Subterranean mole-rats naturally have an impoverished calcio status, yet synthesize calcio metabolites and calbindins

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Mole-rats (Family Bathyergidae) have no obvious source of calcio. They live in an environment devoid of sunlight and consume a herbivorous diet. Calcio status, metabolism and expression were examined in six species of Bathyergids. Serum levels of calcidiol in all species were < 5 µg/l and those of calcitriol were low (18.0 ± 11.0 (sd) ng/l, N = 57) when compared to other rodents. Within 72 h of injecting animals with tritium-labelled calcio, most of the labelled prohormone had been metabolized to more polar metabolites. Three times more tritium-labelled calcitriol (19.3 ± 2.9%) was present than (24R)-hydroxycalcidiol (6.2 ± 10%). The natural absence of detectable circulating concentrations of calcidiol and the threefold greater amount of calcitriol to (24R)-hydroxycalcidiol produced indicate that calcio naturally is in short supply. Calcio-dependent calbindins were absent in the duodenum. Calbindin-D28k was present in the Parkinje cells of the cerebellum and in some collecting ducts and proximal and distal convoluted tubules of the kidney. Calbindin-D9k also was present but was localized uniquely in the juxtaglomerular cells of the five southern African species. These data confirm that Bathyergid mole-rats naturally have an impoverished calcio status. Despite the presence of calbindins in renal tissues, the functional importance of this hormone in calbindin synthesis and normal mole-rat physiology is not known.

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Calcitriol, a pleiotropic hormone that regulates a vast array of biological processes, including mineral metabolism, ionic flux, differentiation and cell signalling (1, 2). Calcio may be synthesized endogenously in the presence of sunlight or acquired dietarily. In avians and mammals, adequate amounts of calcio are synthesized in the skin, utilizing a non-enzymatic photolysis of 7-dehydrocholesterol in the presence of sunlight (3). Dietary sources of calcio are primarily animal tissues (fat and muscle). Calcidiol is the principal circulating calcio metabolite and its concentration is used routinely as an indicator of calcio status (1, 4). Calcidiol is converted in the kidney to the active hormone calcitriol or (24R)-hydroxycalcidiol. The functional significance of this latter metabolite is not known (5).

Calcio-dependent calcium-binding proteins (calbindins) are generally considered good markers of genomic calcitriol activity (6, 7). They occur in all vertebrates studied to date and the proteins are phylogenetically conserved (8). Calbindins are commonly found in tissues involved with calcium transport, namely, the duodenum, kidney and placenta (6, 7, 9). Recently, however, they have been detected in tissues unrelated to mineral metabolism, e.g. cerebellum, pituitary, spinal cord, visual cortex (7). The functional significance of calbindins in these tissues is not yet known. Furthermore, the role of calcio in calbindin synthesis is controversial. Control of intestinal calbindin-D9k by calcio remains undisputed (7); however, its role in calbindin-D9k and calbindin-D28k expression in other tissues is still unclear.

The role of the vitamin endocrine system in strictly subterranean mammals has received scant attention. Mole-rats (Bathyergidae) lead a strictly subterranean existence, living in sealed burrow systems that effectively shield them from sunlight (10). They have inhabited this chthonic milieu for millennia (10) and show a wide range of morphological and physiological adaptations to their underground habitat (10, 11). An

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adaptation that has received little attention is their long-term continued survival and success in an environment devoid of sunlight (12, 13). Previous studies undertaken in our laboratory have shown that mole-rats do have calcitriol receptors (14) and renal hydroxylase enzymes (15). Calcium balance studies in the two species of mole-rat studied to date indicate that dietary calcium absorption is independent of calcitriol (12, 13), although other calcitriol-mediated functions, e.g. bone turnover and reproductive successes, appear to be enhanced by calcitriol supplementation (16).

Given the biological importance of calcitriol in mammalian physiology, and the fact that the chthonic habitat and strictly herbivorous diet of mole-rats preclude an obvious source of this vitamin, we investigated in six species of African mole-rats the calcitriol status, whether these animals have the ability to metabolize calcitriol to more polar metabolites and whether these animals have calbindins in the classical target tissues (duodenum and kidney).

Material and methods

Animals

Five species of freshly caught southern African mole-rats, namely, *Bathyergus janetta*, *Bathyergus suillus*, *Cryptomys damarensis*, *Cryptomys hottentotus* and *Georychus capensis* and captive mole-rats of a sixth species from Kenya, *Heteroecephalus glaber*, were used in this study. Naked mole-rats (*H. glaber*) used in this study were born in captivity and were at least 1 year old. They were kept under dim incandescent lighting and fed their preferred “captive diet” of mixed vegetables. The mole-rats of the other species were freshly caught (of adult mass) and kept out of sunlight until used in this study.

