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**Title:** Post-prandial adjustments in renal phosphate excretion do not involve a gut-derived phosphaturic factor

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**Running Title:** No evidence for an intestinal phosphatonin

**Abstract:**

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**Post-prandial adjustments in renal phosphate excretion do not involve a gut-derived phosphaturic factor**

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**Running title: No evidence for an intestinal phosphatonin**

**Key words:** small intestine, phosphaturia, phosphatonin

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New findings:

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Contrary to earlier reports, rapid signalling between the small intestine and kidney mediated by a gut-derived phosphaturic factor in response to a physiological intestinal phosphate load is not supported by the current findings; moreover, hyperphosphataemia and increased PTH level are likely to be the underlying factors responsible for the phosphaturia following a supraphysiological intestinal phosphate load.

## Abstract

To date, the role of the small intestine in regulating post-prandial phosphate homeostasis has remained unclear and controversial. Previous studies have proposed the presence of a gut-derived phosphaturic factor that acts independently of changes in plasma phosphate concentration or parathyroid hormone (PTH) level; however, these early studies used duodenal luminal phosphate concentrations in the molar range and therefore the physiological relevance of this is uncertain. In the present study, we used both *in vivo* and *in vitro* approaches to investigate the presence of this putative 'intestinal phosphatonin'. Instillation of 1.3M phosphate into the duodenum rapidly induced phosphaturia, but in contrast to previous reports, this was associated with significant hyperphosphataemia and elevated PTH level; however, there was not the expected decrease in abundance of the renal sodium-phosphate cotransporter NaPi-IIa. Instillation of a physiological (10mM) phosphate load had no effect on plasma phosphate concentration, PTH level or phosphate excretion. Moreover, phosphate uptake by opossum kidney cells was unaffected after incubation with serosal fluid collected from intestinal segments perfused with different phosphate concentrations. Taken together, these findings do not support the concept of a gut-derived phosphaturic factor that can mediate rapid signalling between gut and kidney, leading to increased urinary phosphate excretion, as part of normal phosphate homeostasis.

## **Introduction**

Maintaining phosphate homeostasis is of critical biological importance because high plasma phosphate levels (hyperphosphataemia) have been associated with soft tissue calcification and subsequent cardiovascular disease and death (Gutierrez, 2013). The use of plasma phosphate concentration as a marker for future outcomes in chronic kidney disease (CKD) patients has been proposed, with several studies showing that a reduction of plasma phosphate directly improves mortality rates in CKD animal models, as well as in patients (Finch *et al.*, 2013; Russo *et al.*, 2015). The current treatments for hyperphosphataemia in CKD patients are oral dietary phosphate restriction and the use of intestinal phosphate binders; however, these options are less than ideal, because of poor patient compliance, the risk of malnutrition, gastrointestinal side effects, and the relatively poor efficacy of binders (Lee & Marks, 2015; Malberti, 2013). This has led to the development of compounds that specifically inhibit the process of intestinal phosphate absorption, such as nicotinamide and phosphonoformic acid (PFA), which although effective *in vitro*, have varying effects on phosphate transport in *in vivo* models of CKD, as well as in CKD patients (Brooks *et al.*, 1997; Katai *et al.*, 1999; Loghman-Adham & Motock, 1996). Recently, Tenapanor, an inhibitor of the sodium-hydrogen exchanger, NHE3, has been shown to act locally to reduce intestinal phosphate absorption. Uraemic 5/6<sup>th</sup> nephrectomised rats chronically treated with this drug have lower plasma phosphate levels, an improvement in the uraemic markers, creatinine, blood urea nitrogen (BUN), fibroblast growth factor 23 (FGF-23) and albumin, and reduced

ectopic vascular and soft tissue calcification (Labonte *et al.*, 2015). In addition, this compound has been shown to reduce plasma phosphate levels in humans, to have minimal systemic exposure, and to be well tolerated, apart from a tendency to cause loose stool and diarrhoea (Johansson *et al.*, 2016). However, the exact mechanism(s) of action and overall clinical benefits are not known and are still under investigation.

While it is now widely accepted that the intestine is a suitable target for controlling hyperphosphataemia in CKD, our understanding of the cellular mechanisms responsible for dietary phosphate absorption are incomplete (Lee & Marks, 2015) and the role of the intestine in phosphate homeostasis is still debated. In 2007, Berndt and colleagues proposed a novel signalling mechanism in which a duodenal phosphate load triggered acute phosphaturia in rats (Berndt *et al.*, 2007b). This reflex response was apparently independent of several key regulators of phosphate excretion, including elevated levels of plasma phosphate, parathyroid hormone (PTH), FGF-23, and secreted frizzled related protein-4, and renal innervation. Furthermore, infusion of duodenal mucosal homogenates also produced a phosphaturic response, suggesting the presence of a factor(s) within the intestinal mucosa that directly affects renal phosphate excretion. However, almost a decade later, no subsequent publication has confirmed or extended this finding, and so far the proposed phosphaturic factor has not been identified. A confounding factor has been the concentration of phosphate used in original study, which was in the supraphysiological molar,

rather than millimolar, range, and which may also have provided an additional osmotic stimulus (Berndt *et al.*, 2007a). Since the physiological phosphate concentration within the small intestinal lumen is reported to be from 5 to 10mM in the rat (Kirchner *et al.*, 2008; Marks *et al.*, 2015), the aim of our study was to establish whether instilling a physiological phosphate load into the duodenum can trigger the signalling mechanism proposed by Berndt *et al.* In addition, in an effort to identify an intestinally derived and released phosphaturic factor we used an *ex vivo* intestinal perfusion method to collect serosal fluid following luminal exposure to different phosphate loads. Studies were then performed in cultured opossum kidney (OK) cells as a bioassay to detect an acute effect on phosphate uptake when exposed to these collected serosal fluid samples.

## **Methods**

### **Ethical approval**

All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986, Amendment Regulations 2012. The protocols were approved by the University College London (Royal Free Campus) Comparative Biology Unit Animal Welfare and Ethical Review Body (AWERB) committee.

### **Renal clearance of phosphate in anaesthetised rats**

Experiments were performed on 41 male Sprague Dawley (SD) rats purchased from Charles River aged 12–14 weeks (250-300g). After a week of acclimatization, 21 rats were fed *ad libitum* a standard commercial rat chow (Diet RM1, 0.52% Pi, SDS Ltd, Witham, UK) and 20 rats were fed *ad libitum* a low phosphate diet, containing 0.1% Pi (SDS Ltd) for 7 days and allowed free access to water at all times. Rats were then anaesthetized by IP injection with thiobutabarbital (Inactin, 120 mg/kg; Sigma-Aldrich, Dorset, UK) and monitoring of the pedal and corneal reflex was undertaken to ensure that deep anaesthesia was achieved before the femoral artery, jugular, bladder and trachea were cannulated. A cannula-sheathed 19g needle was placed into the duodenum (2-3 cm from the stomach pylorus) for instillation of phosphate or control solutions and secured with a ligature. Once surgery was completed, a 1mL bolus of 0.05% FITC-inulin diluted in isotonic saline was given via the jugular cannula and the same concentration subsequently infused at a rate of 2.4ml/hr throughout the procedure to determine glomerular filtration rate (GFR). A 40 min period was

allowed for surgical recovery and for FITC-inulin equilibration after which rats underwent the following protocol: four consecutive collections of urine were made, each of 15 min. A sample (500uL) of arterial blood was collected via the femoral artery cannula at the start and end of each urine collection period for phosphate and FITC-inulin measurements. After the first control urine collection period, 1ml of 10mM or 1.3M  $\text{KH}_2\text{PO}_4$  in buffer (containing in mM: 16 Na-Hepes, 140 NaCl, 3.5 KCL, pH 5) or isosmolar control buffer without phosphate was administered into the duodenum, the needle removed, and the intestine tied off. Three subsequent collections following the duodenal bolus were made, after which the kidneys were removed and death ensured by incising the heart. The cortex of both kidneys was dissected at 4°C and snap frozen for brush border membrane (BBM) vesicle preparation.

