Current Topics in Steroid Research
Vol. 9, 2012

Original Communication

Correlation between calcium uptake and β-glucuronidase release in isolated intestinal epithelial cells: Dose-response studies

Jordan Hunger, Kaitlyn Biehl, Jacquiline Stuart and Ilka Nemere*

Department of Nutrition, Dietetics and Food Sciences, Utah State University, Logan, UT 84322-8700, USA

ABSTRACT

We have previously reported that TRPV6, and its activating enzyme β-glucuronidase mediate $1,25(OH)_2D_3$-stimulated calcium uptake in isolated chick intestinal epithelial cells. In the current study, we used dose-response analyses for three mediators of calcium uptake, $1,25(OH)_2D_3$, parathyroid hormone (PTH), and forskolin, to compare calcium uptake and enzyme release. For $1,25(OH)_2D_3$, 130 pM-, 300 pM-, 650 pM- and 1200 pM steroid elicited, no increase in calcium uptake, 140% increase, 180% increase and again no increase, respectively, relative to corresponding controls. The same pattern was observed with β-glucuronidase release with 300 pM- or 650 pM $1,25(OH)_2D_3$ eliciting a 170%, and 260% increase relative to corresponding controls, respectively. The peptide bPTH(1-34) was tested at 26 pM-, 65 pM- or 325 pM hormone which also exhibited a biphasic dose-response curve with 65 pM bPTH (1-34) increasing calcium uptake to 180% of controls and β-glucuronidase release to 155% of corresponding controls. Finally, 10 μM forskolin elicited no increase in calcium uptake, while 20 μM and 40 μM agonist increased calcium uptake to 150% of corresponding controls. Similarly, no increase in enzyme release was noted at 10 μM, while 20 μM and 40 μM forskolin resulted in 130% and 125% increase in β-glucuronidase release, respectively, relative to corresponding controls. As a negative control, 100 nM phorbol ester neither increased calcium uptake nor increased β-glucuronidase release, relative to corresponding controls. We conclude that each of the active agonists have closely parallel dose response curves for enhanced β-glucuronidase release, and stimulated calcium uptake.

KEYWORDS: 1,25-dihydroxyvitamin D, calcium uptake, intestine, PTH, forskolin, TRPV 6

*Corresponding author:
ilka.nemere@usu.edu

INTRODUCTION

In 2005, Chang *et al.* [1] reported that the major calcium transporter in kidney cells is TRPV 5, and that this channel is activated by the enzyme β-glucuronidase. They further reported that the predominant form of calcium channel in intestinal cells is TRPV 6. In earlier work we tested the role of TRPV 6 and β-glucuronidase in calcium uptake in isolated chick intestinal cells [2]. Using siRNA we demonstrated that both entities are required for $1,25(OH)_2D_3$-stimulated calcium uptake. In addition, we demonstrated that steroid stimulated calcium uptake, acting through the $1,25D_3$-MARRS receptor [3] is mediated by the protein kinase (PK) A pathway, but not by the PKC pathway (Khanal *et al.* 2008). The peptide parathyroid hormone has also been demonstrated to increase calcium uptake in both rat intestinal cells [4] and the perfused chick duodenal loop [5]. In the current study we undertake dose-response analyses of $1,25(OH)_2D_3$, bovine PTH fragment 1-34 [bPTH(1-34)], and forskolin mediated calcium uptake and β-glucuronidase release.

MATERIALS AND METHODS

Animals and surgical procedures

Chicks were obtained on the day of hatch (Privett Hatchery, Portales, NM) and raised for 3-7 weeks on a vitamin D-replete diet (Nutrena Feeds, Murray UT). On the day of use, chicks were anesthetized with chloropent (0.3 ml/100 g body weight), and the duodenum surgically removed to ice cold saline. After chilling for 15 min, the pancreas was removed, the duodenal loop everted, and rinsed in fresh ice-cold saline. The intestinal segment was then transferred to citrate chelation medium (96 mM NaCl, 27 mM citric acid, 1.5 mM KCl, 5.6 mM Na_2HPO_4, 8 mM KH_2PO_4, pH 5.0). The acidic pH allows retention of viability and morphology in chick intestinal cells [6]. The intestines were stirred for 15 min at room temperature to dissociate epithelial cells, and then the segments transferred to fresh chelation medium. The released cells were poured into 50 ml conical centrifuge tubes and held on ice. Two additional 15 min periods of cell isolation were conducted, the cells pooled and collected by centrifugation at 500 x g, 5 min (4 C). The cell pellets were resuspended in 40 ml of Gey's balanced salt solution (GBSS; containing 119 mM NaCl, 4.96 mM KCl, 0.22 mM KH_2PO_4, 0.84 mM $NaHPO_4$, 1.03 mM $MgCl_2 \bullet 6H_2O$, 0.28 mM $MgSO_4 \bullet 7H_2O$, 0.9 mM $CaCl_2$, pH 7.3).

