicity and the number of osteoclasts per slice remained unchanged. At these concentrations there was no statistical difference in the area of bone resorbed per bone slice, total number of osteoclastic excavations or the mean size of individual excavations at the end of the 18 hour incubation period (Figure 2).

Effects of nifedipine, diltiazem and BAYK8644 on $[Ca^{++}]_i$, cell area, motility and bone resorption:

Nifedipine, diltiazem and BAYK8644 failed to alter $[Ca^{++}]_i$ in single isolated cells at any concentration tested. Nifedipine (25 μM) also failed to inhibit the elevation of $[Ca^{++}]_i$ caused as a result of elevating $[Ca^{++}]_e$ to 15 mM, when added either before or after increasing $[Ca^{++}]_e$ (Figure 1). In addition, the three compounds failed to affect cell spread area, and when motility was computed and analysed using time-series analysis, we found that this was unchanged. The individual components of motility, including pseudopodial protrusion and retraction and cell movement were also found to be unaltered, and this was confirmed by observing time-lapse video recordings of individual cells. When cells incubated on bone were exposed for 18 hours to nifedipine or BAYK8644 (each at 25 μM) there was no evidence of cell toxicity and the number of osteoclasts per bone slice remained unchanged. At these concentrations there was no statistical difference in

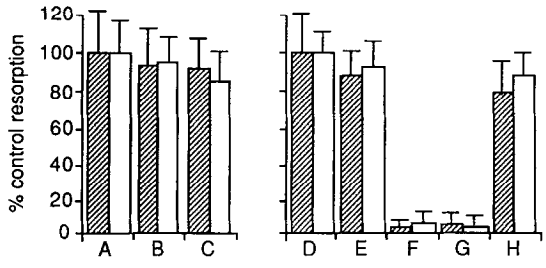

FIGURE 2

The effect of exposure to elevated $[K^+]_e$, elevated $[Ca^{++}]_e$, nifedipine and BAYK8644 on the total area of bone resorbed per bone slice (stippled bars) and number of osteoclastic excavations per bone slice (plain bars), expressed as percentage of control responses ± standard error of mean (n = 6 bone slices per treatment). Left panel: A: Control (untreated cultures); B: $[K^+]_e$ 20 mM; C: $[K^+]_e$ 40 mM. Right panel: D: Control (untreated cultures); E: nifedipine (25 μM); F: $[Ca^{++}]_e$ (20 mM); G: nifedipine (25 μM) and $[Ca^{++}]_e$ (20 mM); H: BAYK8644 (25μM). Except where specifically stated the experiments were carried out at physiological $[Ca^{++}]_e$ (1 mM).

the area of bone resorbed per bone slice, total number of osteoclastic excavations or the mean size of individual excavations at the end of the 18 hour incubation period. The elevation of $[Ca^{++}]_e$ to 20 mM led to a near-complete inhibition of osteoclastic bone resorption, but when the bone-osteoclast cultures were exposed to nifedipine (25 μM) before being exposed to elevated $[Ca^{++}]_e$ (20 mM) the inhibitory effects of Ca^{++}_e on bone resorption still persisted (Figure 2).

DISCUSSION

It has been shown that the extracellular calcium concentration directly controls free cytosolic calcium in isolated osteoclasts (2 - 4). A rise and fall in cytosolic $[Ca^{++}]$ exerts, respectively, a negative and positive control over osteoclastic function (2, 3, 5). For these reasons, it is imperative that we understand the nature of calcium transport into the osteoclast. Generally, calcium-channels of non-excitable cells fall into one of three major types: voltage-sensitive, receptor-operated (or ligand-gated) and G protein-gated. In this study we have used intracellular calcium responses in order to define the nature of the putative 'calcium-activated' calcium channels present in the osteoclast membrane. These appear to be somewhat similar to channels present on PTH-secreting chief cells (14 - 16) and to those on calcitonin-secreting C-cells of the thyroid (17, 18) in terms of their sensitivity to changes in the extracellular calcium concentration. However, whilst C-cells have voltage-dependent channels (17, 18), we have established that in the osteoclast, calcium entry is insensitive to changes in membrane voltage produced as a consequence of elevation in $[K^+]_e$. Depolarising concentrations of $[K^+]_e$ also failed to affect osteoclast spread area and bone resorption.

