Long-term swimming in an inescapable stressful environment attenuates the stimulatory effect of endurance swimming on duodenal calcium absorption in rats

Narattaphol Charoenphandhu · Jarinthorn Teerapornpuntakit · Sarawut Lapmanee · Nitita Dorkkam · Nateetip Krishnamra · Jantarima Charoenphandhu

Received: 17 May 2011/Accepted: 20 July 2011/Published online: 21 August 2011
© The Physiological Society of Japan and Springer 2011

Abstract Endurance swimming is known to increase duodenal calcium absorption in normal rats and bone strength in estrogen-deficient rats. Because the stress resulting from forced training often attenuates the stimulatory effect of exercise, swimming in an inescapable chamber should reveal both the positive effect of the exercise and the negative effect of stress. In the work reported herein, swimming rats showed no signs of stress during 2 weeks of training. However, stress response gradually developed thereafter and peaked at weeks 6 and 7. In rats swimming for 2 weeks, transcellular duodenal calcium transport was enhanced ~2-fold. In contrast, calcium absorption was reduced in rats swimming for 8 weeks, consistent with the absence of swimming-induced upregulation of calcium transporter genes in the 8-week group. In conclusion, prolonged stress hindered the stimulatory effect of swimming on duodenal calcium absorption, and thus endurance exercise should be performed without forced training or stress to retain its beneficial effect on calcium metabolism.

Keywords Calcium transporter · Endurance swimming · Paracellular permeability · Stress · Transcellular transport

Introduction

Swimming, as a non-impact endurance aerobic exercise, is known to benefit total body calcium homeostasis in humans and rodents [1–3]. For example, 12-week endurance swimming helped maintain bone mass and bone strength in estrogen-deficient osteopenic rats [2]. Such beneficial effects could be because of the direct mechanical strain on bone during muscle contraction and exercise-stimulated intestinal calcium absorption [4, 5]. Our recent investigation of female rats clearly showed that 2-week endurance swimming at mild-to-moderate intensity induced adaptation of the intestinal absorptive cells at the molecular level, for example overexpression of calcium transporter genes and nuclear vitamin D receptor (VDR), thereby leading to an enhanced duodenal calcium absorption [6].

However, in rodents, prolonged exposure to an inescapable environment, for example a swim-training chamber, may subtly induce chronic stress, corticosterone release, and/or anxiety-like behavior including fear with inappropriate arousal [7–9]. A significant stress response may not be observed for exercising animals during the first 1–2 weeks of training, but continued training for ~4–8 weeks usually induces such a response [10]. Therefore, swimming exercise in rats should be a suitable model for investigating the positive effects of endurance exercise versus the negative effects of long-term forced
training (i.e., moderate unavoidable stressor). Because chronic stress itself and stress-induced adrenal corticosteroid release have been reported to reduce intestinal calcium absorption [11, 12], it was expected that the stimulatory effects of endurance exercise on intestinal calcium absorption could be attenuated by long-term swimming in an inescapable environment.

With regard to intestinal calcium absorption, calcium is transported across the duodenal epithelium via transcellular and paracellular pathways [13, 14]. Although paracellular calcium flux is usually predominant under normal conditions, especially with calcium-rich diet, transcellular flux becomes more significant under calcium-deficient conditions or high calcium demand, for example in pregnancy, lactation, and exercise [13]. Transcellular calcium transport is a three-step, 1,25(OH)2D3/VDR-dependent active process, i.e.:

1. Apical calcium uptake through transient receptor potential vanilloid family calcium channels (TRPV) 5 and 6;
2. Cytoplasmic, facilitated diffusion after binding to calbindin-D9k; and
3. Basolateral extrusion, ~20% via Na+/Ca2+ exchanger (NCX)-1 and ~80% via the plasma membrane Ca2+-ATPase isoform 1b (PMCA1b) [13–15].

Recently, 2-week endurance swimming was reported to increase mRNA expression of TRPV5, TRPV6, and calbindin-D9k in the rat duodenum [6], whereas 2-week immobilization markedly downregulated these genes [16]. However, whether swimming actually enhanced intestinal calcium absorption through the transcellular pathway had never been demonstrated experimentally.

Paracellular calcium transport, which is also 1,25(OH)2D3-dependent, is partly determined by tight junction permeability to cations [17, 18]. Such cation permeability is controlled by particular isoforms of the 1,25(OH)2D3-dependent tight junction proteins of the claudin family, especially claudin-2, 3, and 12, which have been proposed to form cation-selective pores in the tight junction [18, 19]. In the absence of a transepithelial calcium gradient, paracellular water flow takes with it some free ions, including calcium; this is thus known as solvent drag-induced calcium transport [13]. This water flow is driven by the paracellular hyperosmotic environment created by Na+/K+/ATPase in the lateral membrane [13]. Moreover, calcium also passively traverses the paracellular space down the concentration gradient, especially when the mucosal calcium concentration rises during high-calcium intake [20]. Although our previous investigation revealed increased expression of cation-selective claudin-2 and 3 after 2-week swimming [6], the effects of endurance swimming on paracellular cation permeability and on solvent drag-induced and paracellular passive calcium transport remained unknown.

The principal objectives of this study were:
1. To determine whether long-term endurance swimming of moderate intensity in an inescapable chamber could lead to stress and/or anxiety-like behavior in adult female rats;
2. To investigate the stimulatory effects of endurance swimming on the transcellular and paracellular components of duodenal calcium absorption; and
3. To demonstrate that long-term swimming in a stressful environment was able to attenuate the stimulatory effects of exercise on duodenal calcium absorption and calcium transporter expression.

Materials and methods

Animals

Female Sprague–Dawley rats, weighing 180–200 g (10 weeks old) were obtained from the National Laboratory Animal Centre, Thailand. They were housed in the husbandry unit for at least 7 days before the experiments under a 12/12 h dark/light cycle (light on 6:00–18:00 h; average illuminance of 200 lux), and were fed regular chow, containing 1.0% calcium and 0.9% phosphorus (Perfect Companion, Bangkok, Thailand), and reverse osmosis water ad libitum. The room temperature was 25°C and the humidity 55%. Food and water intake were monitored daily to ensure that all rats had no stress because of insufficient food or water. Animals were cared for in accordance with the “Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences”.

The study was approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Mahidol University, and the Faculty of Medicine, Thammasat University, Thailand.

Experimental design

Unless otherwise specified, the rats were randomly divided into age-matched sedentary groups and exercise groups, the latter of which were subjected to endurance swimming for 2, 4, 6, or 8 weeks. Body weight was recorded before training and every swimming day for all groups. Successful exercise training was confirmed by measuring wet and dry heart weights at the end of each swimming period. These rats were later used in the investigations of stress, anxiety-like behavior, duodenal calcium absorption, and duodenal expression of calcium transporter genes. Each of these was determined in separate experiments. Stress was
continuously monitored by use of the sucrose-intake test, whereas the open-field test was used for assessment of anxiety-like behavior and emotional arousal at the end of the 2, 4, or 8-week swimming periods. Duodenal calcium transport, epithelial charge selectivity, paracellular calcium permeability, and epithelial electrical properties were determined 24 h after the last swimming session by use of an Ussing chamber and dilution potential techniques. In some experiments, the duodenal mucosal cells were collected for study of calcium transporter gene expression by quantitative real-time PCR (qRT-PCR). Duodenum was used in this experiment because it is the efficient site for calcium absorption [20].

