

Trifluoroacetate, a contaminant in purified proteins, inhibits proliferation of osteoblasts and chondrocytes

J. CORNISH,¹ K. E. CALLON,¹ C. Q.-X. LIN,¹ C. L. XIAO,¹
T. B. MULVEY,² G. J. S. COOPER,^{1,2} AND I. R. REID¹

¹Department of Medicine and ²School of Biological Sciences,
University of Auckland, Auckland 1001, New Zealand

Cornish, J., K. E. Callon, C. Q.-X. Lin, C. L. Xiao, T. B. Mulvey, G. J. S. Cooper, and I. R. Reid. Trifluoroacetate, a contaminant in purified proteins, inhibits proliferation of osteoblasts and chondrocytes. *Am. J. Physiol.* 277 (*Endocrinol. Metab.* 40): E779–E783, 1999.—Peptides purified by HPLC are often in the form of a trifluoroacetate (TFA) salt, because trifluoroacetic acid is used as a solvent in reversed-phase HPLC separation. However, the potential effects of this contaminant in culture systems have not been addressed previously. TFA (10^{-8} to 10^{-7} M) reduced cell numbers and thymidine incorporation into fetal rat osteoblast cultures after 24 h. Similar effects were found in cultures of articular chondrocytes and neonatal mouse calvariae, indicating that the effect is not specific to one cell type or to one species of origin. When the activities of the TFA and hydrochloride salts of amylin, amylin-(1–8), and calcitonin were compared in osteoblasts, cell proliferation was consistently less with the TFA salts of these peptides, resulting in failure to detect a proliferative effect or wrongly attributing an antiproliferative effect. This finding is likely to be relevant to all studies of purified peptides in concentrations above 10^{-9} M in whatever cell or tissue type. Such peptides should be converted to a hydrochloride or biologically equivalent salt before assessment of their biological effects is undertaken.

osteoblasts; organ culture; trifluoroacetic acid; peptides

AS A RESULT of rapid advances in the techniques of molecular biology, many new proteins are being identified, purified, and synthesized. Considerable effort is being expended in many areas of biology to determine the activities of these peptides, many of which play roles in cell regulation. Such studies are usually carried out in cell or tissue cultures with either native or synthetic peptides that have been purified by HPLC. Most proteins purified by reversed-phase HPLC contain trifluoroacetate (TFA salt), derived from trifluoroacetic acid (TFA), which is used as a protein-solubilizing and chaotropic agent in HPLC. Peptides purified in this way form salts with TFA, which binds specifically to free NH_2 termini as well as to side chains of exposed lysine, histidine, and arginine residues.

One such peptide is amylin. Amylin consists of 37 amino acids and would be predicted to contain four TFA ions for each peptide molecule. This has been confirmed by direct measurement in one of the widely used preparations that is commercially available (J. Salik, personal communication, Bachem California, Torrance,

CA). In vitro studies of the actions of amylin have typically used peptide concentrations between 10^{-10} and 10^{-7} M, resulting in TFA concentrations in the culture media of four times this level. The potential effects of these concentrations of TFA on biological processes in cells or tissues do not appear to have been considered hitherto. Because TFA is known to have wide-ranging effects on membranes and proteins, it is important that its potential impact as a contaminant in in vitro cultures is understood. The present studies arose from our investigations of the effects of amylin on bone cells, but they address an issue that is potentially of great importance to the assessment of the actions of any peptide purified by HPLC from solutions containing chaotropic anions in any culture system.

