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Molecular and Functional Evidence for Calcineurin-A α and β Isoforms in the Osteoclast: Novel Insights into Cyclosporin A Action on Bone Resorption

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We provide the first molecular evidence for the presence of a functional serine/threonine phosphatase, calcineurin-A (CN-A), in the osteoclast. Polymerase chain reaction (PCR) of an osteoclast cDNA library, together with restriction mapping, revealed two isoform sequences, α and β. We then examined the functionality of the detected CN-A by assessing the effect of a classical antagonist, cyclosporin A (CsA), in the osteoclast resorption (pit) assay. CsA (0.1 and 1 μg ml^{-1}) potently inhibited bone resorption. The presence of lymphocytes, with or without prior exposure to CsA *in vivo,* failed to reverse the CsA-induced resorption–inhibition. Expectedly, CsA had no direct effect on cytosolic Ca^{2+} levels in fura-2-loaded osteoclasts. These studies are a prelude to further investigations into the possible role of CN-A in osteoclast regulation. Finally, mechanistic studies on the bone effects of CsA, a widely used immunosupressant, should proceed from these observations.

Cytosolic Ca^{2+} changes in the osteoclast triggered by a variety of agonists, including Ca^{2+}, are critical in regulating bone resorption (1–3). Nevertheless, the mechanism by which these changes are transduced into effects on resorption is unknown. There is limited evidence to suggest that protein kinase C (PKC) and calmodulin (CAM) kinase are involved in Ca^{2+}-induced resorption-inhibition (4, 5). However, it is unclear whether the same applies to calcineurin (CN), a well-characterized Ca^{2+}-sensitive serine/threonine phosphatase (6).

Calcineurin-A (CN-A) is a 60-kDa subunit consisting of both catalytic and CAM-binding domains (6–8). There are three known isoforms, namely α, β and γ (6–8). CN-A heterodimerizes with a 19-kDa subunit, CN-B, that also has a Ca^{2+}-regulatory site (9). The enzyme is inhibited potently by the two well-known immunosupressants, cyclosporin A (CsA) and FK506 (10). The latter molecules, however, first complex with their respective binding proteins, cyclophilin and FKP12 (11), and then inhibit phosphatase activity.

Both CsA and FK506, when administered to either humans or animals, produce marked effects on bone (12). For example, when administered *in vivo,* both compounds cause bone loss, an effect that we have demonstrated, is mediated by the T-lymphocyte (13). The current study was prompted by contrasting reports suggesting that CsA can inhibit resorption, *in vitro,* both in organ and isolated cell cultures (14, 15). We have therefore examined (a) whether osteoclasts express the CN-A gene and (b) whether CN-A activation by CsA inhibits osteoclastic bone resorption in the pit assay. Furthermore, to understand the cellular basis for the discrepancy between the *in vivo* and *in vitro* bone effects of CsA, we carried out our pit assays in the presence or absence of freshly isolated lymphocytes.

MATERIALS AND METHODS

Reverse transcriptase polymerase chain reaction (RT-PCR). We first designed gene-specific primers based on our analyses of the nucleotide sequences of rat, mouse, human and bovine CN-Aα and CN-Aβ cDNA. These primers have been successfully used to amplify CN-Aα (4 primers) and CN-Aβ (2 primers) (GenBank: RATCNRAA

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and RATCALAB, respectively): CN-A α #1 5′-primer: 5′-CGACAGGAAAAAAACTTGCTGGAT-3′ (nt 424–447); CN-A α #2 5′ primer: 5′-TGTTGACAGAGGGTACTTCAGT-3′ (nt 564–585); CN-A α #3 3′-primer: 5′-GTGAGTGAAATGTTCCTGAGTC-3′ (nt 957–935); CN-A α #4 3′-primer: 5′-GTTTGGCTTTTCCTGTACATG-3′ (nt 1094–1075); CN-A β #5 5′-primer: 5′-AACCATGATAGAAGT-AGAAGCT-3′ (nt 294–315); and CN-A β #6 3′-primer: 5′-CAC-ACACTGCTGGATAGTTATAA-3′ (nt 865–843).