Because stress as a result of animal handling may have interfered with these experiments, all rats were handled by the same researcher throughout the experimental period. Each rat experienced each behavioral test only once to avoid learning and inaccurate behavioral assessment. During the open-field test, animals were subjected to the test one at a time with no other rats in the same room.

Swimming protocol

The swimming protocol, known to be a non-impact endurance exercise with moderate intensity, was modified from the methods of Elhamieur et al. [21] and Teerapornpunakit et al. [6]. In brief, the rats in the swimming groups were assigned to perform endurance swimming for 2, 4, 6, or 8 weeks whereas age-matched controls remained sedentary in a swimming chamber filled with tap water to a depth of 5 cm. The swimming chamber was made of transparent glass with dimensions of $0.4 \times 0.4 \times 0.8$ m filled with tap water 50-cm deep. The water temperature was maintained at 30–32°C. Swimming rats were initially trained for 7 days (i.e., initial training period; starting at 10 min on day 1 with 10 min/day increment) until they were capable of swimming nonstop for 1 h/day. During the endurance swimming period, swimming frequency was 5 days/week (1 h/day; 1500–1600 hours on Monday–Friday). Approximately 15 h after the last swimming session, the animals were subjected to the open-field calcium flux, or gene expression studies.

Sucrose intake test

This behavioral protocol was modified from the method of Calvo-Torren et al. [22], and was performed on a separate set of animals. Briefly, during the sucrose intake test, the swimming and sedentary rats were housed individually in hanging stainless-steel cages ($21 \times 24 \times 18$ cm). At the beginning of the test (i.e., during the 7-day initial training period), all animals first learned to consume 100 mL 2% w/v sucrose solution (Ajax Finechem, Taren Point, NSW, Australia). Then, throughout the 8-week endurance swimming period, the rats were weekly subjected to the same 100-mL sucrose test 1 h/day after food and water deprivation for 4 h. Total consumption was determined by weighing the sucrose bottle before and after each 1-h test session, and the consumed volume was calculated from the density of 1.0098 g/cm³. Because sucrose intake depends on several factors, for example the age of animals, intake volumes for swimming rats were compared with those for age-matched sedentary rats. An increase in sucrose intake (i.e., increased sensitivity to reward) was representative of emotional stress response to unpleasant or stressful environments, whereas physical stress induced by foot shock or pain could reduce sucrose intake (i.e., anhedonic response) [23].

Open-field test

The open-field test has been used to evaluate anxiety-like behavior, emotional arousal after stress, and locomotion in rodents [24, 25]. As previously described [26], the open-field apparatus was a black wooden box (76-cm length $\times$ 57-cm width $\times$ 35-cm height) with a 48-square grid floor (6 $\times$ 8 squares; 9.5 cm/side). The arena was divided into the inner (24 central squares) and the outer zones (24 peripheral squares). The test was performed between 0800 and 1100 hours, during which noise levels were kept low and light dimmed to 20 lux (average illumination in the husbandry unit during daytime is 200 lux). Not only the swimming rats but also normal rats housed separately in an undisturbed environment (i.e., naïve group) were subjected to the test to exclude the effect of age-dependent behavioral changes. Each tested rat was gently placed in one of the four-corner squares of the open-field apparatus and given 5 min to explore. Locomotor performance and behavior were continuously recorded during the 5-min test by use of a high-definition infrared video camera (model HDR-XR 200E; Sony, Tokyo, Japan), and later scored by two well-trained observers. The behavioral data obtained were: time spent in the inner and outer zones; the number of lines crossed in the first 30 s; total number of lines crossed; and number of times rearing was observed. Line crossing was counted when all four paws of a rat had crossed the line marked on the floor of the arena. Rearing reflected exploration behavior when the rat was placed in a novel environment (i.e., the open-field arena), and increased rearing thus suggested arousal or enhanced reactivity to that environment. Because the rat may also experience a conflict between motivation to explore and innate fear of a novel environment, motionless behavior with a decrease in the number of lines crossed in the first 30 s could suggest exaggerated fear. Increased time spent in the outer zone and/or decreased time spent in
the inner zone represented anxiety-like behavior in the rodents whereas the total number of lines crossed in 5 min was a determinant of overall locomotor activity [24, 25].

Tissue preparation

Median laparotomy was performed under 50 mg/kg sodium pentobarbitone i.p. (Abbott, North Chicago, IL, USA) anesthesia. Duodenum (8-cm length) was removed, rinsed out with ice-cold bathing solution, and cut longitudinally along the radix mesenterii to expose the mucosa. Thereafter, the duodenal sheet was mounted in an Ussing chamber to measure calcium flux as described elsewhere [27]. The tissue was equilibrated for 20 min in the chamber before the 50-min calcium flux measurement was carried out. In some experiments, the duodenal epithelial cells were harvested by scraping the mucosal surface once with a sterile ice-cold glass slide [6]. The heart was also collected through median thoracotomy for determination of wet and dry heart weights.

Bathing solution for Ussing chamber study

The bathing solution contained (mmol/L): 118 NaCl, 4.7 KCl, 1.1 MgCl₂, 1.25 CaCl₂, 23 NaHCO₃, 12 d-glucose, and 2 mannitol (all purchased from Sigma). The solution was continuously aerated with humidified 5% CO₂ in 95% O₂, and maintained at 37°C and pH 7.4. The osmolality of the solution ranged between 290 and 292 mmol/kg water, as determined by use of an osmometer (model 3320; Advanced Instruments, Norwood, MA, USA). Deionized water used in calcium-flux measurements had electrical resistance higher than 18.3 MΩ cm and free-ionized calcium concentration less than 2.5 nmol/L.

Measurement of epithelial electrical properties

The electrical data transepithelial potential difference (PD), short-circuit current (Isc), and transepithelial resistance (TER), were determined by use of two pairs of Ag/AgCl electrodes (World Precision Instruments, Sarasota, FL, USA), as previously described [27]. The electrogenic transport of major ions, for example sodium and chloride, is a determinant of PD and Isc, whereas TER represents the tightness of the tight junction [28]. The PD-sensing electrodes were connected to agar bridges (3.0 mol/L KCl in 4 g% agar) located near each surface of the mounted duodenal tissue. The other ends of the PD-sensing electrodes were connected to a pre-amplifier (model EVC-4000; World Precision Instruments), and, finally, to a PowerLab 4/30 operated with the software Chart 5.2.2 for Mac OS X (ADInstruments, Colorado Springs, CO, USA). The current-passing electrodes were located one at each end of the chamber to supply Isc, which was also measured by use of a PowerLab 4/30 connected in series to the EVC-4000 current-generating unit (World Precision Instruments). Electrical resistance of the bathing solution was automatically subtracted by the EVC-4000 system. TER was calculated from PD and Isc by use of Ohm’s equation. The epithelial electrical data were obtained during the solvent drag-induced calcium transport study in 2-week swimming rats, and during the study of transepithelial calcium transport without calcium gradient in 4, 6, and 8-week swimming rats. In all groups, the calcium flux experiments were performed under open-circuit conditions. Isc and TER were quickly determined every 10 min until the end of calcium flux measurement, and the average values are presented. Representative time course data for the 8-week rats are shown in Supplementary Fig. S1.