MATERIALS AND METHODS

Osteoblast-like cell culture. Osteoblasts were isolated by collagenase digestion of 20-day fetal rat calvariae. Calvariae were dissected aseptically, and the frontal and parietal bones were stripped of their periosteum. Only the central portions of the bones, free from suture tissue, were collected. The calvariae were treated twice with phosphate-buffered saline (PBS) containing 4 mM EDTA (pH 7.4) for 15 min at 37°C in a shaking water bath. After being washed once in PBS, the calvariae were treated twice with 3 ml of 1 mg/ml collagenase for 7 min at 37°C. After the supernatants from these two digestions were discarded, the calvariae were treated two more times with 3 ml of 2 mg/ml collagenase (30 min, 37°C). The supernatants of the latter two digestions were pooled and centrifuged, and the cells were washed in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), suspended in further DMEM-10% FCS, and placed in 75-cm² flasks. After 48 h, the media were changed to minimal essential medium (MEM) with 10% FCS. Confluence was reached within 5–6 days, at which time the cells were subcultured. After trypsinization with trypsin-EDTA (0.05%/0.53 mM), the cells were rinsed in MEM with 5% FCS and resuspended in fresh medium and then seeded at 5×10^4 cells/ml in 24-well plates (0.5 ml cell suspension/well, i.e., 2.5×10^4 cells/well). The cells were incubated under 5% CO_2 -95% air at 37°C. Ascorbic acid (50 mg/l) was added to the MEM used throughout. The osteoblast-like character of these cells has been established by demonstration of high levels of alkaline phosphatase activity and osteocalcin production (3) and a sensitive adenyl cyclase response to parathyroid hormone and prostaglandin E_2 (5).

Proliferation studies (cell counts and thymidine incorporation) were performed in subconfluent cell populations. Twenty-four hours after subculturing, cells were changed to serum-free medium with 0.1% bovine serum albumin for a further 24 h before the addition of the experimental compounds. Cell numbers were analyzed 24 h after addition of the peptide or vehicle by the detachment of cells from the wells by exposure to trypsin-EDTA (0.05%/0.53 mM) for ~5 min at 37°C.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Counting was performed in a hemacytometer chamber. Results are expressed per well. [^3H]thymidine incorporation was assessed by pulsing the cells with [^3H]thymidine (0.5 μCi /well) 2 h before the end of the experimental incubation. [^3H]phenylalanine incorporation was assessed by pulsing the cells with [^3H]phenylalanine (1 μCi /well) for 4 h before the end of the experimental incubation. Experiments were terminated at 24 h by washing the cells in MEM, followed by the addition of 10% trichloroacetic acid. The precipitate was washed twice with ethanol-ether (3:1), and the wells were desiccated at room temperature. The residue was redissolved in 2 M KOH at 55°C for 30 min and neutralized with 1 M HCl, and an aliquot was counted for radioactivity. Results are expressed as disintegrations per minute per well. For cell counts and thymidine incorporation, each experiment was performed at least four times with experimental groups consisting of at least six wells.

Chondrocyte culture. Chondrocytes were isolated by the removal of cartilage slices from the articular surfaces of the knee joints of adult dogs under aseptic conditions. Slices were placed in DMEM containing 5% FCS (vol/vol) and antibiotics (penicillin 50 $\mu\text{g}/\text{l}$, streptomycin 50 $\mu\text{g}/\text{l}$, and neomycin 100 $\mu\text{g}/\text{l}$) and chopped finely with a scalpel blade. Tissue was removed and incubated at 37°C first with pronase (0.8% wt/vol for 90 min), followed by collagenase (0.1% wt/vol for 18 h) to complete the digestion. The cells were isolated from the digest by centrifugation (10 min at 1,300 rpm), resuspended in DMEM-5% FCS, passed through a nytex mesh screen of 90- μm pore size to remove any undigested fragments, and recentrifuged. The cells were washed and resuspended twice in the same media and seeded into a 75- cm^2 flask containing DMEM-20% FCS. The cells were incubated under 5% CO_2 -95% air at 37°C until confluence. Chondrocytes were seeded into 24-well plates, as for osteoblasts.

Bone organ culture. Bone resorption studies were carried out in neonatal mouse calvariae as described previously (8). Hemicalvariae were preincubated for 24 h in Medium 199 with 0.1% bovine serum albumin and then changed to fresh medium containing peptide, TFA, or vehicle. Incubation was continued for a further 48 h. To assess DNA synthesis, [^3H]thymidine (0.6 $\mu\text{Ci}/\text{ml}$) was added in the last 4 h of the incubation, as described previously (6). There were 5–7 hemicalvariae in each group.

Conversion of peptides from the TFA salt to the hydrochloride salt. The hydrochloride salt of each peptide was produced by dissolving it in 3 mM hydrochloric acid (10 μmol peptide in 50 μl) and leaving it for 1 h at room temperature before freeze-drying (Savant Instruments, model SVC 100H, Holbrook, NY). This results in the substitution of hydrochloride for TFA salt, the latter being converted to TFA, which is volatile and is removed by lyophilization. Before use, the peptide was redissolved in pure water with sonication (Soni-prep 150, West Sussex, England), cooled on ice for 15 s, and then stored at 4°C until required. The integrity of the peptides after this procedure was confirmed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (P2025A MALDITOFF, Hewlett-Packard).