For RT-PCR of testis RNA (control) and PCR of DNA prepared from the rabbit cDNA library (16, 17), we used the Titan One-Tube RT-PCR System (Boehringer-Mannheim, Indianapolis, IN). This system is designed for the sensitive, rapid, and reproducible analysis of RNA with high fidelity. The one-step reaction system uses RT-AMV for first strand synthesis and a mixture of Taq and Pwo DNA polymerases for the PCR step. Reaction conditions were maintained *per* manufacturer's recommendations. The reaction mixture (50 μl) comprised of 0.2 mM-dNTP, 5 mM-DTT, 1 μg of testicular RNA or 0.4 μg of library DNA, 0.1 μg of each primer (see above), 5 to 10 U of RNase inhibitor, 1× RT-PCR buffer, and 1 μl of the enzyme mix. The GeneAmp PCR System 2400 (Perkin–Elmer, Foster City, CA) was programmed as follows: (i) 50°C/30 min (for RT-PCR only), 94°C/2 min, (ii) 35 cycles of 94°C/30 s, 55°C/30 s, and 68°C/40 s, then 68°C/7 min and subsequent cooling to 4°C. The PCR products were separated by electrophoresis on a 1.5% agarose gel. In separate experiments, the excised bands were treated with *Nco*I, *Pst*I, *Kpn*I (for CN-Aα) and *Hin*dIII (for CN-Aβ) to generate a restriction map.

Bone resorption (pit) assay. Bone resorption was measured using the pit assay (18). Briefly, the bones from 24- to 48-h-old rats were sliced in 3.5 ml Medium 199-H (with Hank's salts; Gibco, Gaithersburg, MD). The resulting cell suspension was settled onto devitalized cortical bone slices (4 × 4 mm) for 30 min. Following the removal of non-adherent cells by gentle rinsing, the slices were transferred to a multi-well dish containing M199-E (with Earle's salts and 10% fetal bovine serum, v/v). Either vehicle or CsA (0.1 or 1 μg ml^{-1}) was applied. In separate experiments, lymphocytes isolated from the rat blood (see below) were dispersed onto the slices before incubation. The slices were then incubated for 24 h in humidified CO_2 (5%) (pH 6.9), following which they were fixed with glutaraldehyde (10% v/v) and stained for the presence of tartrate-resistant acid phosphatase (TRAP) using a kit (Kit 386A, Sigma Chemical Co., St. Louis, MO). The number of osteoclasts with two or more nuclei was determined on each slice using a light microscope (Olympus, Tokyo, Japan). The cells were removed by treating the slices with NaOCl (5 min), and the slices were rinsed with distilled water followed by acetone, and then air-dried. They were then stained with toluidine blue [1% (v/v), in 1% (w/v) borate, 5 min]. The number of resorption pits was determined on each slice by light microscopy. Notably, each experiment was performed with osteoclasts obtained from three animals with five or six bone slices per treatment. The number of pits or osteoclasts per bone slice was expressed as a mean ± SEM. Analysis of Variance with Bonferroni's Correction for Inequality was used to analyze the effect of treatment (significant at $p < 0.05$).

Cytosolic Ca^{2+} measurements. A fura-2-based single cell spectrofluorometric method (19) was used to measure cytosolic [Ca^{2+}] in single osteoclasts. Glass coverslips containing osteoclasts were incubated with 10 μM-fura 2-AM (30 min, 37°C). Cells were then exposed alternately to excitation λ^s of 340 and 380 nm, every second. The emitted fluorescence was filtered at 510 nm and fed into a photomultiplier tube (PM28B, Thorn EMI, London, UK). Single photon currents were counted and recorded (Newcastle Photometric Systems, Newcastle-upon-Tyne, UK). The ratio of emitted fluorescence intensities due to excitation at 340 and 380 nm, F_{340}/F_{380}, was calculated and displayed. Fura-2 was calibrated using a protocol for intracellular calibration as described previously (19).