Calcium flux measurement

Transepithelial calcium flux was determined by the modified method of Chareonphandhu et al. [27]. After 20-min equilibration in an Ussing chamber, the tissue was bathed on both sides with fresh bathing solution. The mucosal solution contained ⁴⁵CaCl₂ with final specific activity of ~450–500 mCi/mol (initial amount of 5 mCi/mL; Amersham, Buckinghamshire, UK). The transepithelial calcium flux from the hot side to the cold side (J_H→C) was calculated by use of Eqs. 1 and 2.

\[ J_{H\rightarrow C} = R_{H\rightarrow C}/(S_H \times A) \]  
\[ S_H = C_H/C_{To} \]

where \( R_{H\rightarrow C} \) was the rate of tracer appearance in the cold side (cpm/h); \( S_H \) was the specific activity in the hot side (cpm/nmol); \( A \) was the surface area of the tissue (0.69 cm²); \( C_H \) was the mean radioactivity in the hot side (cpm); and \( C_{To} \) was the total calcium in the hot side (nmol). Radioactivity of ⁴⁵Ca was analyzed by liquid scintillation spectrophotometry (model Tri-Carb 3100TR; Perkin-Elmer, Boston, MA, USA). Total calcium concentration of the bathing solution was analyzed by atomic absorption spectrophotometry (model SpectrAA-300; Varian Techn, Springvale, Australia).

Unless otherwise specified, transepithelial calcium flux measured in the absence of a calcium gradient (i.e., mucosal and serosal solution had equal calcium concentration of 1.25 mmol/L) represented transcellular calcium flux plus solvent drag-induced calcium flux. The transcellular calcium flux was further determined separately after replacing the mucosal glucose with an equivalent amount of mannitol to abolish solvent drag [27]. Because 2-week swimming has been shown to upregulate a number of transcellular calcium transporters that normally control
vectorial calcium transport in the mucosa-to-serosa direction [6], changes in mucosa-to-serosa calcium flux were investigated in this study.

The solvent drag-induced calcium flux was determined in the presence of 0.1 mmol/L trifluoperazine (in serosal solution; Sigma), which inhibited PMCA activity and thus transcellular calcium transport [29]. The paracellular passive calcium flux was measured when the mucosal solution contained 5, 10, 20, 40, or 80 mmol/L CaCl₂.

Paracellular permeability measurement

The permeability ratio of sodium and chloride (P_{Na}/P_{Cl}), an indicator of the paracellular charge selectivity, was determined by the dilution potential technique [27]. In brief, duodenal epithelium was equilibrated for 20 min in normal bathing solution containing 145 mmol/L NaCl before the mucosal solution was substituted with 72.5 mmol/L NaCl. Osmolality of the mucosal solution was maintained by adding an equivalent amount of mannitol. Changes in PD values before and after solution change (i.e., dilution potential) were recorded every 10 s until stable. P_{Na}/P_{Cl} was calculated from the dilution potential by use of the Goldman–Hodgkin–Katz equation [27]. An increase in P_{Na}/P_{Cl} value usually indicates increased paracellular permeability to cations, e.g., sodium and/or calcium ions. Permeability of calcium (P_{Ca}) was calculated from the paracellular passive calcium flux and the difference between mucosal and serosal calcium concentrations, as described elsewhere [27].

Total RNA preparation and qRT-PCR

By use of TRIzol reagent (Invitrogen, Carlsbad, CA, USA), total RNA was prepared from the duodenal epithelial cells, as described elsewhere [6]. The purity of the total RNA was determined by measurement of absorbance at 260 and 280 nm, the ratio of which fell in the range 1.8–2.0. The integrity of the RNA was analyzed by denaturing agarose gel electrophoresis with the 28S rRNA band appearing approximately twice as intense as the 18S rRNA band. Then, 1 µg total RNA was reverse-transcribed with oligo-dT_{20} primer and the iScript kit (Bio-Rad, Hercules, CA, USA) by use of a thermal cycler (model MyCycler; Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control gene for normalization and for checking the consistency of the reverse transcription (coefficient of variation <5%, n = 10). Primers used in this study were as used in our previous work [6], and are listed in Table 1. qRT-PCR and melting curve analysis were performed by use of the Bio-rad MiniOpticon system with iQ SYBR Green SuperMix (Bio-rad). The mRNA expression level in each sample was calculated from the threshold cycle (C_T). Expression levels in the swimming groups were first normalized by GAPDH expression and later by expression levels in the corresponding sedentary groups. The PCR products were also visualized on 1.5% agarose gel stained with 1.0 µg/mL ethidium bromide under a UV transilluminator (Alpha Innotech, San Leandro, CA, USA). After electrophoresis, PCR products were extracted by HiYield Gel/PCR DNA-extraction kit (Real Biotech, Taipei, Taiwan), and were sequenced by use of the ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). qRT-PCR experiments were performed in triplicate.

Statistical analysis

Unless otherwise specified, results are expressed as mean ± SE. Comparisons between two groups were performed by use of the unpaired Student’s t test, and multiple comparisons were performed by one-way analysis of variance (ANOVA) with Newman–Keuls’ post-test. The level of significance for all statistical tests was P < 0.05. In behavioral experiments (sucrose intake and open-field tests), F values, t values, and/or degree of freedom (df) values are also presented as usual. The qRT-PCR data were compared by the 2^{-ΔΔC_T} method, and the changes, as multiples, are presented as log₂ mean ± SE. Differential mRNA expression was regarded as significant when there was a 2-fold or greater difference in the expression levels between swimming and sedentary groups [6]. All data were analyzed by use of GraphPad Prism 4 for Mac OS X (GraphPad Software, San Diego, CA, USA).

Results

Responses to exercise were present in all rats subjected to endurance swimming for 2–8 weeks

Swimming rats manifested typical responses to exercise, including reduced body weight gain and cardiac hypertrophy. The initial body weights of swimming rats were comparable with those of the age-matched sedentary controls (data not shown), whereas the final body weights of 4, 6, and 8-week swimming rats were significantly lower than those of the corresponding sedentary control rats (Fig. 1a). Wet and dry heart weights normalized by body weight were also increased in all swimming groups compared with the corresponding sedentary groups (Fig. 1b, c).
Emotional stress and/or anxiety-like behavior developed after 4 weeks of swimming in an inescapable chamber.

