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Direct, Rapid Effects of 25-Hydroxyvitamin D_3 on Isolated Intestinal Cells

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Abstract Scattered reports in the literature have suggested that the metabolite 25-hydroxyvitamin D_3 [25(OH)D_3] has biological activity. In the present work, perfusion of isolated duodenal loops of normal chickens with 100 nM 25(OH)D_3 resulted in enhanced transport of ^{45}Ca within 2 min relative to the vehicle controls. We then tested the effect of a range of 25(OH)D_3 concentrations on ^{45}Ca handling by isolated intestinal cells in time course studies. Following a basal uptake period, cell suspensions from 7-week old chicks were treated either with 25, 100, or 300 nM 25(OH)D_3, or the vehicle ethanol (0.01%, final concentration). Both 25 and 100 nM 25(OH)D_3 resulted in a significant ($P < 0.05$) reduction in ^{45}Ca levels, relative to controls, between 1–10 min after treatment, while 300 nM 25(OH)D_3 resulted in a significant increase in ^{45}Ca levels, relative to controls, after 10 min of incubation. The effect of 100 nM 25(OH)D_3 (a physiological level) on cell calcium was abolished by the presence of 6.5 nM 24,25-dihydroxyvitamin D_3. In cell preparations from 14- or 28-week old birds 100 nM 25(OH)D_3 had no effect, relative to vehicle controls. Incubation of cells with 2 μM BAY K8644, a calcium channel activator, stimulated ^{45}Ca uptake within 3 min relative to vehicle controls ($P < 0.05$), while addition of either 20 μM forskolin or 100 nM phorbol ester (stimulators of the PKA and PKC pathways, respectively) resulted in enhanced radionuclide levels after 10 min of incubation ($P < 0.05$, relative to corresponding controls). Finally, cells were treated with 100 nM 25(OH)D_3 or vehicle and samples taken at various times for analyses of protein kinase C and A activities. No effect of 25(OH)D_3 on protein kinase C activity was observed, while protein kinase A activity was stimulated to nearly 200% of controls at 1 min after 25(OH)D_3 addition ($P < 0.05$, relative to corresponding controls) and began declining at 3 min, returning to control levels 5 min after additions. We conclude that 25(OH)D_3 has a direct effect on calcium handling in enterocytes of young animals that may in part be mediated by the protein kinase A signal transduction pathway. J. Cell. Biochem. 90: 287–293, 2003.

Key words: calcium uptake; rapid actions; chick intestine; signal transduction; 1,25-dihydroxyvitamin D_3

Vitamin D_3 is activated through a series of hydroxylations. The first hydroxylation yields [25(OH)D_3], which can then be further metabolized to 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2D_3$]—a compound that is accepted as a hormonally active steroid—or 24,25-dihydroxyvitamin D_3 [24,25(OH)$_2D_3$], which may also have hormonal activity [for review, see Farach-Carson and Nemere, 2003; Tryfonidou et al., 2002]. While 25(OH)D_3 has mainly been regarded as a precursor for the dihydroxylated metabolites, a number of reports have indicated that it has biological activity in perfusion systems [Olson and DeLuca, 1969; Nemere et al., 1984; Yoshimoto and Norman, 1986; Sundell and Bjornsson, 1990; Nemere, 1996], potential clinical signficance [Heaney et al., 1997], and induces calcium ion fluxes in fish enterocytes [Larsson et al., 2002]. Moreover, two independent laboratories have reported the existence of specific binding proteins for 25(OH)D_3 [Gacad et al., 1997; Teegarden et al., 1997, 2000] which may mediate transcriptional effects [Wu et al., 2000].

In an earlier report [Zhao and Nemere, 2002], we found excellent agreement between 1,25(OH)$_2D_3$-stimulated phosphate transport in perfused duodena and ^{32}P uptake in isolated intestinal epithelial cells. We therefore undertook the present study to compare the effect of

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$25(OH)D_3$ on ^{45}Ca transport in perfused duodena with the direct effect (if any) on calcium handling by isolated chick enterocytes. Since a number of signal transduction pathways have been implicated in the action of $1,25(OH)_2D_3$, we also undertook a preliminary investigation into this area with regards to $25(OH)D_3$.

MATERIALS AND METHODS

Animals

White Leghorn cockerels were obtained on the day of hatch from Merrill Poultry (Poul, ID), and raised on a vitamin D-replete diet (Nutrena Feeds, Murray, UT) until use. Unless otherwise specified, chicks were used at 4–7 week of age. On the day of experimentation, animals were anesthetized with chloropent (0.3 ml/100 g body weight). All protocols were approved by the Utah State University Institutional Animal Care and Use Committee.