### **BBM vesicle preparation and Western Blotting**

Preparation of BBM vesicles and subsequent Western blotting has been described previously (Chichger *et al.*, 2016). Blotting was carried out using 40µg of BBM protein heated at 90°C for 2 min. Rabbit polyclonal antibodies to NaPi-IIa and NaPi-IIc were generous gifts from Professor Jurg Biber, University of Zurich, Switzerland. Mouse monoclonal antibody for β-actin (Abcam, Cambridge, UK) was used as a loading control. Blots were visualized with enhanced chemiluminescence on a Fluor-S Multilmager system (BioRad, Hertfordshire, UK), and the abundance of each protein of interest calculated relative to actin and expressed in arbitrary units.

### ***Ex-vivo* intestinal perfusion for collection of serosal fluid**

Experiments were performed on 30 male C57BL/6 mice (approximately 25g), bred at the Royal Free Campus Comparative Biology Unit. Mice were allowed *ad libitum* access to a standard rodent chow containing 0.52% phosphate (RM1 diet; SDS Ltd) and water until the day of experimentation. Mice were terminally anesthetized with 50 mg/kg sodium pentobarbitone via an intraperitoneal (IP) injection. Monitoring of the pedal and corneal reflex was undertaken to ensure that deep anaesthesia was achieved before 10cm segments of the proximal (from the ligament of Treitz) or the last 10cm of distal small intestine was cannulated *in vivo*. Segments were perfused with Krebs buffer containing (in mM): 4.6 KCl, 1 CaCl<sub>2</sub>\*2H<sub>2</sub>O, 1.2 MgCl<sub>2</sub>\*6H<sub>2</sub>O, 25 NaHCO<sub>3</sub>, 118 NaCl, 1.2 KH<sub>2</sub>PO<sub>4</sub> and 10 glucose, oxygenated with 95%O<sub>2</sub> and 5%CO<sub>2</sub> to create a segmental flow at a rate of 1ml/min. The intestine was subsequently removed and suspended in an organ chamber containing liquid paraffin at 37°C. Immediately after removal of the intestinal segment mice were humanly killed by cervical dislocation and death confirmed by cessation of the heartbeat. The suspended intestinal segments were perfused for 30 minutes to allow them to equilibrate. Subsequently, serosal fluid was collected for 45 minutes during which 1.2mM phosphate was perfused through the segment. The solution was then either maintained with 1.2mM phosphate or switched to one containing either 10mM or 50mM phosphate and serosal fluid collected after a further 45 minutes. In total 10 mice were used for each of the different concentrations.

### **Phosphate uptake by OK cells**

Opossum kidney (OK) cells, purchased from Public Health England Culture Collections, were maintained in 80 cm<sup>3</sup> flasks (Nunc, Thermo Fisher Scientific, UK) at 37°C with 5% CO<sub>2</sub> and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum and 2% penicillin streptomycin. The cells were sub-cultured every 7 days. For phosphate uptake experiments, 3 x 10<sup>5</sup> cells were seeded in 6-well culture dishes (Nunc, Thermo Fisher Scientific) and maintained at 37°C with 5% CO<sub>2</sub>. Phosphate uptake was measured 2 days after seeding into the 6-well dishes. The growth medium was aspirated and the cells quickly rinsed twice with pre-warmed (37°C) sodium-free uptake buffer: (in mM) 137 ChCl, 5.4 KCl, 2.8 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub> and 14 HEPES (-Na<sup>+</sup>) (pH 7.4). The cells were then incubated for 30 min at 37°C with either 500µl incubation buffer (containing 137mM Na<sup>+</sup> for total transport, or ChCl for Na<sup>+</sup>-independent transport), or 250µl serosal fluid from proximal or distal small intestine and 250µl incubation buffer (± Na<sup>+</sup>). For experiments using the sodium dependent phosphate transporter inhibitor, phosphoformic acid (PFA), concentrations of 1, 3, 7 and 10mM PFA was included in the incubation buffer. After 30 minutes the incubation buffer was aspirated and cells washed three times with pre-warmed sodium free buffer (-Na<sup>+</sup>). Phosphate transport was then initiated with the addition of 500µl buffer containing 0.1mM KH<sub>2</sub>PO<sub>4</sub> and 1 µCi/ml <sup>33</sup>P ± Na<sup>+</sup>. After 5 mins of incubation at

37°C, the buffer was aspirated and the cells rinsed three times with ice cold 154mM ChCl. Cells were subsequently lysed with 0.1M NaOH and 0.1% SDS. 100µl of cell lysate and 10µl (at 1:100 dilution) of initial solution was used for scintillation counting with a Packard tri-carb 2900tr scintillation counter (Perkin Elmer, Buckinghamshire, UK) Protein concentration was determined using the Bradford method (Bradford, 1976). Results are expressed as nmoles of phosphate/µg of protein/5min (mean ± SD) representing total phosphate transport (uptake in the presence of sodium) or sodium-independent phosphate uptake (uptake in the absence of sodium).

### **Measurements of plasma PTH and phosphate**

From the clearance studies, urinary and plasma phosphate was quantified using a QuantiChrom phosphate assay kit (Bioassay Systems, San Francisco, USA), and PTH in plasma samples taken at the end of the procedure was measured using an ELISA kit targeting rat bioactive PTH (Immunotopics, California, USA). All kits were used according to the manufactures instructions.

### **Statistical analysis**

Data are presented as means ± SD and statistical comparisons were performed using either Student's paired or unpaired t tests with statistical significance taken as  $P < 0.05$ .

## **Results**

### **A 1.3M phosphate load in the duodenum induces phosphaturia in rats maintained on a normal diet**

In keeping with Berndt *et al.* (2007a,b), who also used 1.3M phosphate, we showed that instillation of this dose into the duodenum induces phosphaturia. However, in contrast to their findings, this response occurred later, after 45 minutes, not at 20 minutes (Figure 1A), and was associated with a significant increase in plasma phosphate concentration (Figure 1B) and PTH level (Figure 1C). Interestingly, the phosphaturia was not associated with a change in protein levels of either NaPi-IIa or NaPi-IIc (Figures 1D and 1E, respectively). Representative Western blots for NaPi-IIa and NaPi-IIc can be found in Appendix 1. In addition, the change in urinary phosphate excretion was independent of any change in GFR or mean arterial pressure (Table 1).