Two behavioral assessments, sucrose intake and open-field tests, were used to evaluate stress and anxiety-like behavior, respectively, in rats subjected to swimming in an inescapable chamber. As shown in Fig. 2, the sucrose-intake test revealed no signs of stress in swimming rats during the initial training period (weeks -1 and 0) compared to the age-matched sedentary controls. In the endurance swimming period, the rats showed no significant stress until the end of week 4 when a tendency toward stress response was observed. Significant emotional stress response as indicated by increased sucrose intake was remarkable at the end of weeks 6 and 7 of endurance swim.

Table 1  Rattus norvegicus oligonucleotide sequences used in qRT-PCR experiments

| Gene                | Accession no. | Primer (forward/reverse) | Product length (bp) |
|---------------------|---------------|--------------------------|---------------------|
| Vitamin D-related genes |               |                          |                     |
| VDR                 | NM_017058     | 5'-GACCTTGTACGGTGACGCAC-3' | 141                 |
|                     |               | 5'-AGACTTTGTGGAGCGTAA-3'  |                     |
| Transcellular genes |               |                          |                     |
| TRPV5               | NM_053787     | 5'-CTACGGGTGAACAGCAAC-3'  | 163                 |
|                     |               | 5'-TGCAAGCACAGGCTCTCA-3'  |                     |
| TRPV6               | NM_053686     | 5'-ATCCGGCTATGCA-3'       | 80                  |
|                     |               | 5'-ACTTTTCTGCTACGTGGT-3'  |                     |
| Calbindin-D9k       | X_16635       | 5'-CCGGAAGAATGAAGACAG-3'  | 174                 |
|                     |               | 5'-TCTCCATCCACGTTATACT-3' |                     |
| NCX1                | NM_019268     | 5'-GGTGGGAAGCTATTTT-3'    | 163                 |
|                     |               | 5'-GTTGGGAAGCTTACCTT-3'   |                     |
| PMCA1b              | NM_053311     | 5'-GCCATCTCTGCAAATT-3'    | 109                 |
|                     |               | 5'-AGCCATTTGCTTATAGAAC-3' |                     |
| Paracellular genes  |               |                          |                     |
| NKA                 | X_63375       | 5'-CCACTGCTGACGACACCAT-3' | 79                  |
|                     |               | 5'-CCAGTCTCCAGAATTTCTC-3' |                     |
| ZO-1                | XM_218747     | 5'-GTATCCGATTGTTGTGTC-3'  | 270                 |
|                     |               | 5'-TCACCTTGAGCCACCTG-3'   |                     |
| ZO-2                | NM_053773     | 5'-TGCTAAATGACCCGTAAG-3'  | 169                 |
|                     |               | 5'-GACACTCCGTTGATCGA-3'   |                     |
| ZO-3                | XM_001069839  | 5'-ACCCCTGCTGCA-3'        | 121                 |
|                     |               | 5'-ATGGGAACCTCCAGC-3'     |                     |
| Cingulin            | XM_001059265  | 5'-GCCGGAACCGGTAAAG-3'    | 188                 |
|                     |               | 5'-CCCCCTCAGGCCTT-3'      |                     |
| Occludin            | NM_031329     | 5'-GCTGCTAGGATGTAATG-3'   | 107                 |
|                     |               | 5'-GCACCCACCAAGATCTCT-3'  |                     |
| Claudin-2           | NM_001106846  | 5'-GGCTAGGCTCTGATG-3'     | 246                 |
|                     |               | 5'-GCACCCACAAGATCTCT-3'   |                     |
| Claudin-3           | NM_031700     | 5'-CCACATCTCTGCCGCTT-3'   | 312                 |
|                     |               | 5'-AGGACAGAGGATAGGCT-3'   |                     |
| Claudin-12          | XM_001067932  | 5'-AGTCTGCGCCTGCCAT-3'    | 133                 |
|                     |               | 5'-TCATATTTCAGGTTAC-3'    |                     |

VDR, nuclear vitamin D receptor; TRPV5 and TRPV6, transient receptor potential vanilloid family Ca2+ channels 5 and 6; NCX1, Na+/Ca2+ exchanger 1; PMCA1b, plasma membrane Ca2+-ATPase isoform 1b; NKA, β1-subunit of Na+/K+-ATPase; ZO, zonula occludens; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
training compared with the sedentary controls. However, the swimming rats seemed to eventually adapt and overcome this stress by the end of week 8.

In the open-field test, an increase in the time spent in the outer zone and a decrease in the time spent in the inner zone were observed in 4-week, but not 8-week swimming rats compared with 2-week swimming rats (Fig. 3a). In addition, the number of lines crossed in the first 30 s was less for 8-week swimming rats than for 4-week swimming rats, and the amount of rearing was greater (Fig. 3b, c). Total lines crossed were comparable for all swimming groups (Fig. 3d). The open-field results, therefore, suggested that anxiety-like behavior in swimming rats was present at the end of week 4, and increased levels of arousal and active response to stress, as indicated by the increased amount of rearing, were observed at the end of week 8. To confirm that behavioral reactivity to the open-field test in the swimming rats was not because of the age of the animal, we also performed the open-field test with age-matched naïve rats housed separately in an undisturbed environment. The open-field results from these naïve rats showed no significant changes in any behavior (Fig. 3a–d).

Transcellular active duodenal calcium transport, but not solvent drag-induced calcium transport, was enhanced in 2-week swimming rats. Because neither stress nor anxiety-like behavior was observed in the 2-week swimming rats, the stimulatory effects of endurance swimming on intestinal calcium absorption were investigated at this time point. Our previous study showed that a number of genes related to the transcellular calcium transport were upregulated in the duodenal epithelial cells of 2-week swimming rats, but transcellular calcium flux was not determined in those rats [6]. Here, we demonstrated by use of the Ussing chamber technique that 2-week swimming significantly stimulated transcellular active calcium transport in the duodenum approximately twofold (Fig. 4a). Figure 4b shows the duodenal epithelia of swimming and sedentary rats were cation-selective, because $P_{\text{Na}}/P_{\text{Cl}}$ values in both groups were greater than 0.66, which is the mobility ratio of sodium to chloride in water [28, 30]. This mode of exercise further increased paracellular permeability to cations, as indicated by a $\sim 20\%$ increase in $P_{\text{Na}}/P_{\text{Cl}}$ in swimming rats (Fig. 4b). However, 2-week swimming had no effects on solvent drag-induced paracellular calcium transport (Fig. 4c), paracellular passive calcium transport (with mucosal calcium concentrations of 5–80 mmol/L; Fig. 4d), or paracellular $P_{\text{Ca}}$ (Fig. 4e). The results collectively indicated that transcellular active duodenal calcium transport, but not paracellular calcium transport, was enhanced.
in 2-week swimming rats. Therefore, the increased transepithelial calcium flux in the absence of a calcium gradient (the first value of each graph in Fig. 4d; mucosal and serosal calcium concentrations were 1.25 mmol/L) after 2-week endurance swimming was predominantly because of enhanced transcellular calcium transport.