Calcium uptake studies

For calcium uptake studies with bPTH (1-34), forskolin, or or phorbol myristate acetate (PMA; all from Sigma Chemical Co., St. Louis, MO) aliquots of cell suspension (2.2 ml) were added to 50-ml conical centrifuge tubes containing 1 µCi/ml $^{45}CaCl_2$ (PerkinElmer, Boston MA); this represented the beginning of the incubation period at T = -10 min. Samples (100 µl) were removed at T = -5 and -1 min to establish basal uptake, and test substances or vehicle controls added at T = 0 min. Additional samples were removed at 1, 3, 5, 7, and 10 min during the treated phase. All aliquots were pipetted into 1000 µl ice-cold GBSS to stop uptake and dilute radionuclide, then centrifuged at 1000 x g, 5 min (4°C). After decanting the supernatants, the inside of the tubes-while still inverted-were swabbed with a tissue to remove residual supernatant. Cell pellets were resuspended in 500 µl reagent grade water and analyzed for radioactivity and protein using the Bradford reagent (BioRad, Hercules, CA) against bovine γ-globulin (Sigma) as standard. Data were calculated as cpm/µg protein, and then the specific activity during the treated phase normalized to average basal uptake.

We have previously reported that $1,25(OH)_2D_3$-stimulated calcium uptake is complicated by stimulated extrusion [2]. We subsequently found that culturing cells for 72 h in RPMI 1640 supplemented with 10% fetal bovine serum resulted in a loss of protein kinase (PK) C activity that mediates extrusion [2]. For the present studies we used these culture techniques, collected the cells by centrifugation, and proceeded as described above.

β-glucuronidase release studies

For β-glucuronidase release studies, 12 ml cell suspensions were used, and 1 ml aliquots were removed at selected times before and after addition of test substances, pipetted into tubes on ice, and cells collected by centrifugation. Supernatant fractions were analyzed using 0.5 mM phenolphthalein-β-D-glucuronide (Sigma) as substrate in 0.125 mM Na acetate buffer, pH 4.5. After a 30 min incubation period at 37°C the reaction was stopped by addition of 0.8 mM glycine-NaOH, pH 10.0, and read against phenolphthalein standards.

RESULTS

Effects of $1,25(OH)_2D_3$ on calcium uptake and β-glucuronidase release

Isolated intestinal cells were treated with a range of $1,25(OH)_2D_3$ concentrations and assessed for calcium uptake. Fig. 1 presents the results of these experiments. Treatment of enterocytes with 130 pM $1,25(OH)_2D_3$ resulted in a slight increase in calcium uptake 3 min after addition of steroid, however this was not statistically significant (Fig. 1A). By comparison, addition of either 300- or 650 pM $1,25(OH)_2D_3$ stimulated calcium uptake 130-140% and 175-180% of corresponding controls, respectively (Figs. 1B, 1C). The stimulatory effect of the steroid hormone was lost in cells treated with the supraphysiological dose of 1238 pM $1,25(OH)_2D_3$.

An equivalent series of concentrations were used to treat freshly isolated intestinal cells, and the release of β-glucuronidase enzyme activity assessed. The results shown in Fig. 2 indicate parallel