### **A physiological phosphate load in the duodenum does not induce phosphaturia in rats maintained on a normal phosphate diet**

In contrast to the effect seen with 1.3M phosphate, introduction of a more physiological phosphate load of 10mM into the duodenum did not elicit an acute change in urinary phosphate excretion (Figure 2A). In addition, there was no change in plasma phosphate concentration (Figure 2B) or PTH level (Figure 2C), and NaPi-IIa and NaPi-IIc protein expression was unaffected (Figures 2D and 2E, respectively).

### **A 1.3M phosphate load in the duodenum does not induce phosphaturia in rats maintained on a chronic low phosphate diet**

Several reports have shown that rats chronically adapted to a low phosphate diet and then acutely switched to a high phosphate diet have significantly downregulated protein levels of renal NaPi-IIa, but not NaPi-IIc (Bourgeois *et al.*, 2013; Giral *et al.*, 2009). In the current study rats were chronically adapted to a low phosphate diet for 7 days prior to the renal clearance experiments to establish whether this would exaggerate the renal response to a duodenal phosphate load. However, in contrast to animals on a normal phosphate diet, instillation of 1.3M phosphate did not induce phosphaturia in the low phosphate-adapted animals (Figure 3A), and had no effect on the protein levels of NaPi-IIa or NaPi-IIc (Figure 3D and 3E respectively); but there was a significant increase in plasma phosphate concentration (Figure 3B) and in PTH level (Figure 3C). Animals given a physiological 10mM intestinal phosphate challenge after being maintained on a low phosphate diet showed no changes in phosphate excretion, plasma phosphate concentration or PTH level (Figure 4).

### **Serosal fluid collected from perfused mouse intestine does not alter phosphate uptake by OK cells**

To investigate whether a factor is released from the intestinal mucosa that could affect renal phosphate reabsorption and thereby excretion, we perfused segments of mouse small intestine *ex vivo* with different phosphate

concentrations and collected the serosal fluid. This approach has been used previously to establish the influence of glucose on secretion levels of the gut peptides gluco-insulintropic peptide (GIP), glucagon-like peptide-1 (GLP-1), and peptide tyrosine tyrosine (PYY) (Mace *et al.*, 2012). The presence of a gut peptide in the collected serosal fluid capable of affecting phosphate transport in renal cells was investigated by exposing OK cells to the serosal fluid and performing  $^{33}\text{P}$  uptake studies. Initially, cellular phosphate uptake by OK cells was validated using two methods. First, uptake was measured under sodium and sodium-free conditions to confirm the presence of sodium-dependent phosphate transport in this proximal tubule cell line, as described previously (Malmstrom & Murer, 1986; Thomas *et al.*, 2016b). Sodium-dependent transport accounted for 90.4% of total transport when 0.1mM phosphate was included in the uptake buffer (Figure 5A). Second, PFA, a known blocker of sodium-dependent phosphate transport activity (Villa-Bellosta & Sorribas, 2009), was added to the incubation media. PFA induced a dose-dependent inhibition of total phosphate uptake (Figure 5A), with significant inhibition achieved at 7 and 10mM PFA. In contrast, incubation of OK cells with serosal fluid collected from proximal and distal segments of mouse small intestine perfused with 1.2, 10, and 50mM phosphate had no effect on total (Figure 5B) or sodium-independent (Figure 5C) phosphate uptake by OK cells.

## **Discussion**

Our knowledge of the processes regulating acute post-prandial phosphate homeostasis is limited. In 2007, Berndt and colleagues proposed a mechanism by which the upper small intestine detects the presence of increased dietary phosphate and signals to the kidney to rapidly increase phosphate excretion to maintain phosphate balance. This response was rapid and occurred within 20 minutes, and was independent of changes in PTH and FGF-23 (Berndt *et al.*, 2007b). However, whether a change in renal NaPi-IIa or NaPi-IIc protein abundance was responsible for the phosphaturia was not examined, although many studies have demonstrated that post-prandial downregulation of NaPi-IIa in response to ingestion of a high phosphate diet occurs within 2-4 hours (Bourgeois *et al.*, 2013; Capuano *et al.*, 2005; Giral *et al.*, 2009; Levi *et al.*, 1994), and that increased PTH, but not FGF-23, levels are responsible for the changes seen (Bourgeois *et al.*, 2013).

These differences raise the possibility that there may be two distinct mechanisms occurring in response to dietary phosphate: one that occurs rapidly and one more slowly. However, it is also important to note that the concentration of phosphate used by Berndt and colleagues was 1.3M, and that this phosphate concentration is 1000-fold higher than the documented concentration found in rodent (5-10mM) (Kirchner *et al.*, 2008; Marks *et al.*, 2015) and human upper small intestinal contents (0.5 and 17.5mM) (Davis *et al.*, 1983). While it might be argued that consumption of a meal or soft drink high in phosphate or phosphate

preservatives could generate an intestinal phosphate concentration higher than the millimolar range reported above, it is worth noting that the highest value documented in humans (17.5mM) was measured after consumption of a high phosphate meal (Davis *et al.*, 1983).

In the present study we found, in keeping with the findings of Berndt *et al.*, that urinary phosphate excretion increased in rats given a 1.3M duodenal phosphate bolus. However, in our study this response was associated with a significant increase in plasma phosphate concentration and in PTH level. Instillation of an osmotically balanced buffer had no effect on plasma phosphate or PTH level, or on urinary phosphate excretion, but it was noted, as expected with a hypertonic solution, that the intestinal contents were watery, with an increased presence of mucus, and that the segment became distended. In contrast to the effects seen with 1.3M phosphate, duodenal instillation of the more physiological phosphate load of 10mM did not affect any of the measurements made. Our findings show that although the kidney responds to changes in intestinal phosphate load, the response appears to be only following exposure to very high levels of ingested phosphate. They also confirm previous rodent studies showing that ingestion of a diet high in phosphate (1.2%) (Giral *et al.*, 2009; Bourgeois *et al.*, 2013; Hernandez *et al.*, 1996) or duodenal infusion of phosphate (Martin *et al.*, 2005) induces a rapid increase in plasma phosphate concentration and subsequent release of PTH, and that this response, rather than release of a novel intestinal factor secreted independently of changes in plasma phosphate concentration, is

likely to be responsible for the increase in renal phosphate excretion. In keeping with this suggestion is the finding that in humans, phosphaturia caused by an acute intestinal phosphate load is also associated with changes in plasma phosphate concentration and PTH level (Bevilacqua *et al.*, 2010; Nishida *et al.*, 2006; Scanni *et al.*, 2014). Interestingly, a recent study by Thomas *et al.* has shown that the phosphaturia caused by intravenous infusion of phosphate is associated with elevated plasma phosphate and PTH levels, but that while intragastric administration of the same phosphate load increases urinary phosphate excretion to the same extent as when given intravenously, it is related to elevated plasma PTH and not plasma phosphate levels, highlighting the key role for PTH in the phosphaturic response (Thomas *et al.*, 2016a).