Plasma and tissue collection

Animals were anaesthetized by fluorothane inhalation and thereafter were killed by exsanguination. The plasma fraction of the blood was separated and stored at -70°C for later analysis. Tissue samples of cerebellum, duodenum and kidney were collected, fixed in Bouins fluid for 12 h and stored in 70% alcohol prior to immunohistochemistry studies.

Assay of calcitriol metabolites

Serum total calcitriol and calcidiol metabolite concentrations, respectively, were measured by kidney cytosol receptor (17) and calf thymus receptor assays (18) after metabolite separation using the methods described by Turnbull et al. (19). The sensitivities of the calcitriol and calcidiol assays were 5.0 μg/l and 4 ng/l serum, respectively.

Injection of tritium-labelled calcitriol and its metabolic conversion

Metabolic conversion of tritium-labelled calcitriol to the more polar metabolites was assessed following the methods of Mawer et al. (20).

Eleven *C. damarensis*, three *C. hottentotus* and twelve *H. glaber* animals were each given an intraperitoneal dose of about 5 μCi of tritium-labelled calcitriol (Amersham International, UK). After 48 h, six *C. damarensis* and eight *H. glaber* animals were anaesthetized and killed by cardiac exsanguination. The remaining animals were killed 72 h after receiving the tritium-labelled calcitriol.

Plasma was separated from the red blood cells and subjected to total lipid extraction. The lipid fraction of these plasma samples, thereafter, was subject to preliminary silicic acid column chromatography as described by Delvin and Dussart (21), followed by successive HPLC chromatography using a variety of solvent systems (22, 23).

Chromatography

Silicic acid chromatography was used initially to separate the various calcitriol metabolites. The column, a 0.5 × 8 cm pasteur pipette, was stoppered at the lower end with a cotton-wool plug and packed to a height of 4 cm with a slurry of activated silicic acid in hexane. The column was calibrated using tritium-labelled calcitriol, calcidiol and calcitriol (Amersham International, UK), initially separately and finally in combination. Following calibration chromatography with known metabolites, it was established that calcitriol eluted in the 55% ether/hexane solvent (3–5 ml), calcidiol in the 95% ether/hexane solvent (7–9 ml) and the more polar metabolites in 50% ether/acetone (11–12 ml). The recovery of each of these known metabolites was 85%, 85% and 70%, respectively.

The lipid fraction of serum samples was dissolved in a solvent of 20% ether in hexane and added to the column. Thereafter, the column was eluted successively with 5 ml of 55% ether/hexane, 95% ether/hexane and 50% acetone/ether. Each of the eluted fractions were collected separately. A fraction of each eluted sample was counted for tritium, using a Beckman scintillation cocktail and a beta liquid scintillation counter.

The remainder of the eluted samples were rechromatographed, purified further and separated using five different solvent systems on an HPLC column. A Spherisorb 10-μm, 250-mm silica column (Deeside Industrial Estate, UK) was used for the straight-phase HPLC. The pressure pump was an LDC Constametric (Milton Roy, Laboratory Data Control, Riviera Beach, FL, USA) coupled to an LDC 1203 U III monitor with a 245-nm filter and a Viatron chart recorder. All solvents used were HPLC grade (Walters Millipore, SA). Five different isocratic solvent systems were used: a ternary
solvent system of hexane/isopropanol/methanol in the ratio of 90:6:4 (23) and four binary solvent systems of isopropanol/hexane at concentrations of 4%, 8%, 10% and 15%, respectively (22). Thirty fractions of 1 ml each of the ternary solvent system and forty 1-ml fractions of the binary solvent systems were collected. Each system was calibrated using calciol and the major calciol metabolites (calciol, (24R)-hydroxycalciol and calciol) at a concentration of 100 ng/20 µl hexane. Radioactive peaks that migrated to the same position as authentic metabolites in consecutive solvent systems were used as a means of identification of the metabolite.

Immunohistochemistry of calcium binding proteins

The presence of calbindins was determined using a modified indirect peroxidase immunocytochemical technique in fixed tissues as described by Opperman et al. (24). Tissues were fixed in Bouins solution and were processed routinely through a graded series of alcohols, cleared in chloroform and embedded in paraplast (Histosec Pastilles, Merck, South Africa). Consecutive 5 µm thick sections were used in the immunohistochemical studies. Paraplast was removed from the tissues with xylene and the tissues were rehydrated prior to the immunohistochemical procedures.

