

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

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**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, corticosteroid 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

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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,  $1,25(\text{OH})_2\text{D}_3/\text{VDR}$ -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- $\text{D}_{9k}$ ; and
- 3 basolateral extrusion,  $\sim 20\%$  via  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX)-1 and  $\sim 80\%$  via the plasma membrane  $\text{Ca}^{2+}$ -ATPase isoform 1b (PMCA $_{1b}$ ) [13–15].

Recently, 2-week endurance swimming was reported to increase mRNA expression of TRPV5, TRPV6, and calbindin- $\text{D}_{9k}$  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  $1,25(\text{OH})_2\text{D}_3$ -dependent, is partly determined by tight junction permeability to cations [17, 18]. Such cation permeability is controlled by particular isoforms of the  $1,25(\text{OH})_2\text{D}_3$ -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  $\text{Na}^+/\text{K}^+$ -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 Elhaïmeur et al. [21] and Teerapornpantakit 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  $1.0098 \text{ g/cm}^3$ . 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 illuminance 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 MgCl<sub>2</sub>, 1.25 CaCl<sub>2</sub>, 23 NaHCO<sub>3</sub>, 12 D-glucose, and 2 mannitol (all purchased from Sigma). The solution was continuously aerated with humidified 5% CO<sub>2</sub> in 95% O<sub>2</sub>, 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 Charoenphandhu 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 <sup>45</sup>CaCl<sub>2</sub> 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 ( $J_{H \rightarrow C}$ ) was calculated by use of Eqs. 1 and 2.

$$J_{H \rightarrow C} = R_{H \rightarrow C} / (S_H \times A) \quad (1)$$

$$S_H = C_H / C_{To} \quad (2)$$

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 cm<sup>2</sup>);  $C_H$  was the mean radioactivity in the hot side (cpm); and  $C_{To}$  was the total calcium in the hot side (nmol). Radioactivity of <sup>45</sup>Ca 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 Techntron, 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  $\text{CaCl}_2$ .

#### Paracellular permeability measurement

The permeability ratio of sodium and chloride ( $P_{\text{Na}}/P_{\text{Cl}}$ ), 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.  $P_{\text{Na}}/P_{\text{Cl}}$  was calculated from the dilution potential by use of the Goldman–Hodgkin–Katz equation [27]. An increase in  $P_{\text{Na}}/P_{\text{Cl}}$  value usually indicates increased paracellular permeability to cations, e.g., sodium and/or calcium ions. Permeability of calcium ( $P_{\text{Ca}}$ ) 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  $\mu\text{g}$  total RNA was reverse-transcribed with oligo-dT<sub>20</sub> 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  $\mu\text{g}/\text{mL}$  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  $\pm$  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  $2^{-\Delta\Delta C_T}$  method, and the changes, as multiples, are presented as  $\log_2$  mean  $\pm$  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).