A number of studies have investigated the time-course for changes in the abundance of the renal phosphate transporters in response to dietary phosphate ingestion. Switching from a chronic low phosphate diet acutely to a high phosphate diet induces downregulation in NaPi-IIa protein abundance after 2-4 hours (Bourgeois *et al.*, 2013; Capuano *et al.*, 2005; Katai *et al.*, 1997; Levi *et al.*, 1994; Segawa *et al.*, 2005). In contrast, the time course for NaPi-IIc downregulation has been reported to be longer, ranging from 4-6 hours (Segawa *et al.*, 2005) to 48 hours (Villa-Bellosta *et al.*, 2009). Given that altered NaPi-IIa abundance in response to dietary phosphate is likely to be PTH-mediated (Bourgeois *et al.*, 2013), and that PTH can elicit NaPi-IIa internalization within 5 minutes (Bacic *et al.*, 2006), it can be assumed that reduced NaPi-IIa levels after

an acute phosphate load occurs within minutes, although this has not been definitely confirmed. Interestingly, our study demonstrates that even with a significantly increased plasma phosphate concentration and PTH level, we did not detect changes in either NaPi-IIa or NaPi-IIc protein abundance over the 45-minute time-course of the study. This is in keeping with the recent report that acute phosphaturia in response to intravenous phosphate infusion occurs without any detectable change in type II transporter activity or abundance (Thomas *et al.*, 2016a). Thus the higher tubular load of phosphate, resulting from the elevated plasma phosphate concentration and maintained GFR may explain the phosphaturia, alternatively it has been proposed that changes in membrane lipid composition or NHERF1 phosphorylation may be responsible (Thomas *et al.*, 2016a). Interestingly, rats maintained on a low phosphate diet also had elevated plasma phosphate concentration and PTH level in response to a 1.3M phosphate load, but this did not elicit a change in urinary phosphate excretion, at least over the time-course studied. This finding could be a consequence of the well-documented increase in abundance of NaPi-IIa protein in response to a chronic low phosphate diet, and the potentially slower than expected adaptation of NaPi-IIa protein levels under these experimental conditions.

The presence of the phosphaturic factor proposed by Berndt *et al* was also investigated *in vitro* by performing phosphate uptake studies in OK cells (a widely used bioassay cell culture system to study renal proximal tubular phosphate transport) exposed to serosal fluid. These experiments also aimed to determine

whether NaPi-IIb played a key role in intestinal phosphate sensing, and subsequent release of the putative intestinal phosphatonin(s). We chose to use mouse intestinal segments for the collection of serosal fluid, rather than rat, because the regional profile for NaPi-IIb in mice has been consistently reported, with NaPi-IIb protein almost exclusively located in the mouse ileum (Marks *et al.*, 2006; Radanovic *et al.*, 2005), and transport studies using NaPi-IIb knockout mice have confirmed that phosphate absorption in this region is >90% NaPi-IIb-mediated (Sabbagh *et al.*, 2009). In contrast, in the rat phosphate absorption occurs maximally in the jejunum (Giral *et al.*, 2009; Marks *et al.*, 2006; Walling, 1977), but only ~30% of total transport *in vivo* can be resolved as sodium-dependent and, presumably, NaPi-IIb-mediated (Marks *et al.*, 2015). Therefore, by using defined segments of mouse intestine we believed we could make a clearer interpretation of the potential role of NaPi-IIb as a phosphate sensor. However, although we were able to demonstrate significant sodium-dependent and PFA-sensitive phosphate transport in these cells, no change in uptake was found following treatment with serosal fluid collected from either the proximal or distal mouse small intestine. This lack of response suggests that a modest and physiological intestinal phosphate load does not stimulate the secretion of a phosphaturic factor into serosal fluid.

In summary our *in vivo* and *in vitro* data have been unable to demonstrate the presence of a gut-derived phosphaturic factor that mediates a rapid signalling mechanism between the small intestine and kidney that is independent of

changes in plasma phosphate concentration and/or PTH level. Instead, our data are in keeping with the observation that a dietary phosphate load can cause post-prandial hyperphosphataemia and stimulation of PTH release from parathyroid glands in order to maintain phosphate homeostasis (Bourgeois *et al.*, 2013; Giral *et al.*, 2009; Hernandez *et al.*, 1996; Martin *et al.*, 2005). We also demonstrate that supraphysiological, but not physiological, intestinal phosphate loads elicit changes in plasma phosphate concentration and PTH level and that only when there is positive phosphate balance does this provoke a phosphaturic response. Further studies are needed to establish what intestinal phosphate loads correlate with the phosphate levels found in the human diet.

## **Additional information**

### **Competing interests**

The authors declare that they have no competing financial interests. J Marks received financial research support from Astra Zeneca and Ardelyx unrelated to this project. RJ Unwin is currently also a Chief Scientist at AstraZeneca, Gothenburg, Sweden.

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### **Author contributions**

GJL, ESD, RJU, JM, designed the research; GJL, LMH and JM performed the research and analysed the data. JM, GJL and LMH wrote the paper. All authors approved the final version for publication.

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## **Figure legends**

**Figure 1: Instillation of 1.3M phosphate into the duodenum induces phosphaturia in rats maintained on a normal phosphate diet.** Urinary phosphate excretion (A), plasma phosphate levels (B), and plasma PTH level (C), in rats fed a normal phosphate diet and receiving a duodenal bolus of either 1.3M phosphate or an isosmolar control buffer. Western blot analysis of NaPi-IIa (D) and NaPi-IIc (E) protein in brush border membrane vesicles prepared from kidneys obtained from the rats at the end of the procedure. Results are presented as mean  $\pm$  SD, n = 5-7, \*P<0.05, \*\*P<0.01 compared to control period before instillation of the phosphate bolus (A and B) using a Student's paired t test, or compared with the animals receiving the osmotically balanced control buffer (C) using a Student's unpaired t test.

**Figure 2: Instillation of 10mM phosphate into the duodenum does not induce phosphaturia in rats maintained on a normal phosphate diet.** Urinary phosphate excretion (A), plasma phosphate levels (B), and plasma PTH level (C), in rats fed a normal phosphate diet and receiving a duodenal bolus of either 10mM phosphate or an isosmolar control buffer. Western blot analysis of NaPi-IIa (D) and NaPi-IIc (E) protein in brush border membrane vesicles prepared from kidneys obtained from the rats at the end of the procedure. Results are presented as mean  $\pm$  SD, n = 4-5.

**Figure 3: Instillation of 1.3M phosphate into the duodenum does not induce phosphaturia in rats maintained on a low phosphate diet.** Urinary phosphate excretion (A), plasma phosphate levels (B), and plasma PTH level (C), in rats fed a low phosphate diet for 7 days prior to experimentation and receiving a duodenal bolus of either 1.3M phosphate or an isosmolar control buffer. Western blot analysis of NaPi-IIa (D) and NaPi-IIc (E) protein in brush border membrane vesicles prepared from kidneys obtained from the rats at the end of the procedure. Results are presented as mean  $\pm$  SD, n = 5, \*\*P<0.01 compared with control period before instillation of the phosphate bolus (B) using a paired t test, and \*\*\*P<0.001 compared with the animals receiving the osmotically balanced control buffer (C) using a Student's unpaired t test.