The voltage-independent nature of calcium entry into the osteoclast has also recently been confirmed by patch clamp studies (J. Dixon et al, personal communication). We have also provided further evidence in favour

of the voltage-independent channels by demonstrating the voltage-gated calcium channel blockers, nifedipine and diltiazem failed to block Ca^{++}_e-evoked Ca^{++}_i responses. These compounds also failed to prevent the inhibitory effects of elevated Ca^{++}_e on osteoclast spread area and bone resorption. As expected we also found that compound BAYK8644, a dihydropyridine-type calcium channel agonist failed to elevate Ca^{++}_i, induce cell contraction or inhibit osteoclastic bone resorption.

Therefore, electrophysiologically, osteoclast calcium channels appear to more like voltage-insensitive channels found on parathyroid chief cells. However, there is one important difference. Whilst parathyroid cells respond to much smaller fluctuations (0.1 mM) in extracellular (circulating) calcium (14 - 16), osteoclasts can only sense changes that are much larger in magnitude (2 - 4). Although the concentration of calcium is considerably higher (4 to 20 mM) than that in plasma, this is line with that occuring in the osteoclast resorption lacuna (1). Furthermore, there is strong evidence for the presence of a divalent cation receptor (or "calcium receptor") linked to intracellular calcium-redistribution system on parathyroid chief cells (16, 19); however, it has not yet been confirmed whether such a receptor protein is also expressed on the osteoclast plasma membrane. Nevertheless, if the existence of the putative 'calcium-activated' calcium channel is confirmed by patch clamp studies, it would be interesting to determine whether these channels are modulated directly by a divalent cation receptoror via the intermediacy of GTP-binding proteins.

Acknowledgments: These studies were supported by grants from The Arthritis and Rheumatism Council, UK (MZ), Research into Ageing, UK (MZ), Leverhulme Trust, UK (MZ) and Bartos Foundation, USA (IM). HKD acknowledges the support of The Medical Research Council, UK.

REFERENCES

1. Silver A., Murrills, R.J., Etherington, D.J. (1988) Exptl. Cell Res. 175, 266-276.
2. Zaidi, M., Datta, H.K., Patchell, A., Moonga, B., MacIntyre, I. (1989) Biochem. Biophys. Res. Commun. 163, 1461-1465.
3. Datta, H.K., MacIntyre, I., Zaidi, M. (1989) J. Physiol. 415, 98P.
4. Malgaroli, A., Maldolesi, J., Zambonin-Zallone, A., Teti, A. (1989) J. Biol. Chem. 264, 14342-14347.
5. Datta, H.K., MacIntyre, I., Zaidi, M. (1989) Biosci. Rep. In Press.
6. Zaidi, M., Datta, H.K., Patchell, A., Moonga, B.S., Moss, D.W., Makgoba, M.W., MacIntyre, I. (1989) J. Bone Min. Res. 4, S200.
7. Moonga, B.S., Moss, D.W., Patchell, A., Zaidi, M. (1989) J. Physiol. In Press.
8. Chambers, T.J. (1982) J. Cell Sci. 57, 247-253.
9. Gillespie, J.I., Greenwell, J.R. (1987) J. Physiol 391, 10P.
10. Zaidi, M., Patchell, A., Datta, H.K., MacIntyre, I. (1989) J. Endocrinol. 123, R5-R7.
11. Datta, H.K., Brown, A.F., Dunn, G.A., Zaidi, M. (1990) J. Cell Sci. Submitted.

12. Chambers, T.J., Revell, P.A., Fuller, K., Athanasou, N.A. (1984) J. Cell Sci. 66, 383-399.

13. Zaidi, M., Chambers, T.J., Bevis, P.J.R., Beacham, J.L., GainesDas, R.E., MacIntyre, I. (1988) Quart. J. Exp. Physiol. 73, 471-485.

14. Nemeth, E.F., Scarpa, A. (1986) FEBS Lett. 203, 15-19.

15. Nemeth, E.F., Scarpa, A. (1987) J. Biol. Chem. 262, 5188-5196.

16. Brown, E.M., Enyedi, P., Leeboff, M., Rotberg, J., Preston, J., Chen, C. (1987) FEBS Lett. 218, 113-118.

17. Fried, R.M., Tashijan, A.H. Jr. (1986) J. Biol. Chem. 261, 7669-7674.

18. Muff, R., Nemeth, E.F., Haller-Brem, S., Fischer, J.A. (1988) Arch. Biochem. Biophys. 265, 128-135.

19. Nemeth, E.F. (1987) Ann. N.Y. Acad. Sci. 493, 583-585.