Enhanced calcium transport was abolished after prolonged swimming in an inescapable chamber

In contrast to 2-week swimming, transepithelial calcium transport in the absence of a calcium gradient was not increased in 4 and 6-week swimming rats (Fig. 5a, b), and was markedly reduced by \( \sim 50\% \) in 8-week swimming rats (Fig. 5c). PD and Isc of the duodenal epithelium were also drastically reduced (\( \sim 60\% \)) but only in the 8-week swimming group (Table 2). However, 2–8 weeks of swimming did not affect duodenal TER (Table 2).

Consistent with our previous report for 2-week swimming rats [6], mRNA expression of VDR and genes related to transcellular calcium transport (i.e., TRPV5, TRPV6, NCX1, and PMCA1b), and paracellular ion transport (i.e., ZO-2, ZO-3, cingulin, occludin, claudin-3, and claudin-12) were upregulated by greater than 2-fold in the duodenal mucosal cells of 4-week swimming rats compared with age-matched sedentary rats (Fig. 6). Expression of some genes, i.e., calbindin-D9k, ZO-1, and claudin-2, was unaltered whereas that of the \( \beta\)-subunit of \( \text{Na}^+\text{K}^+\)-ATPase was reduced after 4-week swimming. At the end of week 8, mRNA expression of most altered genes observed in 4-week swimming rats (i.e., VDR, TRPV5, PMCA1b, \( \beta\)-subunit of \( \text{Na}^+\text{K}^+\)-ATPase, ZO-2, ZO-3, occludin, claudin-3, and claudin-12) returned to sedentary levels (i.e., changes were less than 2-fold). Others, for example TRPV5 and NCX1, were still upregulated by more than 2-fold in the 8-week swimming group, but the changes were markedly less than those in the 4-week swimming group. Thus, the results suggested that prolonged swimming (8 weeks) in an inescapable chamber prevented upregulation of several intestinal genes, especially those related to the transcellular calcium transport, thereby leading to a decrease in duodenal calcium absorption.

Discussion

This swimming protocol was a non-impact endurance exercise, previously reported to increase heart weight and citrate synthase activity in the gastrocnemius muscle with no changes in plasma lactate levels [6]. Despite having benefits on various body systems, including cardiovascular, neural, and musculoskeletal systems, exercise can induce physical and emotional stress in individuals, especially during forced and/or high-intensity exercise [10, 31, 32]. However, physical stress should be trivial in swim training with moderate intensity because the protocol usually causes neither pain nor physical injury. Herein, we provided evidence that prolonged endurance swimming in an inescapable environment induced emotional stress and
anxiety-like behavior in female rats. Whereas 2-week swimming stimulated duodenal calcium transport, this stimulatory effect was attenuated after 4 weeks of swim training. The swimming-induced upregulation of calcium transporter genes, for example TRPV6 and PMCA1b, was also abolished after prolonged training, presumably by the exercise-induced stress, which has been known to impair intestinal calcium absorption [12].

Fig. 4  
(a) Transcellular active calcium transport, (b) sodium to chloride permeability ratio ($P_{Na}/P_{Cl}$), (c) solvent drag-induced calcium transport, (d) paracellular passive calcium transport, and (e) paracellular permeability to calcium ($P_{Ca}$) in the duodenum of 2-week swimming and age-matched sedentary rats. Transcellular active calcium flux was measured under mucosal glucose-free conditions, which reduced solvent drag-induced transport. The solvent drag-induced calcium flux was determined in the presence of serosal 0.1 mmol/L trifluoperazine, an inhibitor of PMCA. Paracellular passive calcium flux was determined in the presence of the transepithelial calcium gradient (1.25 mmol/L mucosal calcium). The first value of each paracellular graph was calcium flux in the absence of transepithelial calcium gradient (1.25 mmol/L calcium on both sides); therefore, this value represents the transcellular active calcium flux plus solvent drag-induced calcium flux. $\Delta [Ca]$ denotes the result from subtraction of serosal calcium (1.25 mmol/L) from mucosal calcium. *$P < 0.05$, **$P < 0.01$ compared with its respective sedentary group. Numbers in parentheses are the numbers of animals.

Fig. 5  
Transepithelial calcium transport in the duodenum of 4, 6, and 8-week swimming rats, and their respective age-matched sedentary controls. The epithelium was bathed on both sides with the same bathing solution containing 1.25 mmol/L calcium. ***$P < 0.001$ compared with the age-matched sedentary controls. Numbers in parentheses represent the numbers of experimental animals.
Impact endurance exercise of mild-to-moderate intensity, for example running, has long been reported to benefit bone and calcium metabolism in both humans and rodents [33, 34]. In bone, this mode of exercise can increase bone mineral density and bone strength in humans, perhaps from the increased mechanical strain on bone tissue and the elevated plasma levels of 1,25(OH)$_2$D$_3$ [33]. The impact exercise-induced bone calcium accretion may also be an indirect result of enhanced intestinal calcium absorption. Zittermann et al. [35, 36] demonstrated that the fractional calcium absorption was increased in male athletes with a minimum of 8 h/week of endurance sport activities and after short-term moderate exercise bout in well-trained athletes. A study of female rats also revealed exercise-enhanced duodenal calcium absorption after 13 weeks of 1 h/day, 5 days/week flat-bed treadmill training [5].

On the other hand, little is known regarding the effect of non-impact endurance exercise on bone metabolism and intestinal calcium absorption. Hart et al. [2] showed an increase in bone strength in estrogen-deficient rats subjected to 12-week endurance swim training. We recently reported enhanced calcium absorption in the rat duodenum, proximal jejunum, and cecum after 2-week endurance swimming, but not after single-bout swimming [6]. This current study has further revealed that transcellular active calcium transport was the prominent transport mechanism for the 2-week swimming-enhanced duodenal calcium absorption. This result was consistent with the previous findings of the upregulation of a number of transcellular calcium transporter genes, including TRPV5, TRPV6, NCX1, and calbindin-D$_{9k}$, in the duodenum of 2-week swimming rats [6]. Because calbindin-D$_{9k}$ could increase PMCA activity [37] and the rate of cytoplasmic calcium diffusion [38, 39], the ~37-fold increase in calbindin-D$_{9k}$ mRNA expression in the duodenal epithelial cells of the 2-week swimming rats [6] should contribute substantially to the observed increase in calcium absorption. However, the exact mechanism by which swimming increases mRNA expression of these transporters in rats is unknown, but it could partly be because of concurrent increases in plasma 1,25(OH)$_2$D$_3$ and/or intestinal VDR expression levels during endurance exercise [6, 33, 34]. Nevertheless, it was noted that some intestinal calcium-transporting proteins, especially TRPV6 and calbindin-D$_{9k}$, may be essential for transcellular calcium absorption under physical activity/exercise [6, 16] and some other special conditions with high calcium demand, e.g., pregnancy and lactation [40, 41], but may be of less importance under vitamin D-replete sedentary conditions. The latter notion was on the basis that TRPV6/calbindin-D$_{9k}$ double knockout mice were normocalcemic, and still exhibited intestinal active calcium absorption after 1,25(OH)$_2$D$_3$ administration as determined by everted duodenal sac assay [42].