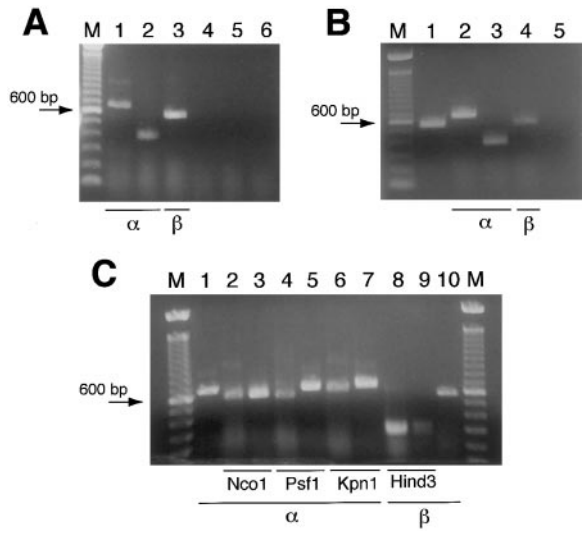

FIG. 1. Identification of calcineurin-A (CN-A) α and β nucleotide sequences by polymerase chain reaction (PCR) in rabbit osteoclast cDNA library (A) Control experiment showing reverse transcriptase-(RT-) PCR analysis of rat testis total RNA. Lanes 1 and 2: PCR products corresponding to CN-Aα amplified with primer pairs 1/4 (670 bp) and 2/3 (413 bp), respectively (for primer sequences, see Materials and Methods). Lane 3: PCR product corresponding to CN-Aβ amplified with primer pair 5/6. Lanes 4 to 6: Negative controls for reactions 1–3, respectively, without added RNA. (B) PCR analysis of phage DNA prepared from rabbit osteoclast cDNA library. Lane 1: PCR product from rat genomic DNA with primers 5/6 (positive control). Lanes 2 and 3: PCR products corresponding to CN-Aα amplified with primer pairs 1/4 and 2/3, respectively. Lane 4: PCR product corresponding to CN-Aβ amplified with primer pair 5/6. Lane 5: Negative control for lane 4 without added DNA. (C) Restriction analysis of PCR fragments corresponding to CN-Aα and CN-Aβ. CN-Aα sequences from osteoclast cDNA library (lanes 1, 3, 5, and 7) or rat testis (lanes 2, 4, and 6). CN-Aβ sequence from the cDNA library (lanes 8 and 10) or testis (lane 9). These PCR fragments were digested with restriction enzymes as indicated. The expected shortening for rat samples are −62 bp (*Nco*I), −108 bp (*Pst*I) and −36 bp (*Kpn*I) (for CN-Aα), and −320 bp for *Hin*dIII (CN-Aβ).

RESULTS

Molecular studies. To analyze CN-A gene expression in the osteoclast, we designed gene-specific primers based on the analysis of nucleotide sequences of rat, mouse, human and bovine CN-Aα and CN-Aβ cDNA. Figure 1A shows a control experiment that establishes specificity of the new primers (see Materials and Methods). Note that, appropriately sized bands were seen upon agarose gel electrophoresis following RT-PCR of rat testis RNA (670 and 413 bp for CN-Aα and 571 bp for CN-Aβ) (Fig. 1A). DNA prepared from the rabbit osteoclast cDNA library was then used for PCR amplification using the same primers. We identified appropriate bands corresponding to both the α (lanes 2 and 3) and β (lane 4) CN-A isoforms (Fig. 1B). To establish their identity further, the bands in lanes 2 and 4 were

digested by the restriction enzymes, *Nco*I, *Pst*I, *Kpn*I (for CN-Aα), and *Hin*dIII (for CAN-β). Expected decrements in size occurred following such digestion (Fig. 1C), suggesting strongly that both CN-A isoform sequences were expressed in the osteoclast.

The expression of CN-A genes is further supported by (a) an identity in sizes of the PCR fragments amplified from both testes and osteoclasts, and (b) the presence of *Nco*I and *Hin*dIII restriction sites, respectively, in the CN-Aα and CN-Aβ PCR fragments. However, the difference in localization of restriction sites for *Pst*I and *Kpn*I in CN-Aα may reflect nucleotide substitutions in the respective rabbit CN-A genes compared with the corresponding rat genes. This is consistent with the same genes from two species being analyzed, thus excluding contamination, during PCR analysis, of the rabbit osteoclast cDNA library.