The primary antibodies to calbindin-D_{28k} and calbindin-D_{28k} were raised in rabbits against antigens prepared from chick, mouse and rat tissues. Rabbit antisera to chick renal calbindin-D_{28k} and mouse and rat intestinal calbindin-D_{9k} were gifts from Dr EM Bruns (Dept Pathology, University of Virginia, Charlottesville). These were used at a dilution of 1:500 for calbindin-D_{28k} and 1:250 for calbindin-D_{9k}. Anti-rabbit peroxidase-conjugated swine antiserum was obtained from Dakopatts, Copenhagen, Denmark. All sections were placed in 5 mmol/l TRIS and 150 mmol/l sodium chloride buffer prior to a 10-min incubation with 10% swine serum in the buffer. Swine serum was removed by washing briefly in the same TRIS/saline buffer. Tissues then were incubated with antibodies for 48 h in a moist environment at 4°C. In each immunocytochemical run, control sections were incubated with preabsorbed non-immune rabbit serum.

Additional positive controls were provided using sections of mouse kidney and duodenum incubated with antibodies to their respective calbindins (8). Controls for antibody specificity consisted of replacing specific antisera with either non-immune rabbit serum at a dilution of 1:250 or with diluent. Furthermore, there was an additional control for calbindin in which the antisera was preabsorbed overnight at 4°C with purified antigen at 400 mg/l undiluted antisera. Both calbindin-D_{9k} antisera and antigens were generous gifts from Dr ME Bruns. Polyclonal antisera to calbindin-D_{28k} were developed from chick duodenal calbindin purified in this laboratory. Purity was confirmed by a single band on SDS-PAGE and specificity of the antibodies confirmed by their ability to be preabsorbed by pure antigen generously provided by Dr S Christakos. The final concentration of antisera/antigen was the same as the primary antibody used in each run.

After the 48 h incubation period, all slides were given three 5-min washes in the TRIS/saline buffer to which 10% horse serum (Highveld Biological Supplies, Johannesburg) was added. The tissues then were incubated with a 1:20 dilution of peroxidase-conjugated swine anti-rabbit immunoglobulin complex for 30 min in a moist environment at room temperature, prior to removing excess peroxidase by three 5-min washes in the TRIS/saline buffer. Diaminobenzidine tetrahydrochloride (Merck, South Africa; 500 mg/l) plus hydrogen peroxide (1 ml/l) was used as a substrate for the peroxidase reaction. This was added to the sections for 5 min at room temperature and thereafter subjected to a 5-min tapwater wash. All sections were stained briefly (15 s) in modified Meyer's haematoxylin then dehydrated and coverslips applied with entelin (Merck, South Africa). Sections were viewed and photographed under the light microscope.

Statistical analyses

Data are presented as means ± sd. Interspecies data for serum metabolites were compared using analyses of variance.

Results

Serum levels of calcitriol metabolites

In all species examined, calcitriol levels were below the limit of detection (< 5 µg/l; Table 1), whereas most (93%) individuals had measurable levels of calcitriol (Table 1). All the individuals (N = 4) with undetectable serum levels of calcitriol belonged to captive H. glaber.

Concentrations of calcitriol were quite variable and thus did not differ significantly between species (Table 1). The mean concentration for all the Bathyrergids examined was 18.0 ± 11.0 ng/l (N = 57).

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>Calcitriol (ng/l)</th>
</tr>
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<tbody>
<tr>
<td>Bathyrergus janetta</td>
<td>4</td>
<td>22.5 ± 7.6</td>
</tr>
<tr>
<td>Bathyr ergus suillus</td>
<td>4</td>
<td>20.8 ± 7.8</td>
</tr>
<tr>
<td>Cryptomys damaren sis</td>
<td>17</td>
<td>17.2 ± 9.6</td>
</tr>
<tr>
<td>Cryptomys hottentotus</td>
<td>18</td>
<td>18.5 ± 10.7</td>
</tr>
<tr>
<td>Georychus capensis</td>
<td>5</td>
<td>15.6 ± 8.0</td>
</tr>
<tr>
<td>Heterocephalus glaber</td>
<td>11</td>
<td>17.2 ± 17.5</td>
</tr>
<tr>
<td>Bathyr ergids</td>
<td>57</td>
<td>18.0 ± 11.0</td>
</tr>
</tbody>
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*Levels of calcitriol were < 5 µg/l.
Conversion of calciol to metabolites

The conversion of tritium-labelled calciol to the more polar metabolites was examined in three species. Plasma collected from mole-rats exsanguinated 48 h after intraperitoneal administration of tritium-labelled calciol and thereafter subjected to silicic acid column chromatography revealed radioactive metabolites in positions characteristic of the injected reference parent compounds.