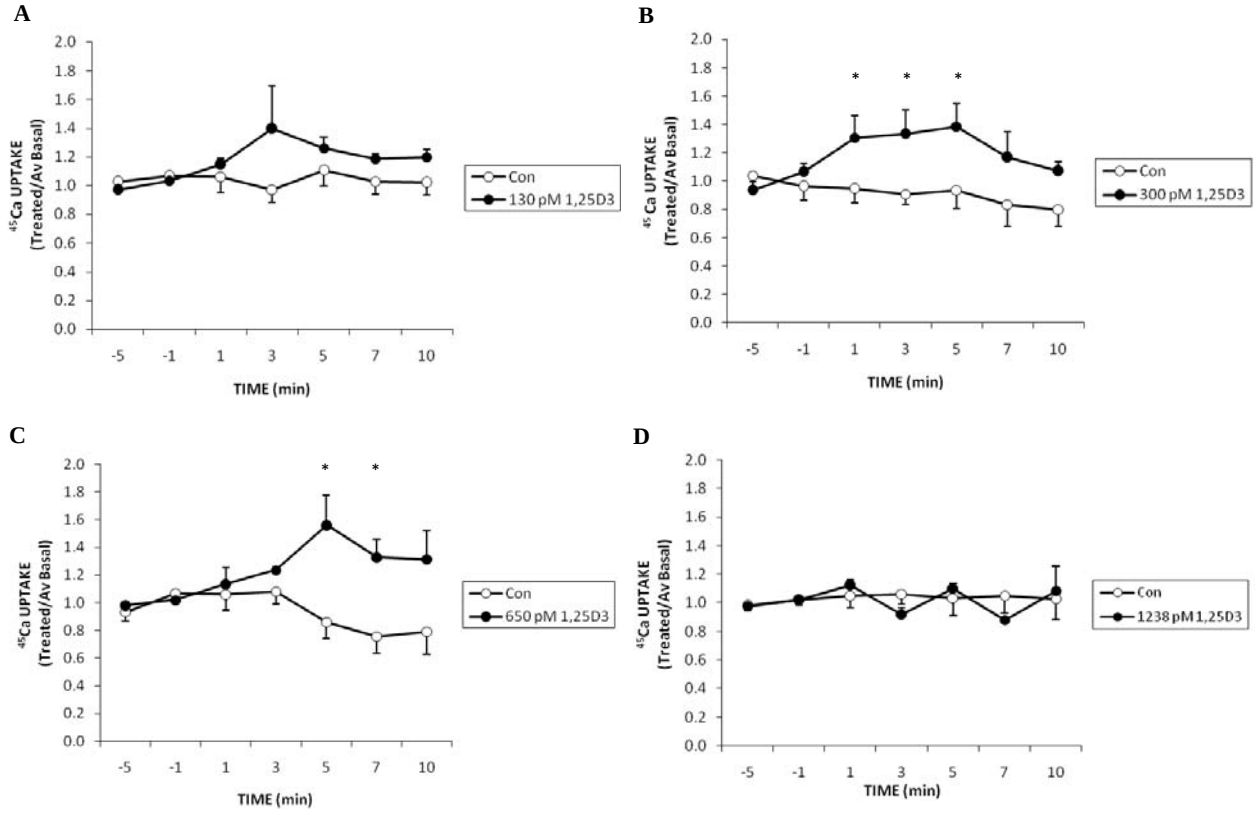

Fig. 1. Dose response analyses of $1,25(OH)_2D_3$-mediated calcium uptake in isolated chick intestinal cells. Cells were isolated by citrate chelation and resuspended in Gey's balanced salt solution (GBSS), then cultured in RPMI 1640 for 3 D. Cells were collected by centrifugation and again resuspended in GBSS for calcium uptake studies. A 2.2-ml cell suspension was added to tubes containing 2.2 μCi $CaCl_2$ at T = -10 min. Aliquots were removed at T = -5 and -1 min to establish basal uptake rates. At T = 0 min, vehicle or the indicated concentrations of $1,25(OH)_2D_3$ were added, and additional aliquots removed at 1, 3, 5, 7, and 10 min. All alilquots were pipetted into 1 ml of ice cold GBSS to stop uptake and dilute radionuclide. Cells were collected by centrifugation, the supernatants decanted, and the inside of the tubes swabbed while still inverted. Pellets were resuspended in reagent grade water and analyzed for protein and radioactivity. Values of cpm/mg protein during the treated phase were normalized to average basal uptake. *indicates significant differences ($P < 0.05$), relative to corresponding controls.

responses: treatment of enterocytes with 130 pM $1,25(OH)_2D_3$ failed to stimulate release of enzyme activity (Fig. 2A); 300- or 650 pM $1,25(OH)_2D_3$ enhanced release as early as 1 min after hormone, reaching 170% and 265% of corresponding controls at 3 min, respectively (Figs. 2B, 2C). At the highest level tested, $1,25(OH)_2D_3$ treatment did not result in enhanced β-glucuronidase release (Fig. 2D). A pilot study with 3 D cultured cells indicated essentially similar results (data not shown).

Effects of PTH on calcium uptake and β-glucuronidase release

In another series of experiments, isolated intestinal cells were treated with 26-, 65-, or 325 pM bPTH (1-34) and analyzed for calcium uptake (Fig. 3) or β-glucuronidase release (Fig. 4). The lowest concentration of peptide hormone failed to stimulate calcium uptake relative to controls (Fig. 3A), while 65 pM bPTH(1-34) stimulated a significant increase within 5 min of addition, reaching 185% of corresponding control levels after 10 min (Fig. 3B). Cells treated with 325 pM bPTH(1-34) failed to respond to hormone with enhanced calcium uptake (Fig. 3C).