Functional studies. To elucidate the functional significance of CN-A in the osteoclast, we studied the effect of its inhibition by CsA (7). We first examined the effects of CsA, both at a low (0.1 μg ml^{-1}) and high (1 μg ml^{-1}) concentration, in the pit assay following a 24- or 48-h incubation. At both time points, CsA inhibited bone resorption significantly ($p < 0.001$ at 0.1 and 1 μg ml^{-1}-CsA) (Fig. 2). However, the inhibition was less marked at 48 h compared with 24 h, suggesting a partial recovery. This resulted in a highly significant difference at both CsA concentrations when the response at 24 h was compared with that at 48 h ($p = 0.02$ at 0.1 μg ml^{-1} and $p = 0.025$ at 1 μg ml^{-1}, respectively). Furthermore, osteoclast number per bone slice remained unchanged, excluding an effect of CsA on osteoclast formation or demise. The potent antiresorptive effect of CsA provides strong functional evidence for CN-A expression in the osteoclast.

Figure 2 also shows that lymphocytes, freshly isolated from rat blood, failed to reverse the CsA-induced osteoclastic inhibition, both at 24 and 48 h. Notably, at either time point, resorption in response to lymphocytes plus CsA was not significantly different from that due to CsA alone (24 h: $p = 0.42$/0.1 μg ml^{-1}; $p = 0.46$/1 μg ml^{-1}; 48 h: $p = 0.187$/0.1 μg ml^{-1}; $p = 0.26$/1 μg ml^{-1}). Note also, that lymphocytes alone did not affect osteoclastic bone resorption. We next studied the effect of lymphocytes, following their *in vivo* conditioning with CsA, on osteoclastic inhibition by exogenous CsA. The cells were isolated freshly from blood drawn from either control rats (unconditioned), or from rats in whom CsA (10 mg kg^{-1} body weight) was administered orally for 28 days (conditioned). Again, at both concentrations, CsA inhibited resorption significantly, despite conditioning (p values in legend to Fig. 2). Thus, a comparison of resorption in the presence of conditioned versus unconditioned lymphocytes revealed no significant difference ($p = 0.25$). We conclude that lymphocytes themselves do not influence the direct antiresorptive effect of CsA.

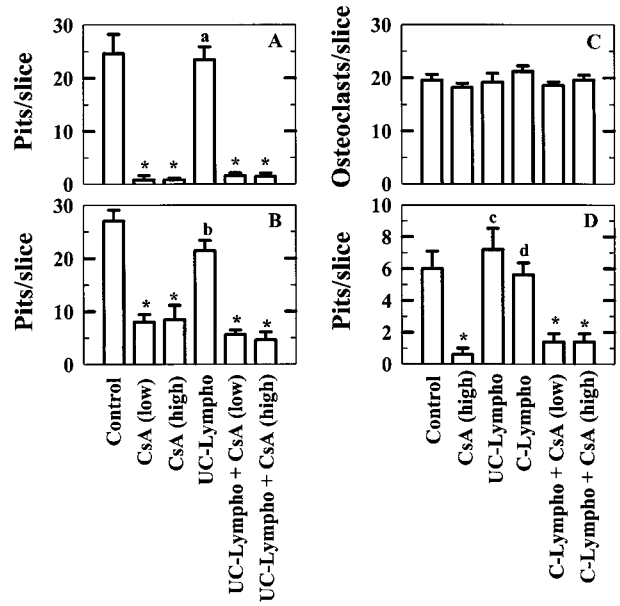

FIG. 2. Pharmacological inhibition of osteoclastic calcineurin-A (CN-A) by a potent antagonist, cyclosporine A (CsA). Effect of CsA (0.1 or 1 μg ml^{-1}) on bone resorption by isolated osteoclasts over a 24 hour (**panels A and D**) or 48 hour (**panel B**) incubation period. Results are expressed as the number of excavations (pits) per bone slice (mean ± SEM), as well as number of osteoclasts per slice (**panel C**). The experiments were carried out in the presence or absence of lymphocytes (**Lympho**) isolated freshly from blood drawn from rats that were untreated (UC) or were conditioned (C) with CsA (for details, see Materials and Methods). Statistics by analysis of variance with Bonferroni's correction for inequality: compared with the respective controls: $^{*}p < 0.001$; $^{a}p = 0.978$; $^{b}p = 0.851$; $^{c}p = 0.975$; $^{d}p = 0.793$.