Materials. Rat amylin, rat calcitonin, and human calcitonin gene-related peptide were from Bachem California. The rat amylin-(1–8) used in this study was a COOH-terminal amide synthesized as described previously (2). TFA was obtained from BDH Laboratory Supplies (Poole, Dorset, England). EDTA and collagenase were obtained from Sigma (St. Louis, MO). Trypsin-EDTA, MEM, DMEM medium, Medium 199, and FCS were from GIBCO (Grand Island, NY). [^3H]thymidine and [^3H]phenylalanine were from Amersham International (Little Chalfont, Buckinghamshire, UK).

Statistical analysis. Data are presented as means \pm SE. The significance of differences between groups was determined with Student's *t*-test. The comparisons to be made in each experiment were specified a priori, so no adjustment for multiple comparisons was necessary. Where several experiments have been shown in one figure, the data are expressed as the ratio of results in treatment groups to those in the control group and the *P* values shown were calculated with the data from the individual experiments before the data were pooled.

RESULTS

The effects of TFA in concentrations of 10^{-9} to 10^{-7} M were studied in cultured osteoblasts. There was a concentration-dependent reduction in cell number, thymidine incorporation, and phenylalanine incorporation at 24 h (Fig. 1). This suggests that both cell proliferation and protein synthesis were impaired, although it

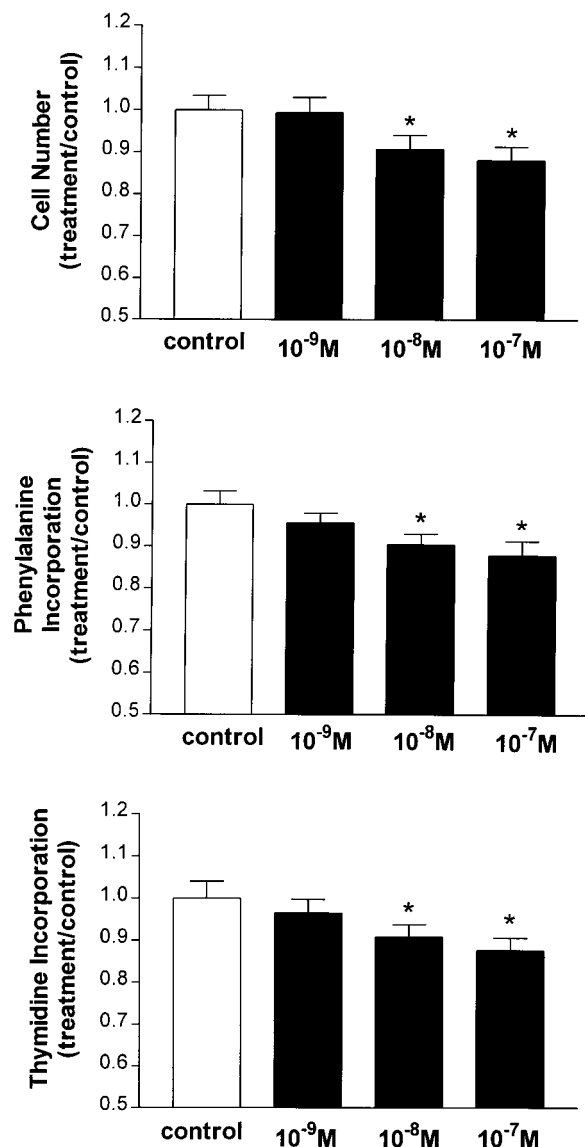


Fig. 1. Effects of addition of trifluoroacetic acid (TFA) in concentrations shown on indexes of growth in cultures of fetal rat osteoblasts studied over a period of 24 h. Significantly different from control, **P* < 0.05.