**Figure 4: Instillation of 10mM phosphate into the duodenum does not induce phosphaturia in rats maintained on a low phosphate diet.** Urinary phosphate excretion (A), plasma phosphate levels (B), and plasma PTH level (C), in rats fed a low phosphate diet for 7 days prior to experimentation and receiving a duodenal bolus of either 10mM phosphate or an isosmolar control buffer. Western blot analysis of NaPi-IIa (D) and NaPi-IIc (E) protein in brush border membrane vesicles prepared from kidneys obtained from the rats at the end of the procedure. Results are presented as mean  $\pm$  SD, n = 5.

**Figure 5: Phosphate uptake in OK cells.** The ability of the phosphate uptake assay to detect sodium-dependent phosphate transport in OK cells was

confirmed using sodium-free buffers and the phosphate transport inhibitor, phosphoformic acid (PFA) (A). Total phosphate uptake (B) and sodium-independent phosphate uptake (C) was measured in OK cells exposed to serosal fluid collected from proximal and distal intestinal segments perfused with 1.2, 10, or 50mM phosphate. Results are presented as mean  $\pm$  SD, n = 4-5, \*\*P<0.01, \*\*\*P<0.005 compared with uptake in the presence of sodium using a Student's unpaired t test.

**Table 1.** Glomerular filtration rate (GFR) and mean arterial pressure (MAP) were unchanged during the surgical procedure. Results are presented as mean  $\pm$  SD, n=4-7.

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Figure 1

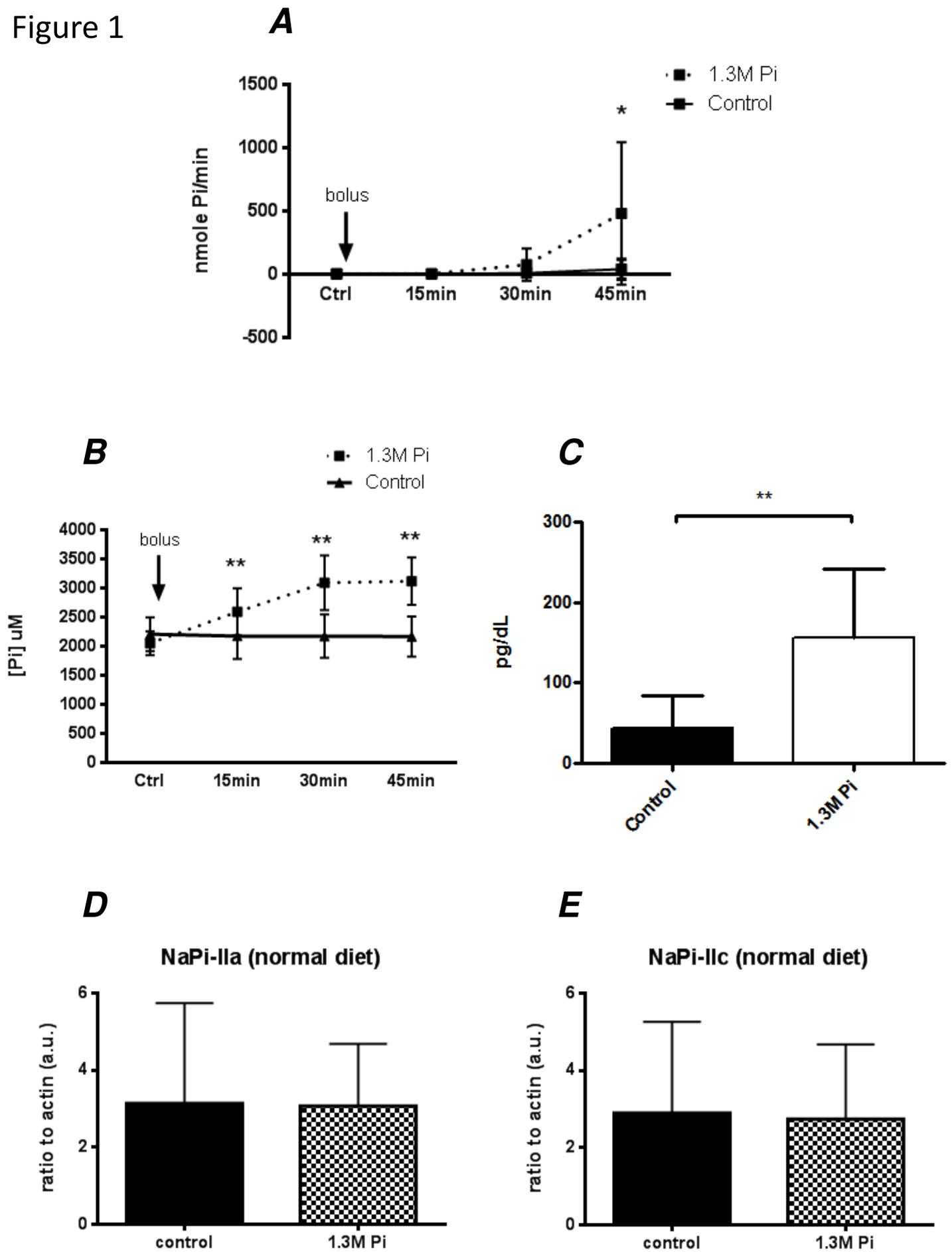
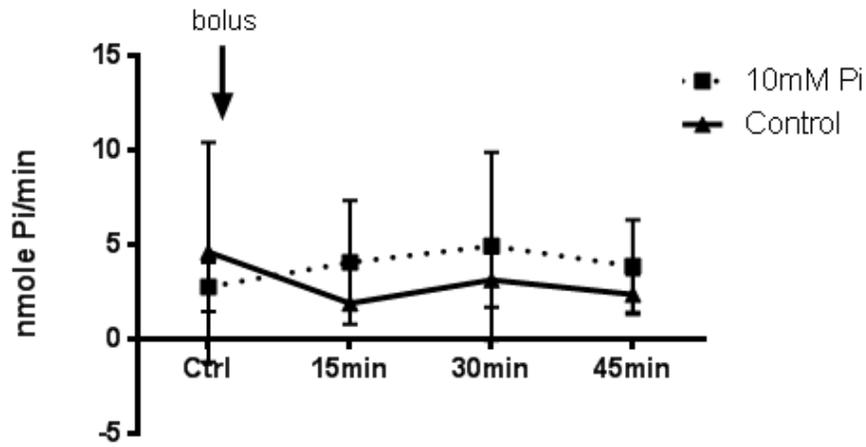
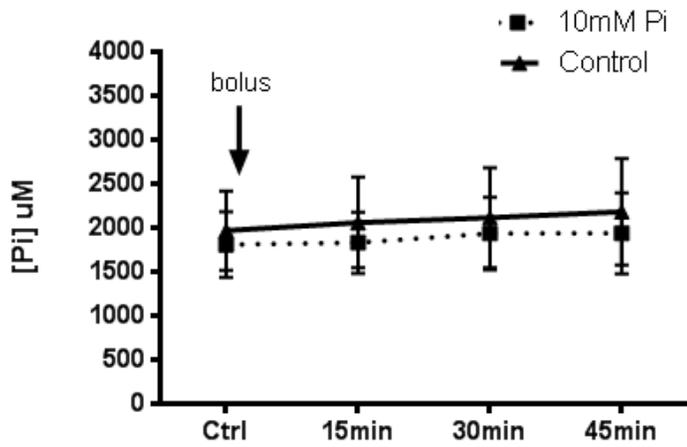