Fig. 4 depicts the results of experiments in which β-glucuronidase release was determined in response to equivalent concentrations of bPTH(1-34). Addition of 26 pM hormone to cell suspensions failed to enhance the release of the enzyme activity

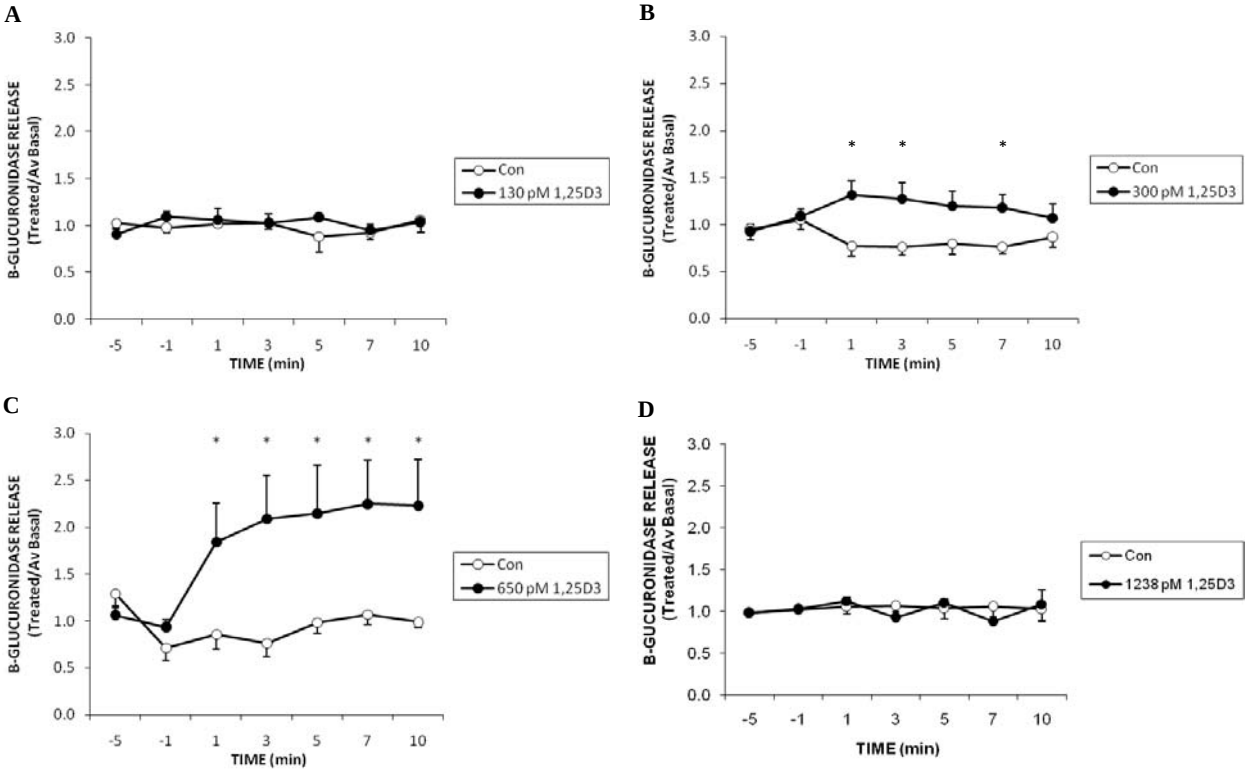

Fig. 2. Dose response analyses of 1,25$(OH)_2D_3$-mediated β-glucuronidase release from isolated chick intestinal cells. Cells were isolated and resuspended in GBSS as described in the legend to Fig. 1, but 12-ml suspensions were used immediately thereafter. Aliquots of 1-ml were pipetted into microfuge tubes on ice at the indicated times, with additions made at T = 0. Cells were centrifuged, supernatants decanted and used to assess release of enzyme activity against phenolphthalein standards. Pellets were resuspended as described and analyzed for protein. *indicates significant differences ($P < 0.05$), relative to corresponding controls.

(Fig. 4A), while 65 pM bPTH(1-34) stimulated β- glucuronidase within 1 min of addition, reaching 155% of corresponding controls at 7 min of incubation with hormone (Fig. 4B). The highest concentration of peptide appeared to give a slight increase at 7 and 10 min (Fig. 4C), although this was not statistically significant.