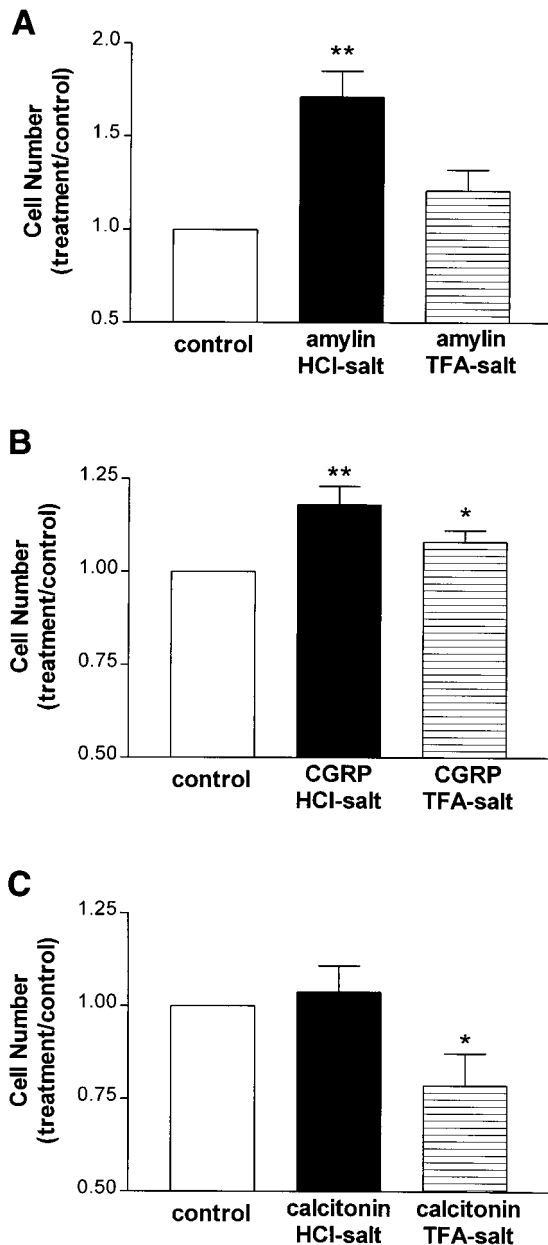


Fig. 2. Effects of rat amylin (A: 10^{-8} M), human calcitonin gene-related peptide (CGRP; B: 10^{-8} M), and rat calcitonin (C: 10^{-7} M), as either TFA salt or HCl salt, on cell number in cultures of fetal rat osteoblasts studied over a period of 24 h. Significantly different from control, * $P \leq 0.03$, ** $P < 0.01$.

should be noted that the decrease in phenylalanine incorporation paralleled the reduction in cell number, indicating that protein synthesis per cell was unchanged.

We then assessed the interaction of the effects of TFA with those of an osteoblast mitogen, in this case rat amylin (Fig. 2A). The hydrochloride salt produced a clear stimulation of osteoblast proliferation. However, the TFA salt produced only an upward trend in cell number, which did not reach statistical significance (Fig. 2A). Similar results were produced with calcitonin gene-related peptide (10^{-8} M), which increased cell number by 8% when used as a TFA salt but by 18%

when used as the hydrochloride (Fig. 2B). Similar experiments were performed with rat calcitonin. When formulated as the hydrochloride salt it had no effect on osteoblast proliferation, but in the TFA form it significantly reduced cell number (Fig. 2C). Studies of the addition of HCl alone showed no effect on cell number (e.g., control, $2.66 \pm 0.04 \times 10^4$ cells/well; HCl, 3×10^{-7} M, $2.5 \pm 0.13 \times 10^4$ cells/well).

Figure 3 shows similar experiments with the weaker osteoblast mitogen amylin-(1–8). This peptide as a hydrochloride salt significantly stimulated both cell number and thymidine incorporation, although as the TFA salt it had no effect on either parameter.

To determine whether the effect of TFA was specific to osteoblasts, we repeated the studies in primary cultures of chondrocytes (Fig. 4). As with osteoblasts, there was a concentration-dependent inhibition of thymidine incorporation and a reduction in cell number.

Finally, we assessed the effects of TFA in an organ culture model, the neonatal mouse calvaria. TFA reduced thymidine incorporation in this model, as did the TFA salt of amylin-(1–8) (Fig. 5). However, as in the isolated osteoblasts, the hydrochloride salt of amylin-(1–8) stimulated thymidine incorporation. In mouse calvaria, we also studied ^{45}Ca release from prelabeled bones, an index of osteoclastic bone resorption. Release of ^{45}Ca was unaffected by TFA in concentrations of up to 2×10^{-7} M, and amylin inhibited bone resorption whether it was added as the TFA salt or the hydrochloride salt (Fig. 6).