| Table 2 | Epithelial electrical properties of the duodenum of sedentary and swimming rats |
|---------|-----------------------------------|
|         | PD (mV)                | Isc (μA/cm$^2$) | TER (Ω cm$^2$) |
| Sedentary (n = 4) | Swimming (n = 8) | Sedentary (n = 4) | Swimming (n = 8) | Sedentary (n = 4) | Swimming (n = 8) | Sedentary (n = 4) | Swimming (n = 8) |
| 2 weeks | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 |
| 4 weeks | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 |
| 6 weeks | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 |
| 8 weeks | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 | 6.25 ± 0.57 |

*P < 0.001 compared with the corresponding sedentary group.
In addition to the transcellular calcium transport, 2-week swimming also increased the paracellular cation permeability, as indicated by a *20% higher* \( P_{Na}/P_{Cl} \) value for trained rats than for sedentary controls. The increased \( P_{Na}/P_{Cl} \) could be partly explained by the altered mRNA expression of tight junction genes in 2-week swimming rats [6], particularly claudin-2 and 12, both of which are known to be under the regulation of 1,25(OH) \(_2\)D\(_3\) and are capable of forming sodium/calcium-permeable tight junction pores [18, 43]. However, such an increase in cation permeability was too small to enhance either the solvent drag-induced or paracellular passive calcium transport. Although solvent drag-induced calcium flux was unchanged, the 2-fold increase in transcellular calcium transport was still large enough to be observed as a significant increase in the transepithelial calcium absorption, especially when the mucosal calcium was relatively low (Fig. 4d).

Because repeated exposure to an inescapable environment can lead to chronic emotional stress in rodents [7, 8, 25], the beneficial effect of swim training longer than 4 weeks on intestinal calcium absorption was questionable. Long-term exercise training (~4–8 weeks) has long been recognized as a stress that elevated corticosteroid levels [10, 32]. The absence of supportive platform and water exposure might further aggravate fear and anxiety-like behavior [44]. In our study, as demonstrated by the sucrose intake test, a significant increase in emotional stress was observed for swimming rats compared with their age-matched sedentary controls.Sucrose hyperphagia and preference for sweetened water seems to be a common response to moderate unavoidable stress or emotional stress in rats [23, 45]. In the open-field test, an increase in outer zone time and a decrease in inner zone time confirmed the presence of anxiety-like behavior in 4-week swimming rats. Moreover, increased rearing and a decrease in number of lines crossed in the first 30 s by 8-week swimming rats suggested inappropriate arousal and exaggerated fear, respectively. Although some behavioral changes in rats, e.g., behavioral despair, were age-related [46], changes in anxiety-like behavior in swimming rats should result from the training, because no such responses were observed for naïve rats housed separately in an undisturbed environment (Fig. 3).

Stress has long been known to suppress intestinal calcium absorption [11, 12]. The mechanism responsible is often explained as stress-induced corticosteroid release from the adrenal glands [9]. Prolonged exposure to corticosteroids (>3 days) either from exogenous (e.g., dexamethasone) or endogenous sources (e.g., corticosterone in rats) can reduce intestinal calcium absorption [11], probably by reducing intestinal response to 1,25(OH) \(_2\)D\(_3\) and \( \text{TRPV6} \) mRNA expression [47, 48]. In contrast, 1–2 days of corticosteroid exposure transiently increased intestinal calcium transport by upregulating \( \text{TRPV6} \) and \( \text{PMCA}^{1b} \) expression [11, 48]. In the current study the benefit of endurance swimming disappeared after 4 weeks of swimming, concurrently with appearance of stress and anxiety-like behavior that became more intense after week 4. Because \( \text{NCX1} \) contributes only ~20% of the basolateral calcium extrusion [15], marked \( \text{NCX1} \) mRNA upregulation in the 4-week swimming rats did not alter the overall transepithelial calcium flux. A decrease in duodenal calcium absorption observed at week 8 of swimming could be explained, in part, by a decrease in the upregulation of
several duodenal genes related to calcium absorption, e.g., TRPV6 and PMCA1b (Fig. 6). Furthermore, the increases in TRPV5 (~73-fold compared with sedentary controls) and calbindin-D28k (~37-fold) mRNA expression previously reported for 2-week swimming rats [6] were also diminished in this prolonged training (4 and 8-week swimming; Fig. 6), and may thus cancel out the stimulatory effect of exercise on calcium absorption in the 4–8-week swimming rats.

Besides calcium transport, decreases in the PD and Isc of the duodenal epithelium of the 8-week swimming rats suggested a decrease in the electrogenic transport of other ions, especially sodium transport via Na\(^+\)/K\(^+\)-ATPase. Isc is generally a representative of electrogenic ion flux. Conversely, PD of the duodenal epithelium is mostly dependent on the electrogenic sodium transport that generates charge separation between the two sides of the epithelial sheet, and also on the ion-restrictive property of the tight junction (paracellular tightness), which helps maintain this charge separation [28, 30]. Although it has previously been shown that stress, corticotrophin-releasing factor, and adrenal corticosteroids could reduce mucosal barrier function and/or tight junction resistance [50–52], this impaired paracellular barrier did not contribute to a decrease in PD in the 8-week swimming rats, because there was no change in TER, an indicator of paracellular tightness [28, 30]. Therefore, low PD was not simply a result of the back-leak of transported ions through the low-resistance tight junction, but should have resulted from a decrease in electrogenic sodium transport, which is a determinant of Isc in the duodenum [29]. In other words, low PD and Isc in the 8-week swimming rats could be explained, in part, by an exercise-induced and/or stress-induced decrease in intestinal sodium absorption [53].

Nevertheless, there was a discrepancy between rat behavior and duodenal adaptation in the 8-week swimming rats. As indicated by the sucrose intake test, emotional stress was pronounced at week 7, but not at week 8, of swimming (Fig. 2), whereas a decrease in duodenal calcium absorption was clearly observed in the 8-week group. We speculated that the absence of emotional stress response in 8-week swimming rats might be because of habituation or learning to cope with repetitive stressful stimuli [54]. But the stressful stimuli were actually present all the time when swimming rats were exposed to temperature change in water or forced exercise. Thus, the normal body responses to stress (e.g., adrenal corticosteroid release) might persist, and eventually led to a decrease in calcium absorption.

In conclusion, we have demonstrated in this study that endurance swimming had a beneficial effect on calcium metabolism by stimulating transcellular active duodenal calcium absorption. However, the inescapable stressful environment imposed on the swimming rats during a prolonged training period gradually induced stress and anxiety-like behavior, especially during weeks 4–7. Because stress can reduce intestinal calcium absorption [11, 12], the stimulatory effect of endurance swimming was neutralized at the end of week 4. Swimming for 8 weeks eventually led to a decrease in duodenal calcium absorption compared with aged-matched sedentary controls, presumably by preventing the upregulation of genes related to calcium absorption. Although further experiments are required to demonstrate the molecular and cellular mechanisms by which stress and/or anxiety impaired calcium absorption in long-term swimming rats, these results provided evidence that forced training in a stressful environment should be avoided to retain the beneficial effect of endurance swimming on body calcium homeostasis.