Figure 2

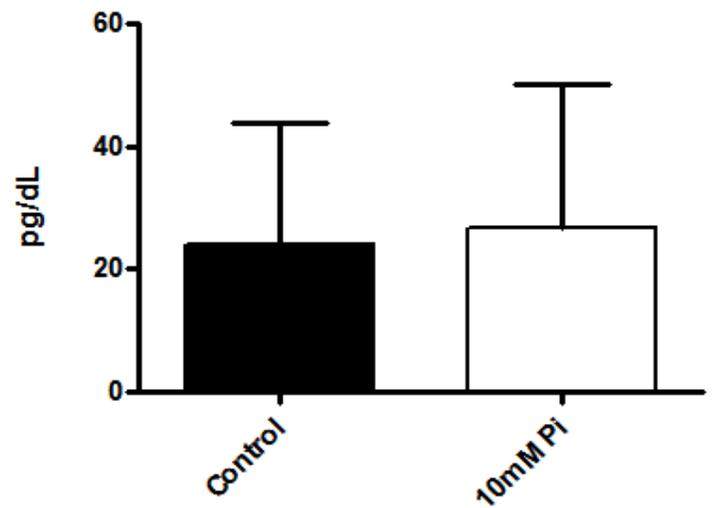
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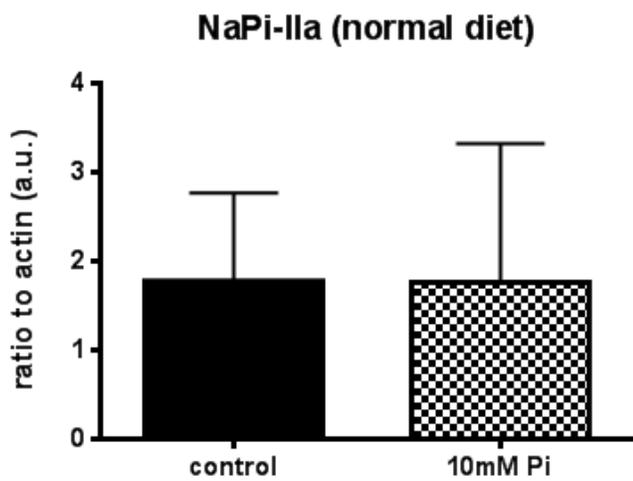
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**C**



**D**



**E**

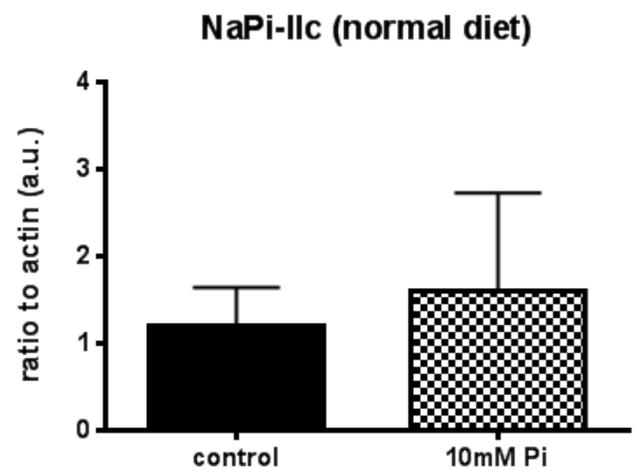
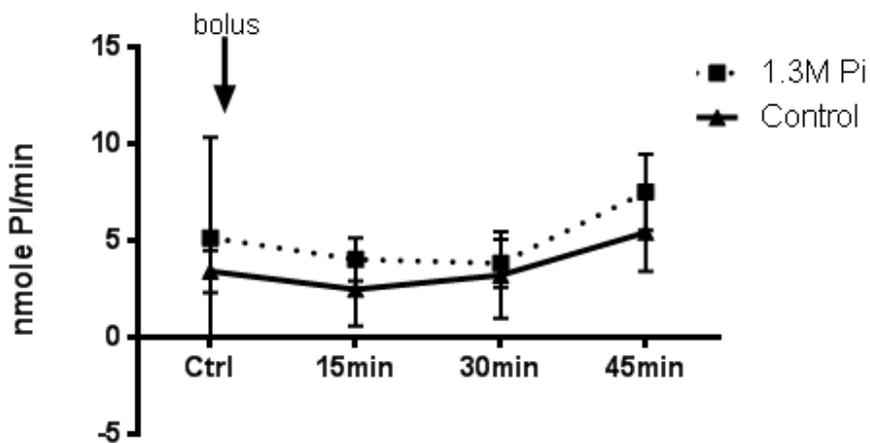
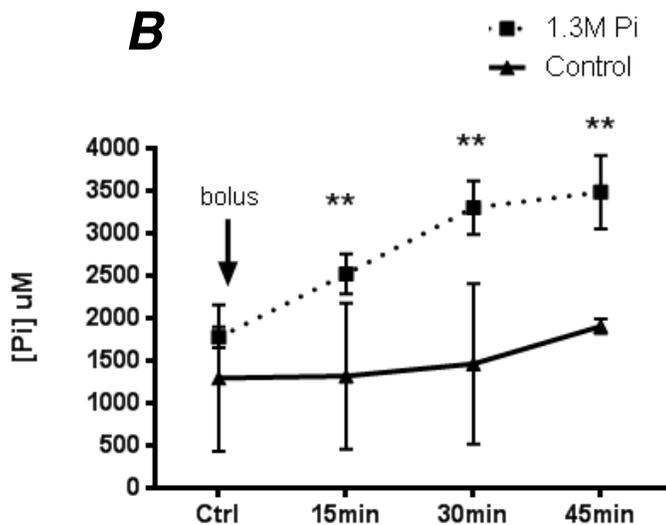