Finally, we attempted to examine for any effect of CsA on cytosolic Ca^{2+} levels in the osteoclast. CsA (1 μg ml^{-1}) failed to trigger cytosolic Ca^{2+} signals in fura-2-loaded osteoclasts, virtually excluding a direct role for Ca^{2+} in the CsA-induced resorption-inhibition. Taken together, the molecular and functional data suggest strongly that CsA inhibits bone resorption independently of cytosolic Ca^{2+} changes, most likely, *via* a direct action on CN-A.

DISCUSSION

We provide molecular evidence, through PCR of library cDNA, that CN-A sequences are present in the osteoclast. We have further used restriction analysis to identify the two known isoforms, α and β. We believe, however, that it is unlikely that CN-A activation mediates the inhibitory effects of high Ca^{2+} on bone re-

sorption. A cytosolic Ca^{2+} increase would normally be expected to activate CN-A (via CAM-binding) and thus inhibit resorption (3, 7, 20). However, the present study indicates that it is the inhibition rather than stimulation of CN-A that is associated with reduced resorption. Thus, whilst it is unlikely that Ca^{2+}-induced resorption-inhibition uses a CN-A pathway, a Ca^{2+} change could still modulate CN-A activity.

The physiological role of osteoclastic CN is presently unclear. Its known substrates include CAM kinase II, nitric oxide synthase (NOS), GTPase, heat shock protein (*hsp* 25), and the transcription factor *Elk-1* (21–24). Of these, NOS is found in abundance in the osteoclast (25, 26). The latter expresses both its constitutive (e) and inducible isoforms (25, 26). Furthermore, NO itself is a critical regulator of osteoclast function (27). Should CN-A dephosphorylate and hence, activate the Ca^{2+}-sensitive eNOS, this would indicate a direct role of CN-A in osteoclastic NO formation. This attractive, though speculative hypothesis, merits further studies.

More relevant to CN-A action in the osteoclast, however, is its inhibition by the widely used immunosupressant, CsA. It is well known that CsA, administered *in vivo,* produces accelerated bone remodeling resulting in rapid bone loss (12). However, as indicated above, CsA paradoxically inhibits bone resorption *in vitro.* We have also demonstrated earlier that T-lymphocytes are critical for the CsA-induced bone loss seen *in vivo* (12). To understand the mechanistic basis of the apparently discrepant *in vitro* and *in vivo* actions of CsA, we cultured lymphocytes with osteoclasts in the pit assay. Specifically, lymphocytes derived from either control or CsA-conditioned rats were incubated with freshly isolated rat osteoclasts on bone slices for 24 or 48 h. Lymphocytes were expectedly found not to affect basal resorption. More importantly, however, they failed to reverse the anti-resorptive action of CsA. The latter was true whether or not the cells were conditioned by prior *in vivo* exposure to CsA. Taken together, the findings suggest that lymphocytes, alone, do not reverse the CsA-induced resorption-inhibition seen *in vitro.*

In conclusion, we provide complementary evidence, molecular and functional, for the presence of CN-A in the osteoclast. Specifically, we also show that the CN-A antagonist, CsA, inhibits bone resorption by isolated osteoclasts in the pit assay. We also demonstrate that the anti-resorptive effect of CsA is not reversed by the sole presence of lymphocytes. Thus, it is likely that the known CsA-induced resorption-stimulation and bone loss *in vivo* requires other cell types and/or soluble factors. These interesting possibilities warrant further investigation.