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

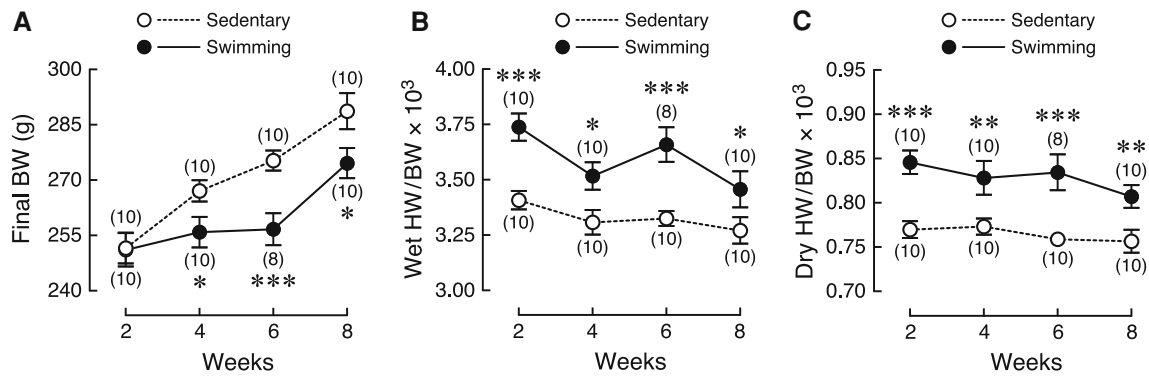
| Gene                      | Accession no. | Primer (forward/reverse)                                       | Product length (bp) |
|---------------------------|---------------|--|---------------------|
| Vitamin D-related genes   |               |  |                     |
| VDR                       | NM_017058     | 5'-GACCTGTGTCAGTTACAGCATC-3'<br>5'-AGACTGGTTGGAGCGTAA-3'       | 141                 |
| Transcellular genes       |               |  |                     |
| TRPV5                     | NM_053787     | 5'-CTTACGGGTTGAACACCACCA-3'<br>5'-TTGCAGAACACAGAGCCTCTA-3'     | 163                 |
| TRPV6                     | NM_053686     | 5'-ATCCGCCGCTATGCAC-3'<br>5'-AGTTTTTCTGGTCACTGTTTTTGG-3'       | 80                  |
| Calbindin-D <sub>9k</sub> | X_16635       | 5'-CCCGAAGAAATGAAGAGCATTTT-3'<br>5'-TTCTCCATCACCGTTCCTATCCA-3' | 174                 |
| NCX1                      | NM_019268     | 5'-GTTGTGTTTCGCTTGGGTTGC-3'<br>5'-CGTGGGAGTTGACTACTTTC-3'      | 163                 |
| PMCA <sub>1b</sub>        | NM_053311     | 5'-CGCCATCTTCTGCACAATT-3'<br>5'-CAGCCATTGTTCTATTGAAAGTTC-3'    | 109                 |
| Paracellular genes        |               |  |                     |
| NKA                       | X_63375       | 5'-CCACTGCTGAGCAGACACCAT-3'<br>5'-CCGAGTTCAGATGAATTTCTTC-3'    | 79                  |
| ZO-1                      | XM_218747     | 5'-GTATCCGATTGTTGTGTTCC-3'<br>5'-TCACTGTAGCACCATCCGC-3'        | 270                 |
| ZO-2                      | NM_053773     | 5'-TCTGAAGGTGAACACACAA-3'<br>5'-CCAGGATGTCTCTATACACG-3'        | 134                 |
| ZO-3                      | XM_001069839  | 5'-TGCTAATGAGACCGCTAAAG-3'<br>5'-GACACTCCGTTGATCTGTAA-3'       | 169                 |
| Cingulin                  | XM_001059265  | 5'-ATCGGGAACCTCCAGTCAAC-3'<br>5'-TGACGGGAACGGCTAAAG-3'         | 121                 |
| Occludin                  | NM_031329     | 5'-CACGTTGACCAATGC-3'<br>5'-CCCGTCCATAGGCTC-3'                 | 188                 |
| Claudin-2                 | NM_001106846  | 5'-GCTGCTGAGGGTAGAATGA-3'<br>5'-GCTCGCTTGATAAGTGTCC-3'         | 107                 |
| Claudin-3                 | NM_031700     | 5'-GCACCCACCAAGATCCTCTA-3'<br>5'-AGGCTGTCTGCTCTTCCA-3'         | 246                 |
| Claudin-12                | XM_001067932  | 5'-CCTCAAGTCTTCGGTGCC-3'<br>5'-CAGGAGGATGGGAGTACAG-3'          | 312                 |
| Housekeeping gene         |               |  |                     |
| GAPDH                     | NM_017008     | 5'-AGTCTACTGGCGTCTTCAC-3'<br>5'-TCATATTTCTCGTGGTTCAC-3'        | 133                 |

VDR, nuclear vitamin D receptor; TRPV5 and TRPV6, transient receptor potential vanilloid family Ca<sup>2+</sup> channels 5 and 6; NCX1, Na<sup>+</sup>/Ca<sup>2+</sup> exchanger 1; PMCA<sub>1b</sub>, plasma membrane Ca<sup>2+</sup>-ATPase isoform 1b; NKA, β<sub>1</sub>-subunit of Na<sup>+</sup>/K<sup>+</sup>-ATPase; ZO, zonula occludens; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Emotional stress and/or anxiety-like behavior developed after 4 weeks of swimming in an inescapable chamber