Effects of forskolin on calcium uptake and β-glucuronidase release

We tested three concentrations of the adenylate cyclase activator, forskolin on suspensions of isolated intestinal epithelial cells. As shown in Fig. 5, 10 µM forskolin did not alter calcium uptake relative to controls (Fig. 5A), while 20 µM and 40 µM stimulated calcium uptake to a similar extent, reaching 150-155% of corresponding controls (Figs. 5B, 5C, respectively). Fig. 6 illustrates a similar pattern for β-glucuronidase release: No increase in released enzyme activity in cells treated with 10 µM forskolin, relative to corresponding controls (Fig. 6A), and an increase in β-glucuronidase activity released to a similar extent for 20- and 40 µM forskolin (130% and 125% relative to corresponding controls, respectively, Figs 6B, 6C).

Lack of effect of PMA on calcium uptake and β-glucuronidase release

To confirm our hypothesis that agonists promoting calcium uptake act through β-glucuronidase release, we isolated intestinal epithelial cells and treated them with vehicle or 100 nM PMA, a compound known to activate PKC. As shown in Fig. 7, we confirmed that this concentration of phorbol ester does not stimulate calcium uptake (Fig. 7A), and likewise we report that it does not stimulate release of β-glucuronidase activity (Fig. 7B).

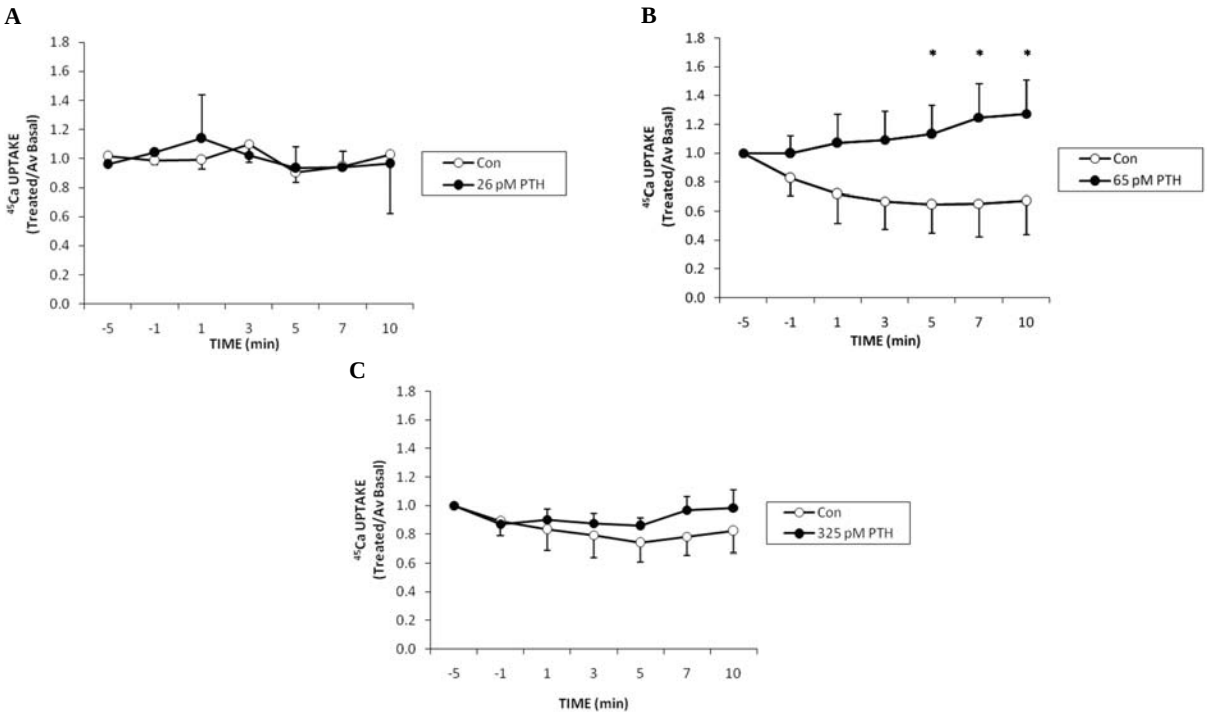

Fig. 3. Dose response analyses of bPTH(1-34)-mediated calcium uptake in isolated chick intestinal cells. Methods were as described in the legend to Fig. 1, but without the 3 D incubation. *indicates significant differences ($P < 0.05$), relative to corresponding controls.