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REFERENCES

1. Malgaroli, A., Meldolesi, J., Zabonin-Zallone, A., and Teti, A. (1989) *J. Biol. Chem.* **264,** 14342.
2. Zaidi, M., Moonga, B. S., Moss, D. W., and MacIntyre, I. (1989) *Biochem. Biophys. Res. Commun.* **159,** 68.
3. Moonga, B. S., Moss, D. W., Patchell, A., and Zaidi, M. (1990) *J. Physiol. (London)* **429,** 29.
4. Su, Y., Chakraborty, M., Nathanson, M., and Baron, R. (1992) *Endocrinology* **131,** 1497.
5. Moonga, B. S., Stein, L. S., Kilb, J. M., and Dempster, D. W. (1996) *Calcif. Tiss. Int.* **59,** 105.
6. Klee, C. B., Ren, H., and Wang, X. (1998) *Minirev. Calcineurin Regul.* **273,** 13367.
7. Klee, C. B., Draetta, G. F., and Hubbard, M. (1988) *Adv. Enzymol. Mol. Biol.* **61,** 149.
8. Kincaid, R. L. (1993) *Adv. Second Messenger Phosphoprotein Res.* **27,** 1.
9. Aitken, A., Klee, C. B., and Cohen, P. (1984) *Eur. J. Biochem.* **139,** 663.
10. Schreiber, S. L., and Crabtree, G. R. (1992) *Immunol. Today* **13,** 136.
11. Liu, J., Albers, M. W., Wandless. T. J., Luan, S., Alberg, D. G., Belshaw, P. J., Cohen, P., MacKintosh, C., Klee, C. B., and Schreiber, S. L. (1992) *Biochemistry* **31,** 3896.
12. Cvetkovic, M., Mann, G. N., Romero, D. F., Liang, X. G., Ma, Y., Jee, W. S., and Epstein, S. (1994) *Transplantation* **57,** 1231.
13. Buchusky, F. J., May, F., and Mann, G. N. (1996) *Endocrinology* **137,** 2278.
14. Orcell, P., Denne, N., and De Vernejoal, M. C. (1991) *Endocrinology* **128,** 1638.
15. Chowdhury, M. H., Shen, V., and Dempster, D. W. (1991) *Calcif. Tiss. Int.* **49,** 275.
16. Kameda, T., Mano, H., Yamada, Y., Takai, H., Amizuka, N., Kobori, M., Izumi, N., Kawashima, H., Ozawa, H., Ikeda, K., Kameda, A., Hakeda, Y., and Kumegawa, M. (1998) *Biochem. Biophys. Res. Commun.* **245,** 419.
17. Tezuka, K., Sato, T., Kamioka, H., Nijweide, P. J., Tanaka, K., Matsuo, T., Ohta, M., Kurihara, N., Hakeda, Y., and Kumegawa, H. (1992) *Biochem. Biophys. Res. Commun.* **186,** 911.
18. Dempster, D. W., Murrills, R. J., Horbert, W. R., and Arnett, T. R. (1987) *J. Bone Min. Res.* **2,** 443.
19. Shankar, V. S., Huang, C. L.-H., Adebanjo, O. A., Simon, B. J., Alam, A. S. M. T., Moonga, B. S., Pazianas, M., Scott, R. H., and Zaidi, M. (1995) *J. Cell. Physiol.* **162,** 1.
20. Stemmer, P. M., and Klee, C. B. (1994) *Biochemistry* **33,** 6959.
21. Dawson, T. M., Steiner, J. P., Dawson, V. L., Dinerman, J. L., Uhl, G. R., and Snyder, S. H. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9808.
22. Herskovits, J. S., Burgess, C. C., Obar, R. A., and Vallee, R. B. (1993) *J. Cell Biol.* **122,** 565–578.
23. Sugimoto, T., Stewart, S., and Guan, K-L. (1997) *J. Biol. Chem.* **272,** 29415.

24. Gaestel, M., Benndorf, R., Hayess, K., Priemer, E., and Engel, F. (1992) *J. Biol. Chem*. **267,** 21607.

25. Brandi, M. L., Hukkanen, M., Umeda, T., Moradi-Bidhendi, N., Bianchi, S., Gross, S. S., Polak, J. M., and MacIntyre, I. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2954.

26. Sunyer, T., Rothe, L., Kirsch, D., Jiang, X., Anderson, F., Osdoby, P., and Collin-Osdoby, P. (1997). *Endocrinology* **136,** 2148.

27. MacIntyre, I., Zaidi, M., Alam, A. S. M. T., Datta, H. K., Moonga, B. S., Lidbury, P. S., Hecker, M., and Vane, J. R. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2936.