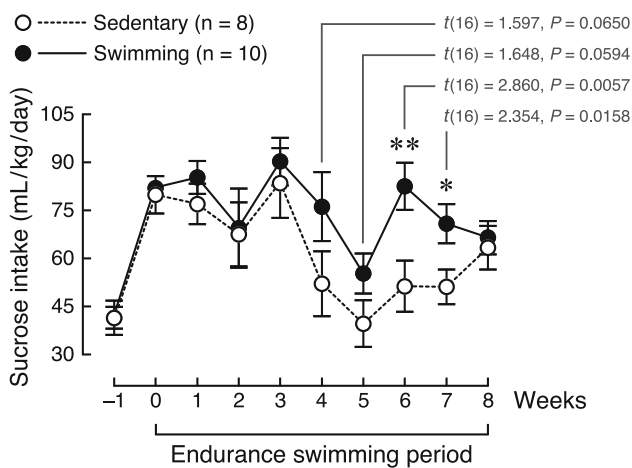
Two behavioral assessments, sucrose intake and open-field tests, were used to evaluate stress and anxiety-like behavior, respectively, in rats subjected to swimming in an inescapable chamber. As shown in Fig. 2, the sucrose-

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



**Fig. 1** a Final body weight (BW), b wet heart weight (HW) normalized by BW, and c dry HW normalized by BW in 2, 4, 6, and 8-week swimming rats and their age-matched sedentary controls. Final BW was measured at the ends of 2, 4, 6, or 8-week swimming

sessions. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with the corresponding age-matched sedentary controls. Numbers in parentheses are the numbers of animals



**Fig. 2** Sucrose intake by swimming and age-matched sedentary rats. All rats were subjected to this behavioral assessment before swim training (week -1) and then weekly during the 8-week endurance swimming period. The initial training period was the period between weeks -1 and 0. Sucrose intake is usually greater in stressed rats. \* $P < 0.05$ , \*\* $P < 0.01$  compared with age-matched sedentary controls. Some data are presented with their corresponding *t* and *P* values

training compared with the sedentary controls. However, the swimming rats seemed to eventually adapt and overcome this stress by the end of week 8.

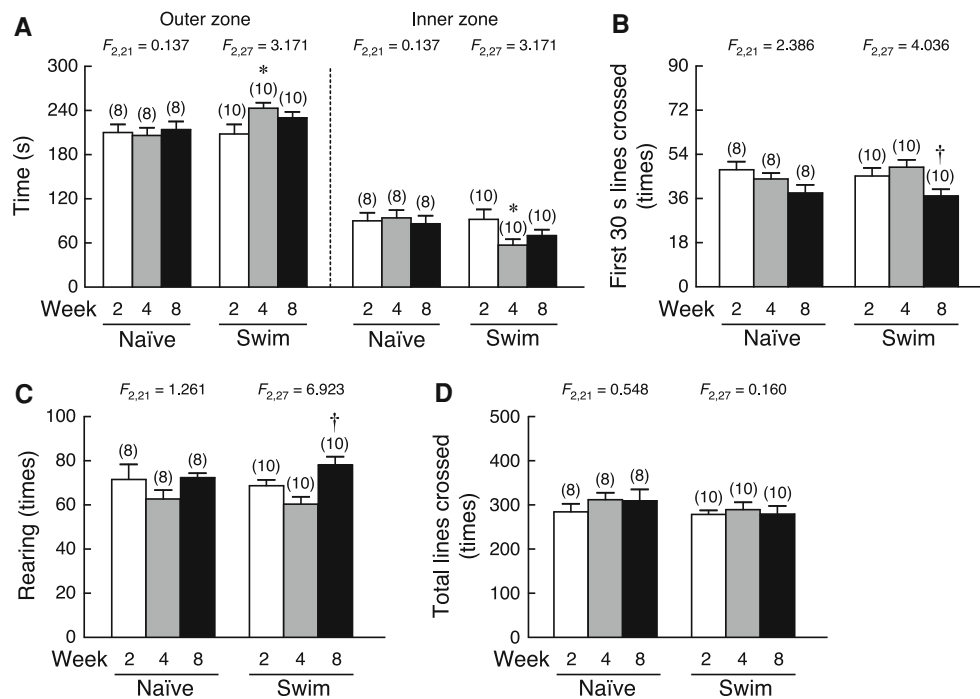
In the open-field test, an increase in the time spent in the outer zone and a decrease in the time spent in the inner zone were observed in 4-week, but not 8-week, swimming rats compared with 2-week swimming rats (Fig. 3a). In addition, the number of lines crossed in the first 30 s was less for 8-week 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  $P_{Na}/P_{Cl}$  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 ~20% increase in  $P_{Na}/P_{Cl}$  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  $P_{Ca}$  (Fig. 4e). The results collectively indicated that transcellular active duodenal calcium transport, but not paracellular calcium transport, was enhanced