With the exception of the one breeding female, most of the radioactivity in naked mole-rats (H. glaber) remained as calciol (71.8 ± 13.2%) 48 h after administration (Fig. 1). Only 18.9 ± 9.8% had been converted to calcidiol and even less (9.4 ± 3.8%) to more polar metabolites. By 72 h more of the tritiated calciol in these rats (H. glaber) had been converted to calcidiol (43.7 ± 7.3%) and more polar metabolites (18.3 ± 4.1%). Both species of Cryptomys showed similar conversion rates to naked mole-rats at 48 and 72 h (Figs. 1 and 2).

The single breeding naked mole-rat female was not included in the mean as she had converted most of the injected tritiated calciol to calcidiol (59.0%) and more polar substances (13.7%) within the 48-h period after injection (Fig. 1). This might reflect different metabolite turnover rates and requirements at pregnancy.

At 72 h there were threefold more counts present in the calciol fraction than in the (24R)-hydroxy-calcidiol, with the bulk of the counts found as calcidiol (Fig. 2).

Presence of calcium-binding proteins

All six species of mole-rats, irrespective of the serum concentrations of calcidiol and calcitriol, stained positively for calbindins in specific areas of the cerebellum and kidney tissues (Fig. 3A–C). Duodenal tissues showed no specific binding to calbindin-D9k or calbindin-D28k (Fig. 3D).

In the cerebellum, calbindin-D28k was present in the Purkinje cells only. The stain occurred throughout the perikarya and extended into the dendritic process (Fig. 3A). Both calbindin-D9k and calbindin-D28k were present in kidney tissue. Calbindin-D28k showed a wider distribution than calbindin-D9k. The larger protein was present in the distal and proximal convoluted tubules and collecting ducts of the cortical region of the kidney (Fig. 3B). No reaction was seen in the loops of Henle or glomeruli. Positive stained cells of the collecting tubules extended into medullary rays. Not
Fig. 3. Immunohistochemical localization of calbindins in cells counterstained with haematoxylin. (A) Immunohistochemical localization of calbindin-D_{28k} in mole-rat cerebellum (e.g. *Bathyergus janetta*). Positive staining was present in the Purkinje cells and their dendritic processes of all six species investigated. Photographs taken using light microscopy at: (i) ×10; (ii) ×40. (B) Immunohistochemical localization of calbindin-D_{28k} in the kidney (e.g. *Heterocephalus glaber*). In all six species investigated, positive staining occurred in the proximal and distal convoluted tubules and in the collecting ducts. Not all the collecting tubules and convoluted tubules in the kidney showed positive staining and even within positively stained tissue there were variable degrees of staining. Photographs were taken using light microscopy at: (i) ×10; (ii) ×40. (C) Immunohistochemical localization of calbindin-D_{9k} in the kidney of the southern African Bathyergids (e.g. *Cryptomys damarensis*). Positive staining occurred in the juxtaglomerular cells only. This calbindin-D_{9k} was not present in the kidney of the naked mole-rat. Photographs taken using light microscopy at: (i) ×10; (ii) ×40. (D) Duodenal cells in all six mole-rat species showed no immunoreactivity with either calbindin-D_{9k} or calbindin-D_{28k}. Photographs taken using light microscopy at: (i) ×10; (ii) ×40.
Discussion

Mole-rats differ markedly from most other mammalian species (25) in that, in their normal physiological state (as determined by freshly caught animals), serum levels of calcidiol are below 5 μg/l (Table 1). Concentrations of calcidiol below 5 μg/l are generally indicative of calcidiol deficiency (4) and thus the data from this study might indicate a natural impoverished calcidiol status in mole-rats. Recently, however, several phylogenetically distinct species (26, 27) have exhibited similar undetectable natural serum concentrations, with no evidence of pathological problems associated with calcidiol deficiency (4). The reliability, therefore, of serum calcidiol concentrations as a marker of calcidiol status is now questioned (28, 29).

Many non-ruminant herbivores have undetectable levels of calcidiol, and it is speculated that they do not utilize calcidiol-mediated intestinal absorption (26). Similarly mole-rats show extremely efficient apparent intestinal mineral absorption (> 85%) despite undetectable levels of calcidiol (12, 30). Furthermore, the apparent fractional absorption efficiency is not enhanced by calcidiol supplementation (30, 31).