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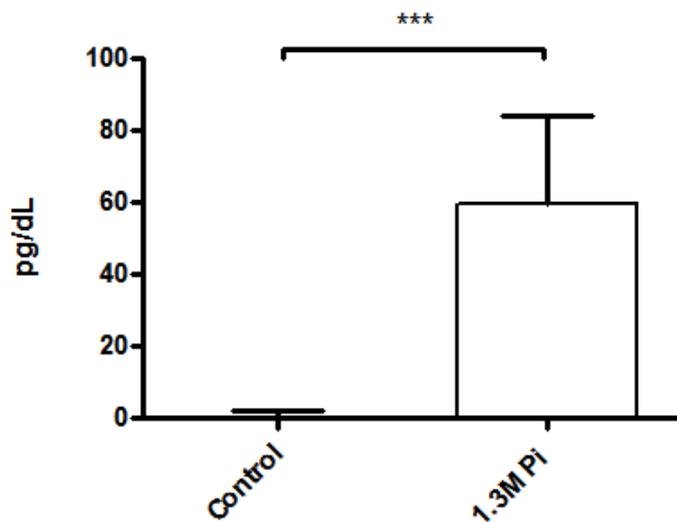
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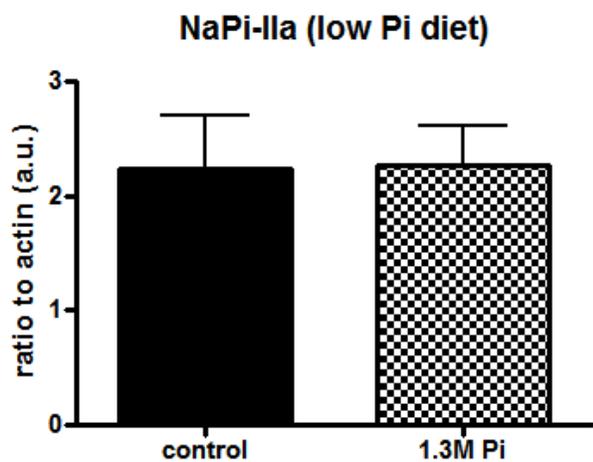
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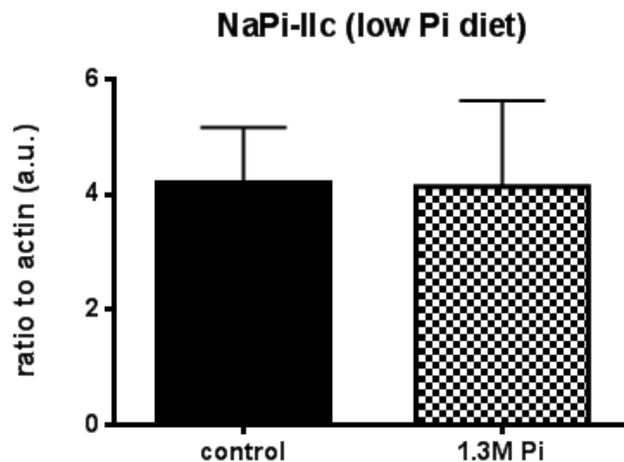
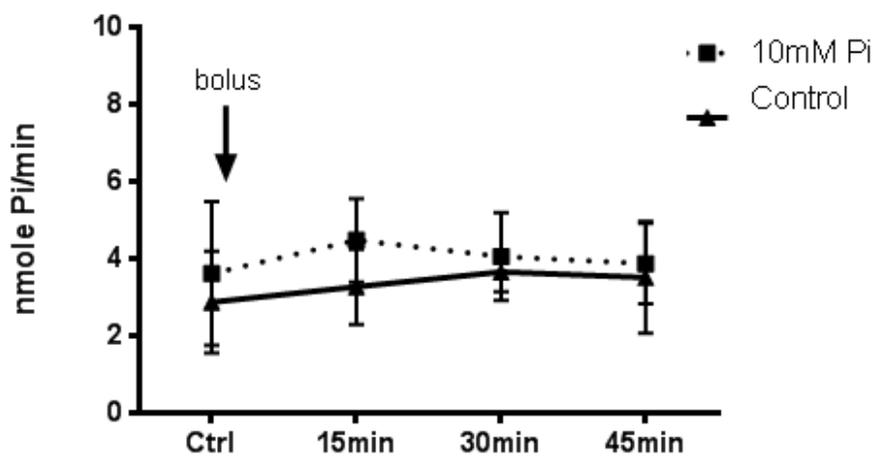
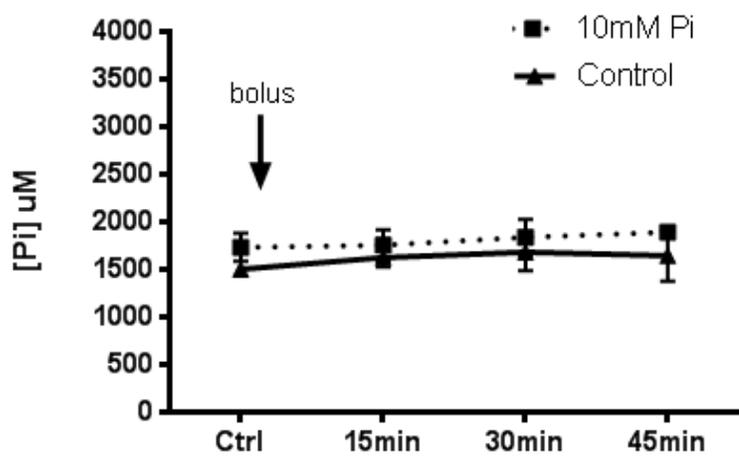