**Fig. 3** **a** Time spent in the outer and inner zones of the open-field arena, **b** lines crossed in the first 30 s, **c** rearing, and **d** total lines crossed by 2, 4, and 8-week swimming rats. Naïve rats were age-matched, and were housed separately in their cages without exposure to unnecessary handling or any stressful conditions (i.e., not being placed in the water-filled chamber). All results were obtained from the open-field test. *F* values and degree of freedom values are also presented for each behavioral datum. \**P* < 0.05 compared with 2-week swimming group. †*P* < 0.05 compared with 4-week swimming group. Numbers in parentheses represent the numbers of animals



in 2-week swimming rats. Therefore, the increased trans-epithelial 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 ~50% in 8-week swimming rats (Fig. 5c). PD and Isc of the duodenal epithelium were also drastically reduced (~60%) but only in the 8-week swimming group (Table 2). However, 2–8 weeks of swimming did not affect duodenal TER (Table 2).

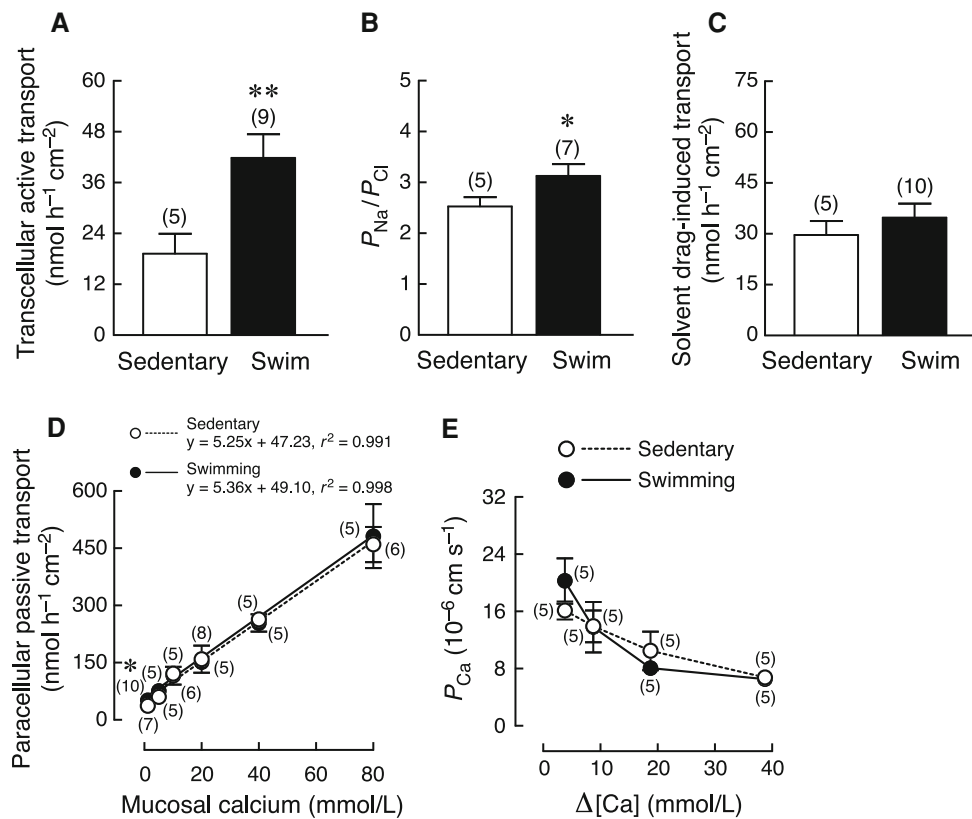
Consistent with our previous report for 2-week swimming rats [6], mRNA expression of VDR and genes related to transcellular calcium transport (i.e., TRPV5, TRPV6, NCX1, and PMCA<sub>1b</sub>), and paracellular ion transport (i.e., ZO-2, ZO-3, cingulin, occludin, claudin-3, and claudin-12) were upregulated 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-D<sub>9k</sub>, ZO-1, and claudin-2, was unaltered whereas that of the  $\beta$ -subunit of Na<sup>+</sup>/K<sup>+</sup>-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, TRPV6, PMCA<sub>1b</sub>,  $\beta$ -subunit of Na<sup>+</sup>/K<sup>+</sup>-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