Acknowledgments We thank Amporn Nuntapornsak, Kanogwun Thongchote, and Parinya Lertsintai for excellent technical assistance. This work was supported by grants from the Faculty of Science, Mahidol University (SCY52-02 to N. Charoenphandhu), the King Prajadhipok and Queen Rambhai Barni Memorial Foundation (to S. Lapmanee), the Royal Golden Jubilee Program (PHD/0042/2551 to J. Teerapornpuntakit), and the Thailand Research Fund (RSA5180001 to N. Charoenphandhu). N. C. designed the research; N. C., J. T., S. L., N. D., and J. C. performed the research; N. C., N. K., and J. C. analyzed the data; N. C. and N. K. wrote the paper.

Conflict of interest None.

References

1. Falk B, Bromstein Z, Zigel L, Constantini N, Eliakim A (2004) Higher tibial quantitative ultrasound in young female swimmers. Br J Sports Med 38:461–465
2. Hart KJ, Shaw JM, Vajda E, Hegsted M, Miller SC (2001) Swim-trained rats have greater bone mass, density, strength, and dynamics. J Appl Physiol 91:1663–1668
3. Melton SA, Hegsted M, Keenan MJ, Morris GS, O’Neil CE, Zablah-Pimentel EM (2004) Water exercise prevents femur density loss associated with ovariectomy in the retired breeder rat. J Strength Cond Res 18:508–512
4. Hubal MJ, Ingalls CP, Allen MR, Wenke JC, Hogan HA, Bloomfield SA (2005) Effects of eccentric exercise training on cortical bone and muscle strength in the estrogen-deficient mouse. J Appl Physiol 98:1674–1681
5. Yeh JK, Aloia JF, Yasumura S (1989) Effect of physical activity on calcium and phosphorus metabolism in the rat. Am J Physiol Endocrinol Metab 256:E1–E6
6. Teerapornpuntakit J, Dorkkam N, Wongdee K, Krishnamra N, Charoenphandhu N (2009) Endurance swimming stimulates transepithelial calcium transport and alters the expression of genes related to calcium absorption in the intestine of rats. Am J Physiol Endocrinol Metab 296:E775–E786
7. Armario A, Gaivaldà A, Martí J (1995) Comparison of the behavioural and endocrine response to forced swimming stress in five inbred strains of rats. Psychoneuroendocrinology 20:879–890
8. Avital A, Richter-Levin G, Leschiner S, Spanier I, Veenman L, Weizman A, Gavish M (2001) Acute and repeated swim stress effects on peripheral benzodiazepine receptors in the rat hippocampus, adrenal, and kidney. Neuropsychopharmacology 25:669–678
9. Korte SM (2001) Corticosteroids in relation to fear, anxiety and psychopathology. Neurosci Biobehav Rev 25:117–142
10. Droste SK, Collins A, Lightman SL, Linhorst AC, Reul JM (2009) Distinct, time-dependent effects of voluntary exercise on circadian and ultradian rhythms and stress responses of free corticosterone in the rat hippocampus. Endocrinology 150: 4170–4179
11. Fox J, Ross R, Care AD (1985) Effects of acute and chronic treatment with glucocorticoids on the intestinal absorption of calcium and phosphate and on plasma 1, 25-dihydroxyvitamin D levels in pigs. Clin Sci (Lond) 69:553–559
12. Islam N, Chanda S, Ghosh TK, Mitra C (1998) Cold stress facilitates calcium mobilization from bone in an ovarioectomized rat model of osteoporosis. Jpn J Physiol 48:49–55
13. Charoenthong, N, Wongkeek, K, Krishnamurthy, N (2010) Is prolactin the cardinal calcitropic maternal hormone? Trends Endocrinol Metab 21:395–401
14. Hoenderop JG, Nilius B, Bindels RJ (2005) Calcium absorption between enterocytes. Mol Biol Cell 19:1912–1921
15. Ghijzen WE, De Jong MD, Van Os CH (1983) Kinetic properties of Na+/Ca2+ exchange in basolateral plasma membranes of rat small intestine. Biochim Biophys Acta 730:85–94
16. Sato T, Yamamoto H, Sawada N, Nishiaki K, Tsuji M, Nikawa T, Arai H, Morita K, Taketani Y, Takeda E (2006) Immobilization decreases duodenal calcium absorption through a 1, 25-dihydroxyvitamin D-dependent pathway. J Bone Miner Metab 24:291–299
17. Chirayath MV, Gadzik LL, Hulla W, Graf J, Cross H, Peterlik M (2011) Vitamin D increases tight-junction conductance and paracellular Ca2+ transport in Caco-2 cell cultures. Am J Physiol Gastrointest Liver Physiol 274:G389–G396
18. Fujita H, Sugimoto K, Inatomi S, Maeda T, Caruso NM, Ehlers LB, Fleschner M, Spencer RL, Moore RL (2007) Short-term treadmill running in the rat: what kind of stressor is it? J Appl Physiol 103:1979–1985
19. Moraska A, Deak T, Spencer RL, Roth D, Fleschner M (2000) Treadmill running produces both positive and negative physiologic adaptations in Sprague-Dawley rats. Am J Physiol Regul Integr Comp Physiol 279:R1321–R1329
20. Maimoun L, Sultan C (2009) Effect of physical activity on calcium homeostasis and calcitropic hormones: a review. Calcif Tissue Int 85:277–286
21. Elhami E, Courderot-Masuyer C, Nicod L, Bobillier-Chauveau C, Elhami E, Courderot-Masuyer C, Nicod L, Bobillier-Chauveau C (2004) Evidence for an acute rise of intestinal calcium absorption in response to aerobic exercise. Eur J Nutr 42:208–214
22. Walters JR, Howard A, Charpin MV, Gniecko KC, Brodin P, Thulin E, Forsén S (1990) Stimulation of intestinal basolateral calcium-pump activity by recombinant synthetic calbindin-D9k and specific mutants. Biochem Biophys Res Commun 170:603–608
23. Yeh JK, Aloia JF (1990) Effect of physical activity on calcitropic hormones and calcium balance in rats. Am J Physiol Endocrinol Metab 258:E263–E268
24. Zittermann A, Sabatusch O, Jantzen S, Platen P, Dohr A, Dimitriou T, Scheld K, Klein K, Stehle P (2000) Exercise-trained young men have higher calcium absorption rates and plasma calcitriol levels compared with age-matched sedentary controls. Calcif Tissue Int 67:215–219
25. Zittermann A, Sabatusch O, Jantzen S, Platen P, Dohr A, Stehle P (2002) Evidence for an acute rise of intestinal calcium absorption in response to aerobic exercise. Eur J Nutr 41:189–196
26. van Cromphaut SJ, Rummens K, Stockmans I, Van Herck E, van Cromphaut SJ, Rummens K, Stockmans I, Van Herck E, van Cromphaut SJ, Rummens K, Stockmans I (eds) Comprehensive human physiology: from cellular mechanisms to integration, 1st edn. Springer, Berlin, pp 1217–1232