The presence of calcitriol, albeit in low concentrations (Table 1), relative to other rodents (32) is in itself conclusive evidence that calcitriol is metabolized by these chthonic mammals. Administration of tritium-labelled calcitriol and the subsequent analyses of mole-rat plasma for radioactive calcitriol metabolites 48 and 72 h later, showed that like all vertebrates studied to date (20, 33) calcitriol is converted to its more polar metabolites (Fig. 2).

Three times as much calcitriol was produced compared to (24R)-hydroxycalcidiol (Fig. 2). The relative proportion of each dihydroxy metabolite is similar to what one would expect in calcitriol-deficient animals (5). Absence of calcitriol from field and captive animals gives credence to our hypothesis that these animals are normally in an impoverished calcidiol state.

Substantial evidence exists that calcitriol elicits some of its genomic biological responses through protein synthesis of calbindins (7). As it is beyond dispute that duodenal calbindin expression is mediated by calcitriol (7), absence of calbindin in mole-rat duodenal tissues (Fig. 3D) might therefore indicate that these animals are deficient in calcitriol. Possibly these animals lack sufficient calcitriol receptors in the duodenum (14) to induce calcitriol-mediated calbindin synthesis. Furthermore, lack of duodenal calbindins (12, 13) supports our view that duodenal calcium transport is not mediated by calcitriol. Alternatively, the lack of calbindins in duodenal tissues may suggest that mole-rats do not employ calbindin-mediated active transport of calcium in this gastrointestinal region. Indeed, the mode of calcium absorption in the duodenum of the damara mole-rat has been shown to be passive, independent of calcidiol status and more than adequate to maintain a positive mineral balance (30).

Despite the very low serum concentrations of both calcidiol and calcitriol in all six species, calbindin-D28k was present in the kidney and cerebellum (Fig. 3A–C) and showed similar distribution to that documented for other mammals (7, 24, 34). Calbindin-D28k is found in all animals examined to date (i.e. from molluscs to mammals) (34) and is thought to have widespread functional significance, although its precise role is unknown (7). The role of calcidiol in renal calbindin expression is unclear. Hall and Norman (35) have shown that these proteins are tightly regulated by calcidiol whereas Harrison et al. (36) and Bruns et al. (6) demonstrated that renal calbindin-D28k was unaffected by the administration of calcitriol. Furthermore diabetic rats, with their concomitant decrease in serum calcitriol concentrations show pronounced changes in duodenal calbindin content (37) but not in renal calbindin content (38). It is therefore unclear, at this stage, whether the renal calbindin-D28k presence in mole-rats is a marker of calcidiol activity or whether its expression, like that of calbindins in the cerebellum, is independent of calcidiol status (6). Given the absence of duodenal calbindins, we speculate that the latter is the case.

Calbindin-D9k is found in mammalian tissues only (34). This protein was present in kidneys of the five southern African mole-rat species, but was absent in the northeast African species (H. glaber). The calbindin-D9k was not co-localized with calbindin-D28k, but rather showed a unique distribution restricted to juxtaglomerular cells only (Fig. 3C). This is the first report, to date, of detection of calbindins in juxtaglomerular cells. These might reflect the importance of calcium signalling, buffering and transduction in mole-rat juxtaglomerular cells. The unique presence of calbindin-D9k in southern African mole-rat juxtaglomerular cells and functional significance of calbindin-D9k in mole-rat kidneys is not known. Heterocephalus glaber differs from the other mole-rat species investigated in that it does not show positive staining to calbindin-D9k. This aberration is in keeping with other physiological differences shown by this unusual mammal (11). Interspecies differences in the mole-rats family might reflect different calbindin function and/or may reflect their different evolutionary histories and degree of geographical isolation.

Our hypothesis that mole-rats, living in an underground habitat, naturally have low amounts of circulating calcidiol was correct. However, the speculation that mole-rats do not have the cellular mechanisms
for calcium metabolism and metabolism did not hold true. Indeed, calcium metabolism followed a similar enzymatic pathway to that of most mammals. While mole-rats do possess renal calbindins, the role of calcium in mole-rat calbindin expression and normal mole-rat physiology is not known.

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References
33. Feinblatt JD. The comparative physiology of calcium regulation in sub-mammalian vertebrates. Adv Comp Physiol Biochem 1982;8:73–110
34. Rhoten WB, Bruns MEH, Christsakos S. Presence and localization
of two vitamin D-dependent calcium binding proteins in kidneys of higher vertebrates. Endocrinology 1985;117:674–783
35. Hall AK, Norman AW. Regulation of calbindin-D_{28k} gene expression by 1,25-dihydroxyvitamin D_{3} in chick kidney. J Bone Miner Res 1990;5:325–30

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