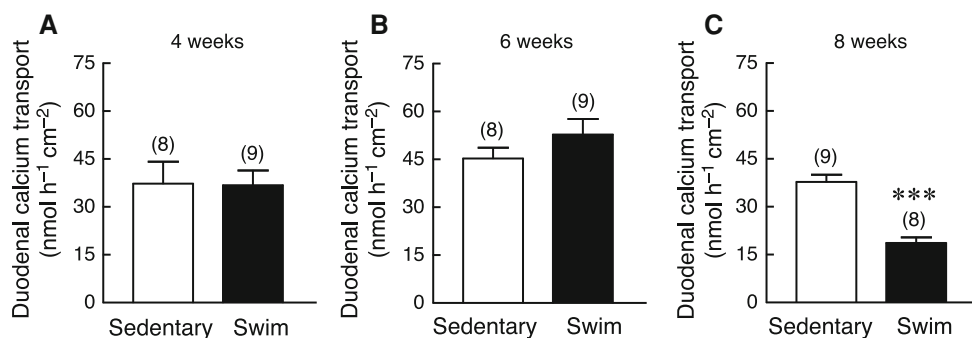
This swimming protocol was a non-impact endurance exercise, previously reported to increase heart weight and citrate synthase activity in the gastrocnemius muscle with no changes in plasma lactate levels [6]. Despite having benefits on various body systems, including cardiovascular, neural, and musculoskeletal 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





**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 serosal calcium; 5, 10, 20, 40, and 80 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

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 PMCA<sub>1b</sub>, was also abolished after prolonged training, presumably by the exercise-induced stress, which has been known to impair intestinal calcium absorption [12].

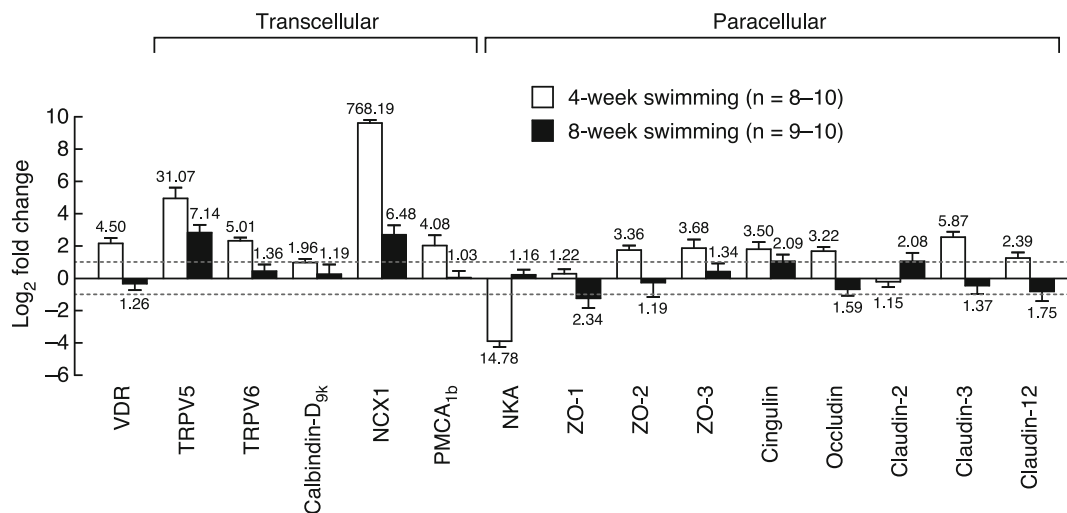
**Table 2** Epithelial electrical properties of the duodenum of sedentary and swimming rats