27. Benn BS, Ajbade D, Porta A, Dhawan P, Hediger M, Peng JB, Jiang Y, Oh GT, Jeung EB, Lieben L, Bouillon R, Carmeliet G, Christakos S (2008) Active intestinal calcium transport in the absence of transient receptor potential vanilloid type 6 and calbindin-D9k. Endocrinology 149:3196–3205
28. Charoenthong, N, Tudor, P, Pulsook N, Krishnamurthy N (2006) Chronic metabolic acidosis stimulated transcellular and solvent drag-induced calcium transport in the duodenum of female rats. Am J Physiol Gastrointest Liver Physiol 291:G446–G455
29. Gregory R (1996) Epithelial transport. In: Gregory R, Windhorst U (eds) Comprehensive human physiology: from cellular mechanisms to integration, 1st edn. Springer, Berlin, pp 1217–1232
30. Chirayath MV, Gadzik LL, Hulla W, Graf J, Cross H, Peterlik M (2011) Vitamin D increases tight-junction conductance and paracellular Ca2+ transport in Caco-2 cell cultures. Am J Physiol Gastrointest Liver Physiol 274:G389–G396
31. Brown DA, Johnson MS, Armstrong CJ, Lynch JM, Caruso NM, Ehlers LB, Fleschner M, Spencer RL, Moore RL (2007) Short-term treadmill running in the rat: what kind of stressor is it? J Appl Physiol 103:1979–1985
32. Zittermann A, Sabatusch O, Jantzen S, Platen P, Dohr A, Dimitriou T, Scheld K, Klein K, Stehle P (2000) Exercise-trained young men have higher calcium absorption rates and plasma calcitriol levels compared with age-matched sedentary controls. Calcif Tissue Int 67:215–219
33. Zittermann A, Sabatusch O, Jantzen S, Platen P, Dohr A, Stehle P (2002) Evidence for an acute rise of intestinal calcium absorption in response to aerobic exercise. Eur J Nutr 41:189–196
34. Walters JR, Howard A, Charpin MV, Gniecko KC, Brodin P, Thulin E, Forsén S (1990) Stimulation of intestinal basolateral membrane calcium-pump activity by recombinant synthetic calbindin-D9k and specific mutants. Biochem Biophys Res Commun 170:603–608
35. Feher JJ, Fullmer CS, Wasserman RH (1992) Role of facilitated diffusion of calcium by calbindin in intestinal calcium absorption. Am J Physiol Cell Physiol 244:4179–4186
36. Feher JJ, Fullmer CS, Wasserman RH (1992) Role of facilitated diffusion of calcium by calbindin in intestinal calcium absorption. Am J Physiol Cell Physiol 244:4179–4186
37. Christakos S (2008) Active intestinal calcium transport in the absence of transient receptor potential vanilloid type 6 and calbindin-D9k. Endocrinology 149:3196–3205
38. Benn BS, Ajbade D, Porta A, Dhawan P, Hediger M, Peng JB, Jiang Y, Oh GT, Jeung EB, Lieben L, Bouillon R, Carmeliet G, Christakos S (2008) Active intestinal calcium transport in the absence of transient receptor potential vanilloid type 6 and calbindin-D9k. Endocrinology 149:3196–3205
39. Christakos S (2008) Active intestinal calcium transport in the absence of transient receptor potential vanilloid type 6 and calbindin-D9k. Endocrinology 149:3196–3205
40. Benn BS, Ajbade D, Porta A, Dhawan P, Hediger M, Peng JB, Jiang Y, Oh GT, Jeung EB, Lieben L, Bouillon R, Carmeliet G, Christakos S (2008) Active intestinal calcium transport in the absence of transient receptor potential vanilloid type 6 and calbindin-D9k. Endocrinology 149:3196–3205
41. Benn BS, Ajbade D, Porta A, Dhawan P, Hediger M, Peng JB, Jiang Y, Oh GT, Jeung EB, Lieben L, Bouillon R, Carmeliet G, Christakos S (2008) Active intestinal calcium transport in the absence of transient receptor potential vanilloid type 6 and calbindin-D9k. Endocrinology 149:3196–3205
43. Van Itallie CM, Anderson JM (2006) Claudins and epithelial paracellular transport. Annu Rev Physiol 68:403–429
44. Schulz D, Buddenberg T, Huston JP (2007) Extinction-induced “despair” in the water maze, exploratory behavior and fear: effects of chronic antidepressant treatment. Neurobiol Learn Mem 87:624–634
45. Bertiere MC, Sy TM, Baigts F, Mandenoff A, Apfelbaum M (1984) Stress and sucrose hyperphagia: role of endogenous opiates. Pharmacol Biochem Behav 20:675–679
46. de Chaves G, Moretti M, Castro AA, Dagostin W, da Silva GG, Boeck CR, Quevedo J, Gavioli EC (2009) Effects of long-term ovariectomy on anxiety and behavioral despair in rats. Physiol Behav 97:420–425
47. Chan SD, Chiu DK, Atkins D (1984) Mechanism of the regulation of the 1α, 25-dihydroxyvitamin D3 receptor in the rat jejunum by glucocorticoids. J Endocrinol 103:295–300
48. Kim MH, Lee GS, Jung EM, Choi KC, Jeung EB (2009) The negative effect of dexamethasone on calcium-processing gene expressions is associated with a glucocorticoid-induced calcium-absorbing disorder. Life Sci 85:146–152
49. Kim MH, Lee GS, Jung EM, Choi KC, Oh GT, Jeung EB (2009) Dexamethasone differentially regulates renal and duodenal calcium-processing genes in calbindin-D9k and -D28k knockout mice. Exp Physiol 94:138–151
50. Meddings JB, Swain MG (2000) Environmental stress-induced gastrointestinal permeability is mediated by endogenous glucocorticoids in the rat. Gastroenterology 119:1019–1028
51. Santos J, Saunders PR, Hanssen NP, Yang FC, Yates D, Groot JA, Perdue MH (1999) Corticotropin-releasing hormone mimics stress-induced colonic epithelial pathophysiology in the rat. Am J Physiol Gastrointest Liver Physiol 277:G391–G399
52. Smith F, Clark JE, Overman BL, Tozel CC, Huang JH, Rivier JE, Blökslager AT, Moeser AJ (2010) Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am J Physiol Gastrointest Liver Physiol 298:G352–G363
53. Barclay GR, Turnberg LA (1988) Effect of moderate exercise on salt and water transport in the human jejunum. Gut 29:816–820
54. Mansi JA, Drolet G (1997) Chronic stress induces sensitization in sympathoadrenal responses to stress in borderline hypertensive rats. Am J Physiol Regul Integr Comp Physiol 272:R813–R820