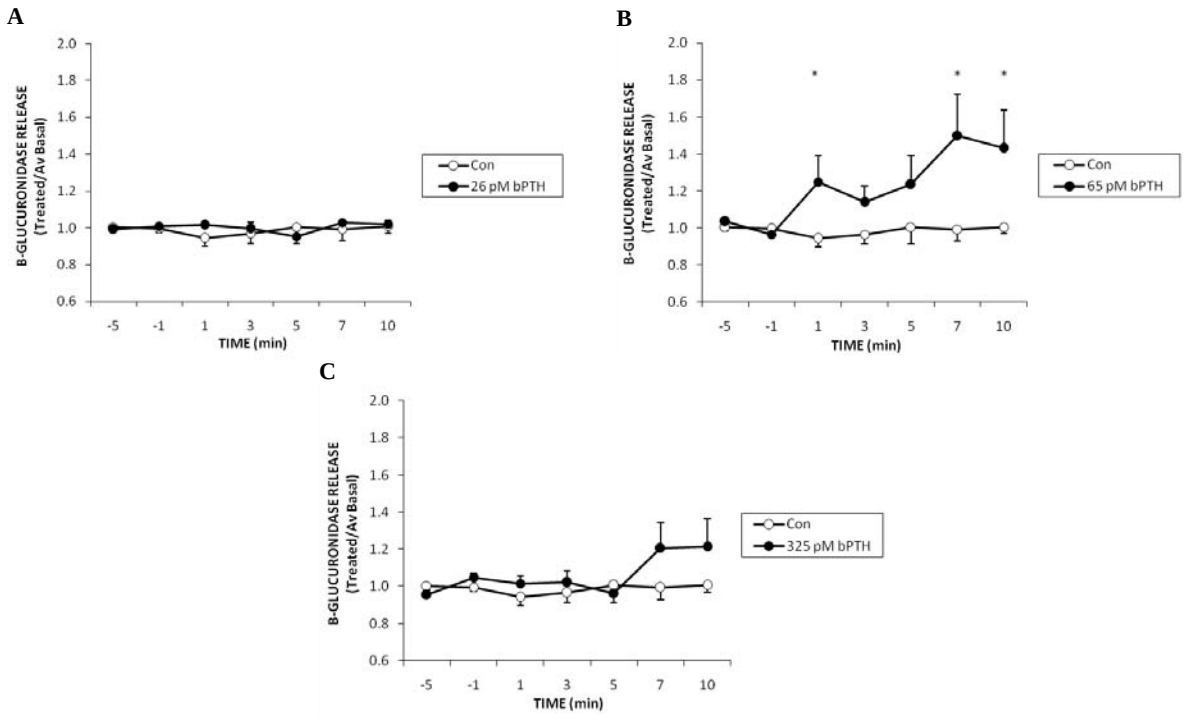

Fig. 4. Dose response analyses of bPTH(1-34)--mediated β-glucuronidase release from isolated chick intestinal cells. Methods were as described in the legend to Fig. 2. *indicates significant differences ($P < 0.05$), relative to corresponding controls.