| Electrical property       | 2 weeks           |                  | 4 weeks           |                  | 6 weeks           |                  | 8 weeks           |                  |
|---------------------------|-------------------|------------------|-------------------|------------------|-------------------|------------------|-------------------|------------------|
|                           | Sedentary (n = 4) | Swimming (n = 4) | Sedentary (n = 8) | Swimming (n = 8) | Sedentary (n = 8) | Swimming (n = 8) | Sedentary (n = 6) | Swimming (n = 8) |
| PD (mV)                   | 6.74 ± 0.51       | 6.23 ± 0.65      | 6.02 ± 1.23       | 6.25 ± 0.57      | 7.32 ± 0.36       | 5.93 ± 0.69      | 7.40 ± 0.72       | 4.30 ± 0.33***   |
| Isc (μA/cm <sup>2</sup> ) | 66.79 ± 8.04      | 48.99 ± 4.76     | 52.48 ± 9.86      | 62.71 ± 7.47     | 70.08 ± 7.91      | 57.21 ± 9.43     | 63.39 ± 3.37      | 37.76 ± 4.92***  |
| TER (Ω cm <sup>2</sup> )  | 108.03 ± 11.03    | 130.92 ± 6.97    | 120.74 ± 8.92     | 106.38 ± 8.49    | 111.29 ± 8.39     | 119.85 ± 14.77   | 118.39 ± 7.14     | 122.02 ± 6.20    |

\*\*\*  $P < 0.001$  compared with the corresponding sedentary group

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)<sub>2</sub>D<sub>3</sub> [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<sub>9k</sub>, in the duodenum of 2-week swimming rats [6]. Because calbindin-D<sub>9k</sub> could increase PMCA activity [37] and the rate of cytoplasmic calcium diffusion [38, 39], the ~37-fold increase in calbindin-D<sub>9k</sub> 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)<sub>2</sub>D<sub>3</sub> 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<sub>9k</sub>, 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<sub>9k</sub> double knockout mice were normocalcemic, and still exhibited intestinal active calcium absorption after 1,25(OH)<sub>2</sub>D<sub>3</sub> administration as determined by everted duodenal sac assay [42].



**Fig. 6** Expression of genes related to the transcellular and paracellular transport in duodenal epithelial cells after 4 and 8 weeks of endurance swim training. The levels of mRNA expression were determined by qRT-PCR, and were expressed as  $\log_2$  mean  $\pm$  SE. The data were plotted with the corresponding changes, as multiples of the control values. The *two*

*dashed lines* indicate 2-fold upregulation or downregulation. *VDR*, nuclear vitamin D receptor; *TRPV5/6*, transient receptor potential vanilloid family  $\text{Ca}^{2+}$  channels 5/6; *NCX1*,  $\text{Na}^+/\text{Ca}^{2+}$  exchanger 1; *PMCA<sub>1b</sub>*, plasma membrane  $\text{Ca}^{2+}$ -ATPase isoform 1b; *NKA*,  $\beta_1$ -subunit of  $\text{Na}^+/\text{K}^+$ -ATPase; *ZO*, zonula occludens

In addition to the transcellular calcium transport, 2-week swimming also increased the paracellular cation permeability, as indicated by a  $\sim 20\%$  higher  $P_{\text{Na}}/P_{\text{Cl}}$  value for trained rats than for sedentary controls. The increased  $P_{\text{Na}}/P_{\text{Cl}}$  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  $1,25(\text{OH})_2\text{D}_3$  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 ( $\sim 4$ –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  $1,25(\text{OH})_2\text{D}_3$  [47] and TRPV6 mRNA expression [48, 49]. In contrast, 1–2 days of corticosteroid exposure transiently increased intestinal calcium transport by upregulating TRPV6 and PMCA<sub>1b</sub> 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 NCX1 contributes only  $\sim 20\%$  of the basolateral calcium extrusion [15], marked 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 PMCA<sub>1b</sub> (Fig. 6). Furthermore, the increases in TRPV5 (~73-fold compared with sedentary controls) and calbindin-D<sub>9k</sub> (~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 I<sub>sc</sub> 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<sup>+</sup>/K<sup>+</sup>-ATPase. I<sub>sc</sub> 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 I<sub>sc</sub> in the duodenum [29]. In other words, low PD and I<sub>sc</sub> 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.

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**Conflict of interest** None.

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