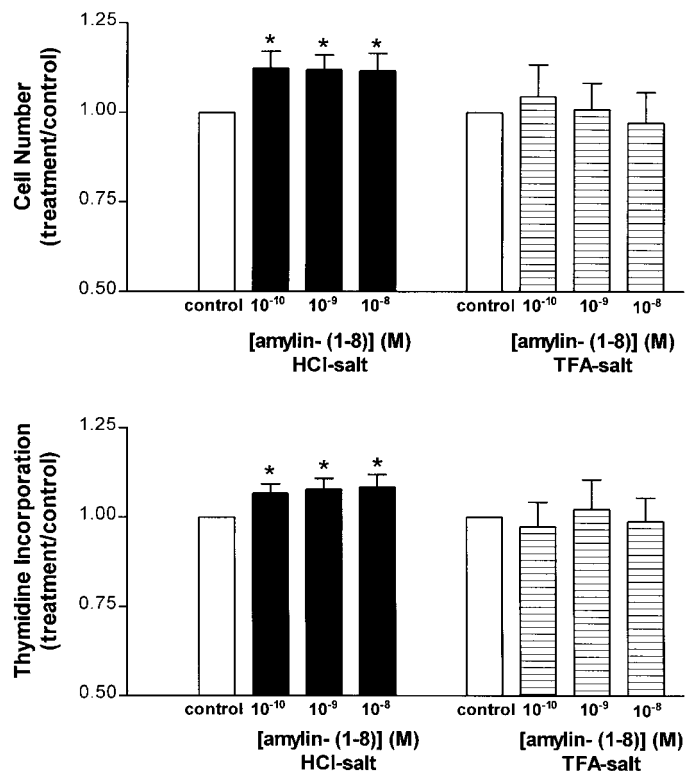


Fig. 3. Effects of amylin-(1–8) as either TFA salt or HCl salt on cell number (top) and thymidine (bottom) incorporation in cultures of fetal rat osteoblasts studied over a period of 24 h. Significantly different from control, * $P < 0.05$.

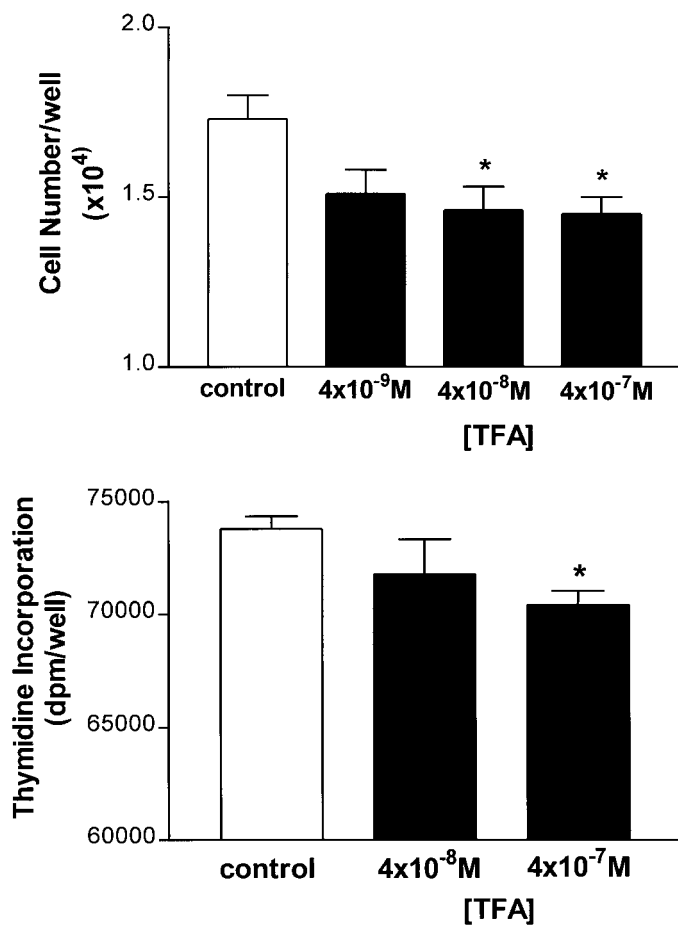


Fig. 4. Effects of addition of TFA in concentrations ([TFA]) shown on indexes of growth in cultures of canine articular chondrocytes studied over a period of 24 h. Significantly different from control, * $P < 0.02$.