Figure 4

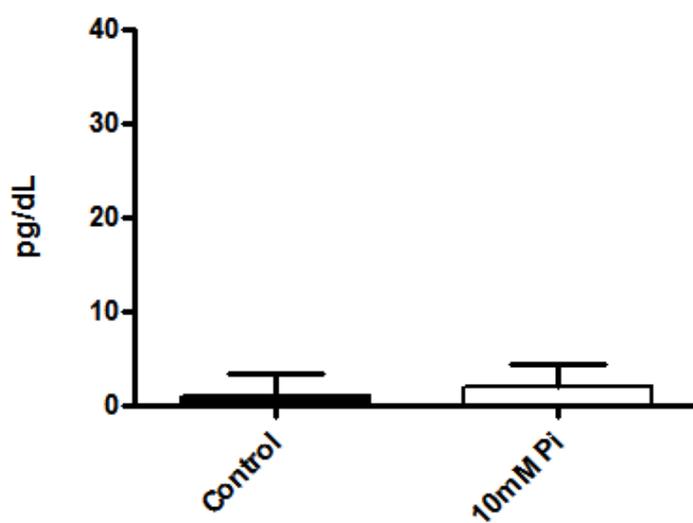
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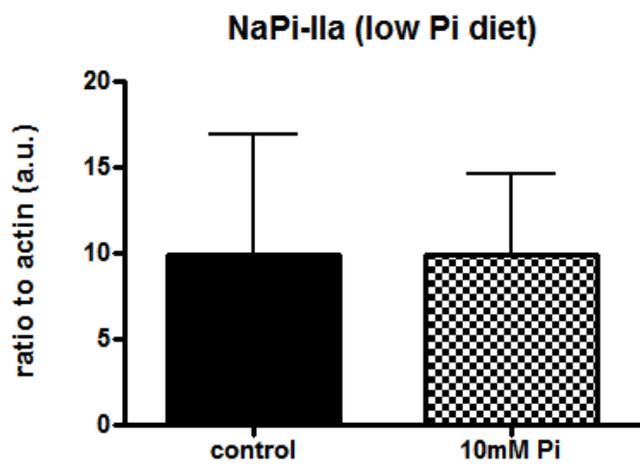
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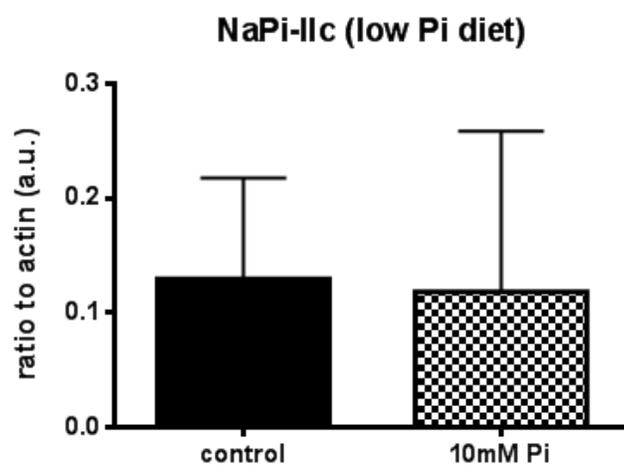
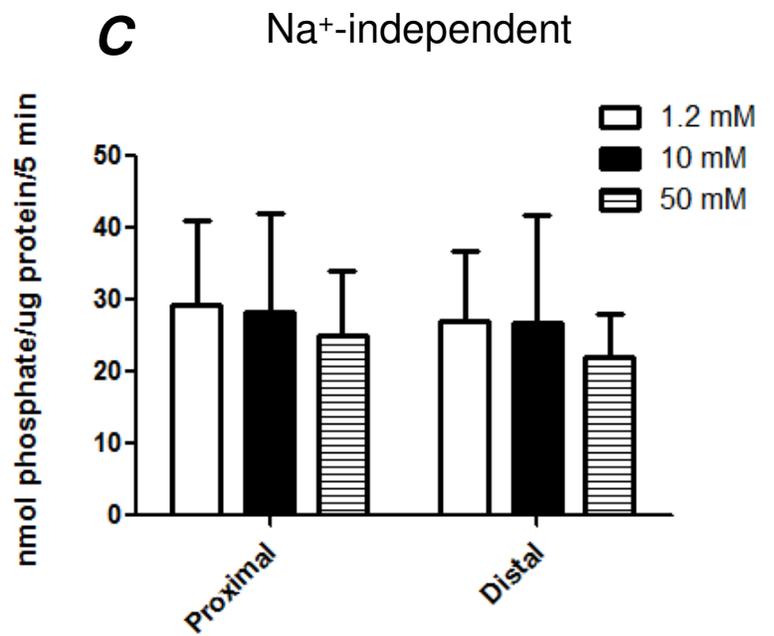
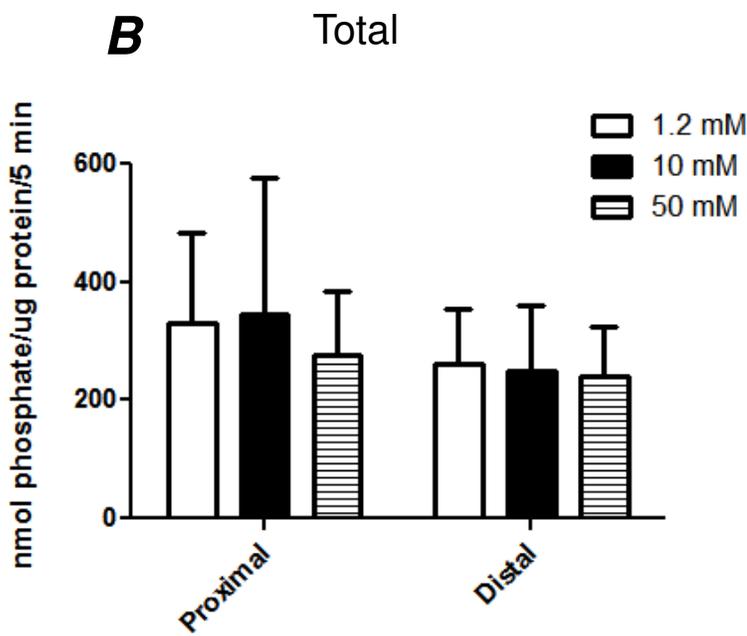
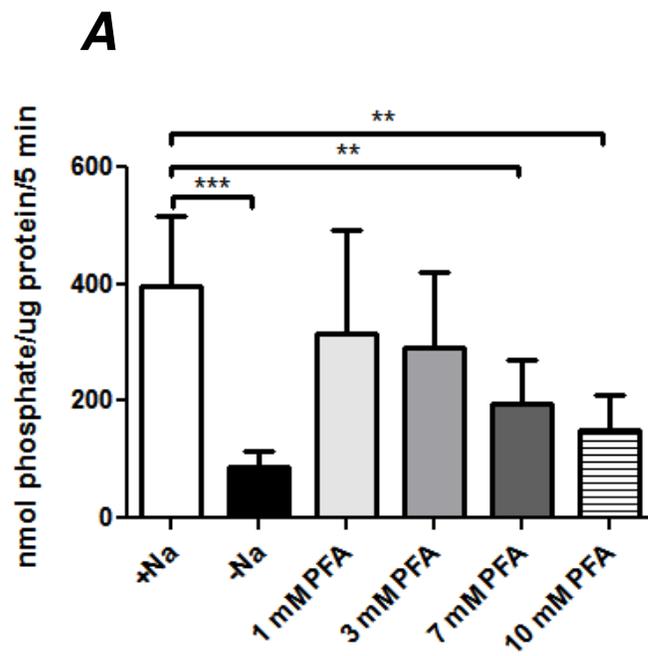


Figure 5



GFR (ml/min)

MAP (mmHg)

	Group (n)	Ctrl	15 min	30 min	45 min	Ctrl	15 min	30 min	45 min
<b>Normal diet</b>	Control (4)	2.52 ± 0.55	3.38 ± 0.96	3.92 ± 0.85	3.57 ± 1.22	109.25 ± 2.65	108.03 ± 2.92	104.49 ± 3.73	100.78 ± 3.10
	10mM phosphate (5)	3.51 ± 1.15	3.44 ± 0.63	3.08 ± 1.29	3.84 ± 0.52	112.22 ± 5.22	109.63 ± 2.60	107.07 ± 2.46	101.48 ± 1.09
	High control (5)	3.34 ± 1.38	3.75 ± 1.81	3.63 ± 1.34	4.18 ± 1.30	114.48 ± 5.90	108.70 ± 5.48	104.77 ± 5.06	101.04 ± 4.81
	1.3M phosphate (7)	2.80 ± 0.31	3.23 ± 0.33	2.79 ± 0.55	3.09 ± 1.03	108.20 ± 4.04	104.92 ± 9.22	101.84 ± 4.87	100.12 ± 6.54
<b>Low phosphate diet</b>	Control (5)	3.55 ± 1.17	3.57 ± 1.32	4.10 ± 0.36	3.53 ± 0.93	102.38 ± 8.32	103.66 ± 8.70	99.55 ± 7.06	98.28 ± 5.96
	10mM phosphate (5)	2.77 ± 1.19	4.64 ± 2.62	3.41 ± 0.84	4.06 ± 1.21	104.55 ± 3.11	102.68 ± 4.64	99.47 ± 4.30	97.34 ± 2.29
	High control (5)	3.53 ± 1.25	4.13 ± 2.00	3.61 ± 1.85	4.56 ± 1.35	101.86 ± 5.56	103.19 ± 3.43	102.67 ± 2.31	100.84 ± 1.39
	1.3M phosphate (5)	3.00 ± 1.47	3.64 ± 1.47	3.07 ± 0.44	4.71 ± 1.28	106.42 ± 11.4	102.76 ± 10.4	97.00 ± 9.79	96.71 ± 9.38