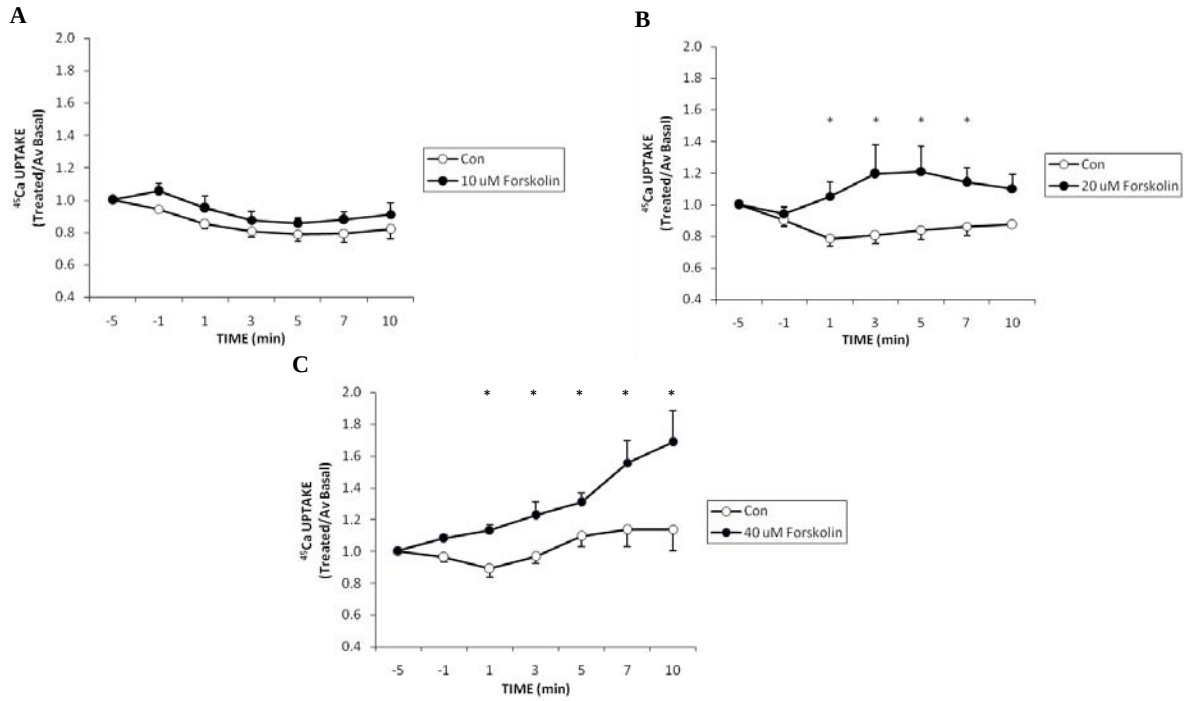

Fig. 5. Dose response analyses of forskolin-mediated calcium uptake in isolated chick intestinal cells. Procedures were as described in the legend to Fig. 1, but without the 3 D incubation in RPMI 1640. *indicates significant differences ($P < 0.05$), relative to corresponding controls.

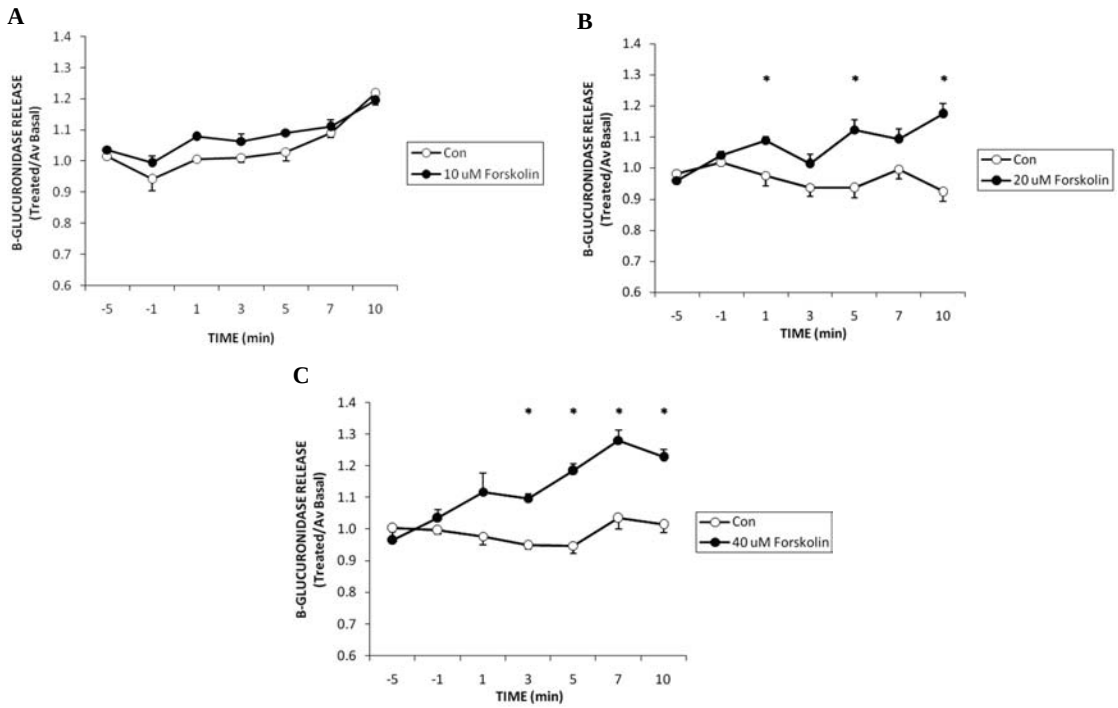

Fig. 6. Dose response analyses of forskolin-mediated β-glucuronidase release from isolated chick intestinal cells. Procedures were as described in the legend to Fig. 2. *indicates significant differences ($P < 0.05$), relative to corresponding controls.