DISCUSSION

The present studies establish that TFA in concentrations of the order of 10^{-8} to 10^{-7} M reduces cell proliferation in cultured osteoblasts, chondrocytes, and neonatal mice calvariae. Thus the effect is not specific to one cell type or to one species of origin, and it is seen in both cell and tissue culture. This raises the possibility that it may be a general phenomenon that should be considered when the effects of any purified peptide are being assessed in any culture system. It is probably only relevant when the peptide is being added at concentrations of $>10^{-9}$ M, because lower peptide levels will result in TFA concentrations that are below those found to have effects in the present studies. However, it is possible that cell types may vary in their sensitivity to TFA, so it is an issue that should be addressed in any culture system used to assess the effects of purified or synthetic proteins.

The consequences of failing to take into account the effects of the accompanying anion of a peptide salt are demonstrated in the present study. Weak proliferative effects can be overlooked because they are counteracted by the TFA contaminating the peptide [as with amylin-(1-8) in the present studies], or a peptide that has no

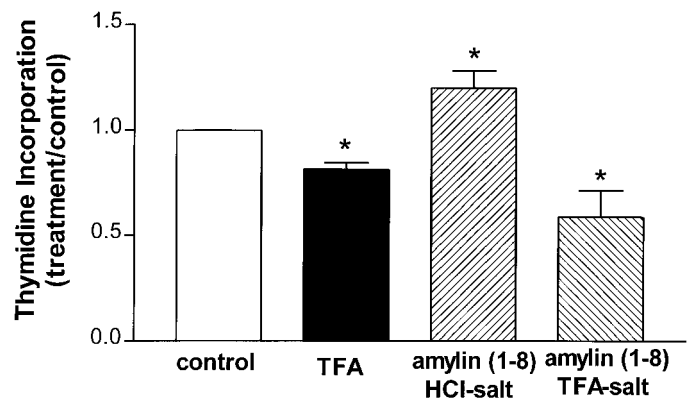


Fig. 5. Effects of addition of 2×10^{-7} M TFA or amylin-(1-8) as either TFA salt or HCl salt on thymidine incorporation in neonatal mouse calvariae. Significantly different from control, * $P < 0.05$.

effect on proliferation might be found to apparently inhibit cell growth, as we found with calcitonin. These effects, however, seem to be specific to cell replication because protein synthesis was only reduced in parallel with cell number and cell-mediated bone resorption was not affected by TFA. These two negative findings do not provide any guarantee that all other cellular processes are not influenced by TFA, and the onus remains on investigators to establish this fact for the end points being assessed in a given study. The use of vehicle controls has not addressed this issue to date, because virtually no one has been adding TFA to their control cultures because of a general unawareness of the presence of this contaminant in purified peptides.

The mechanisms of the difference in effects of TFA and hydrochloride salts remain to be determined. They clearly are effects of TFA and not of HCl, because they are seen when TFA is added alone and because the addition of 10^{-7} M H^+ or Cl^- ions could not produce such biological effects. This amount of H^+ ion is well within the buffering capacity of the media. Cl^- is the principal anion of the media and is already present in a concentration of more than 100 mM.

The biological effects of TFA have already been investigated in two other contexts. TFA is the principal

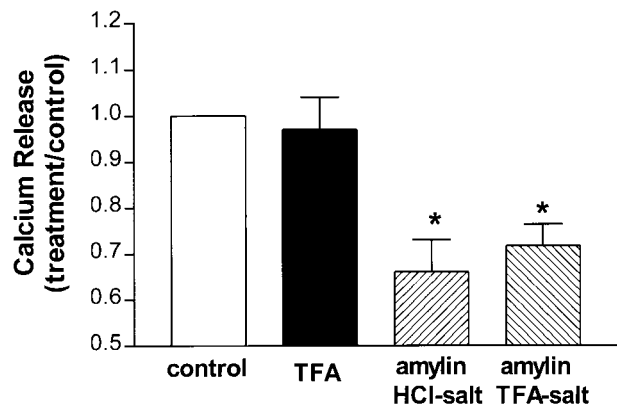


Fig. 6. Effects of addition of 2×10^{-7} M TFA or amylin as either TFA salt or HCl salt on ⁴⁵Ca release from neonatal mouse calvariae. Significantly different from control, * $P < 0.002$.

metabolite of the general anesthetic agent halothane. In vivo, it causes the formation of trifluoroacetylated forms of many proteins, a process thought to underlie the development of halothane hepatitis (4). Effects have also been found in cultures of glioma cells and are suggested to underlie halothane-associated neurotoxicity (7). This widespread modification of cell proteins could underlie the effects found in the present study. Possibly related to this effect is the fact that TFA tends to break down the intermolecular structure of water; that is, it is a chaotropic anion. Such anions have been shown to impact on membrane function, enzymatic catalysis, the secondary structure of proteins, and protein stability (1). These actions provide potential explanations for the suppression of cell proliferation observed in the present studies.

In conclusion, we have demonstrated significant biological effects of TFA, which is present as a contaminant in many commercially available peptides. This finding casts doubt on the results of any studies of purified peptides in concentrations above 10^{-9} M in which measures were not taken to remove TFA from the peptides used or to add it in appropriate concentrations to controls. Fortunately, the problem is easily circumvented by the production of hydrochloride salts from the TFA salts generally available.

We are grateful to Dr. P. Wiggins for helpful discussions regarding this work and to Dr. C. A. Poole for advice regarding chondrocyte preparations.

This work was funded by the Health Research Council of New Zealand and the Endocore Trust.

Address for reprint requests and other correspondence: J. Cornish, Dept. of Medicine, Univ. of Auckland, Private Bag 92019, Auckland 1001, New Zealand (E-mail: j.cornish@auckland.ac.nz).

Received 18 September 1998; accepted in final form 14 June 1999.

REFERENCES

1. **Collins, K. D., and M. W. Washabaugh.** The Hofmeister effect and the behaviour of water at interfaces. *Q. Rev. Biophys.* 18: 323–422, 1985.
2. **Cornish, J., K. E. Callon, C. Q.-X. Lin, C. L. Xiao, T. B. Mulvey, D. H. Coy, G. J. S. Cooper, and I. R. Reid.** Dissociation of the effects of amylin on osteoblast proliferation and bone resorption. *Am. J. Physiol.* 274 (*Endocrinol. Metab.* 37): E827–E833, 1998.
3. **Groot, C. G., J. K. Danes, J. M. van der Meer, and M. P. M. Herrmann-Erlee.** Osteocalcin antigenicity in cultured osteoblast-like cells after stimulation with 1,25-vitamin D3. *Cell Biol. Int. Rep.* 9: 528, 1985.
4. **Gut, J., U. Christen, N. Frey, V. Koch, and D. Stoffler.** Molecular mimicry in halothane hepatitis: biochemical and structural characterization of lipoylated autoantigens. *Toxicology* 97: 199–224, 1995.
5. **Herrmann-Erlee, M. P. M., and J. M. van der Meer.** The effect of PTH and PGE2 on growth and differentiation of primary fetal rat osteoblast-like cells, on UMR 106 osteosarcoma cells, and on a SV40 transformed "osteoblast-like" cell line. *Int. Conf. IX on Calcium Regulating Hormones and Bone Metabolism, 1986*, p. 409.
6. **Lowe, C., J. Cornish, T. J. Martin, and I. R. Reid.** Effects of leukemia inhibitory factor on bone resorption and DNA synthesis in neonatal mouse calvaria. *Calcif. Tissue Int.* 49: 394–397, 1991.
7. **Ma, T. G., Y. H. Ling, G. D. McClure, and M. T. Tseng.** Effects of trifluoroacetic acid, a halothane metabolite, on C6 glioma cells. *J. Toxicol. Environ. Health* 31: 147–158, 1990.
8. **Reid, I. R., C. Lowe, J. Cornish, S. J. M. Skinner, D. J. Hilton, T. A. Willson, D. P. Gearing, and T. J. Martin.** Leukemia inhibitory factor: a novel bone-active cytokine. *Endocrinology* 126: 1416–1420, 1990.