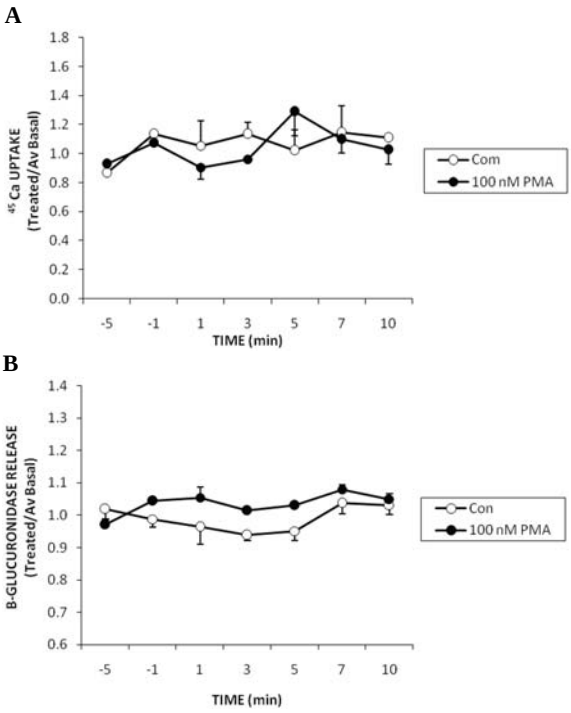

Fig. 7. Lack of effect of phorbol ester in calcium uptake or β-glucuronidase release from isolated chick intestinal cells. Procedures were as described in the legend to Fig. 2.

DISCUSSION

In the current work, we demonstrate a parallel dose-response relationship with agonists known to increase calcium uptake in isolated intestinal epithelial cells and β-glucuronidase release. We have previously reported that both $1,25(OH)_2D_3$ and PTH stimulate calcium transport in perfused duodenal loops from chick [5]. In the case of the steroid hormone, 130 pM $1,25(OH)_2D_3$ was an effective concentration in perfused duodenal loops [5], while in the isolated cell system it was not (Fig. 1A), suggesting that the targeted delivery of hormone through the vasculature is more efficient. However, both systems provided agreement that 650 pM $1,25(OH)_2D_3$ is the optimal concentration for stimulating calcium transport [7] or uptake (Fig. 1B). As previously also reported, both the seco-steroid hormone and PTH yield biphasic dose response curves in which concentrations higher than optimal result in inhibition of calcium transport [5, 8].

Forskolin, an activator of adenylate cyclase, also demonstrated a close correlation in activating calcium uptake through β-glucuronidase release. We have previously found that in chick [2] and mouse [9] intestinal cells that PKA mediates calcium uptake, and can be inhibited by rpcAMP [2]. In chick, we have further determined that the PKC β isotype is involved in calcium extrusion [10], while PKCα mediates phosphate uptake [11].

The importance of TRPV6 in regulating intestinal calcium absorption has recently been corroborated by Cui *et al.* [12]. This group used transgenic mice expressing human TRPV6 to demonstrate an increase in intestinal calcium absorption, serum calcium, and bone mineral density relative to wild type controls [12] calbindin-D_{9K} was also upregulated in duodenum and jejunum of transgenic mice relative to wild type controls [12], suggesting that the protein may be more influenced by calcium than $1,25(OH)_2D_3$ levels, although plasma concentrations of the steroid hormone were not measured.

Others [13] have reported that TRPV6 knockout mice are still able to transport calcium across the intestine, indicating that other vitamin D-regulated pathways have yet to be discovered.

CONCLUSION

The close parallels between β-glucuronidase release and calcium uptake for three agonists indicates TRPV 6 is the likely calcium transporter in intestinal cells.

ACKNOWLEDGEMENTS

This work was supported by the Utah Agricultural Experiment Station, Utah State University. Approved as journal paper number 8505.

REFERENCES

1. Chang, Q., Hoefs S., van der Kemp, A. W., Topala, C. N., Bindels, R. J. and Hoenderop, J. G. 2005, Science, 310, 490.
2. Khanal, R., Sterling, T. M., Smith, N. M. and Nemere, I. 2008, J. Cell. Biochem., 105, 1109.
3. Khanal R. and Nemere, I 2007, Current Medicinal Chemistry, 14, 1087.
4. Nemere, I. and Szego, C. M. 1981, Endocrinology, 108, 1450.

5. Nemere I. and Norman A. W. 1986, Endocrinology, 119, 1406.
6. Sterling, T. M. and Nemere, I. 2007, Steroids, 72, 151.
7. Nemere, I., Yoshimoto, Y. and Norman A. W. 1984, Endocrinology, 115, 1476.
8. Yoshimoto, Y., Nemere, I. and Norman A. W. 1986, Endocrinology, 118, 2300.
9. Nemere, I., Garbi, N., Hammerling, G. J. and Khanal, R. C. 2010, J. Biol. Chem., 285, 31859.
10. Tunsophon, S. and Nemere, I. 2011, Anat Physiol. Si:002 doi 10.4172/2161-0940.S1-002
11. Tunsophon, S. and Nemere, I. 2010, Steroids, 75, 307.
12. Cui, M., Li, Q., Johnson, R. and Fleet, J. C. 2012, J. Bone Mineral Res., 27, 2097.
13. Christakos, S., Dhawan, P., Ajibade, D. and Feng, J. J. 2012, J. Steroid Biochem. Mol. Biol., 121, 183.