Perfusion Studies

Duodena were vascularly perfused through the celiac artery, as previously described [Nemere, 1996], with either 100 nM $25(OH)D_3$ ($n = 3$) or control medium (0.005% ethanol, final concentration; $n = 3$). The luminal perfusate contained 1 μCi/ml of $^{45}CaCl_2$ (New England Nuclear Life Sciences, Boston, MA). The effluent from the celiac vein was collected and analyzed for transported radionuclide during the latter 10 min of the basal equilibration phase and during the 40 min treated phase.

Cell Isolation

Duodena (two per experiment) were surgically removed to ice-cold saline and chilled for 15 min prior to removal of the pancreas. The loops were everted, rinsed in saline, and placed in citrate chelation medium [Zhao and Nemere, 2002] for three, 15-min incubation periods at 23°C with stirring. The released cells were collected by centrifugation for 5 min at 500*g* (4 C), and resuspended in 20 ml of Gey's balanced salt solution containing 0.1% bovine serum albumin (GBSS-BSA), pH 7.35 [Zhao and Nemere, 2002]. Five millilitres of the cell suspension were then mixed with 5 μCi of $^{45}CaCl_2$ and then 2-ml aliquots removed to separate 12-ml polyethylene tubes (Fisher Scientific, Houston, TX). Duplicate 100 μl samples were removed to 900 μl of ice cold GBSS at $T = -5$ and -1 min prior to addition of test substances. Duplicate 100 μl samples were then removed to ice cold GBSS at $T = 1$, 3, 5, 7, and 10 min. The samples were centrifuged, the supernatants decanted, and while still in the inverted position, the insides of the tubes swabbed with a Kimwipe. The cell pellets were disrupted in 1 ml of ice cold, double-distilled, deionized water prior to determination of radioactivity by liquid scintillation spectrophotometry and protein with the Bradford reagent (Bio-Rad, Hercules, CA).

Protein Kinase Assays

Cells were prepared without radioactivity as described above and the cell pellets stored at −20°C until used. All procedures were according to the kit manufacturer's (InVitrogen-Gibco, Carlsbad, CA) instructions and described in detail elsewhere [Zhao and Nemere, 2002; Larsson and Nemere, 2003].

Statistical Analyses

For ^{45}Ca uptake studies, cpm were related to mg of protein in the corresponding sample prior to normalization to the average basal value determined from $T = -5$ and -1 min. Specific activities for protein kinases A and C were deter-

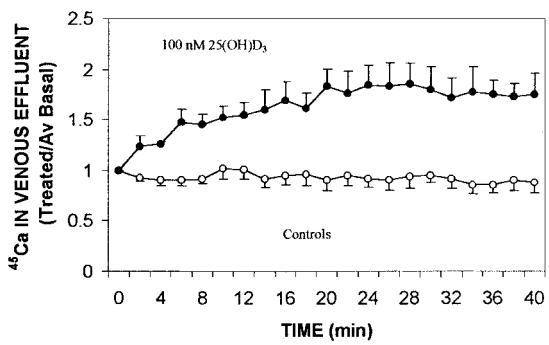

Fig. 1. Vascular perfusion of isolated duodenal loops with 100 nM $25(OH)D_3$ stimulates calcium transport. Duodenal loops were cannulated through the celiac artery and perfused with Gey's balanced salt solution (GBSS), aerated with 95% O_2/5% CO_2. Luminal perfusion was performed with GBSS lacking bicarbonate and containing 1 μCi/ml of $^{45}CaCl_2$. Basal perfusion occurred for 20 min and samples collected every 2 min during the latter 10 min. Duodena were then vascularly perfused with control medium (0.005% ethanol, final volume) or medium containing 100 nM $25(OH)D_3$ for an additional 40 min. Samples of venous effluent were collected at 2 min intervals for determination of radioactivity and cpm during the treated phase normalized to average basal cpm. Values in this and subsequent figures represent mean ± SEM. Data from controls are depicted as open circles ($n = 3$) and data from steroid treated preparations as closed circles ($n = 3$). A statistically significant ($P < 0.05$) increase in transport for duodena vascularly perfused with $25(OH)D_3$ was observed for $T = 2$–40 min relative to corresponding controls.

mined as differences between extract incubations with activators versus inhibitors, as detailed in the kit instructions. Statistical significance was determined by Student's *t*-test for paired observations.

RESULTS

Perfusion Studies

Figure 1 demonstrates the results of studies in which duodena (3 per group) were vascularly perfused with either 100 nM 25(OH)D_3 or vehicle (0.005% ethanol, final concentration) and the effect on calcium transport determined. Radioactivity in the venous effluent of controls maintained a steady level over the 40 min treated phase while duodena, vascularly perfused with the vitamin D metabolite, exhibited enhanced transport of ^{45}Ca as early as 2 min ($P < 0.05$), increasing to 200% of controls at T = 40 min ($P < 0.05$ for all time points during the treated phase).

Dose-Response Studies With Isolated Cells

Experiments were then conducted with isolated cells and a range of 25(OH)D_3 concentrations

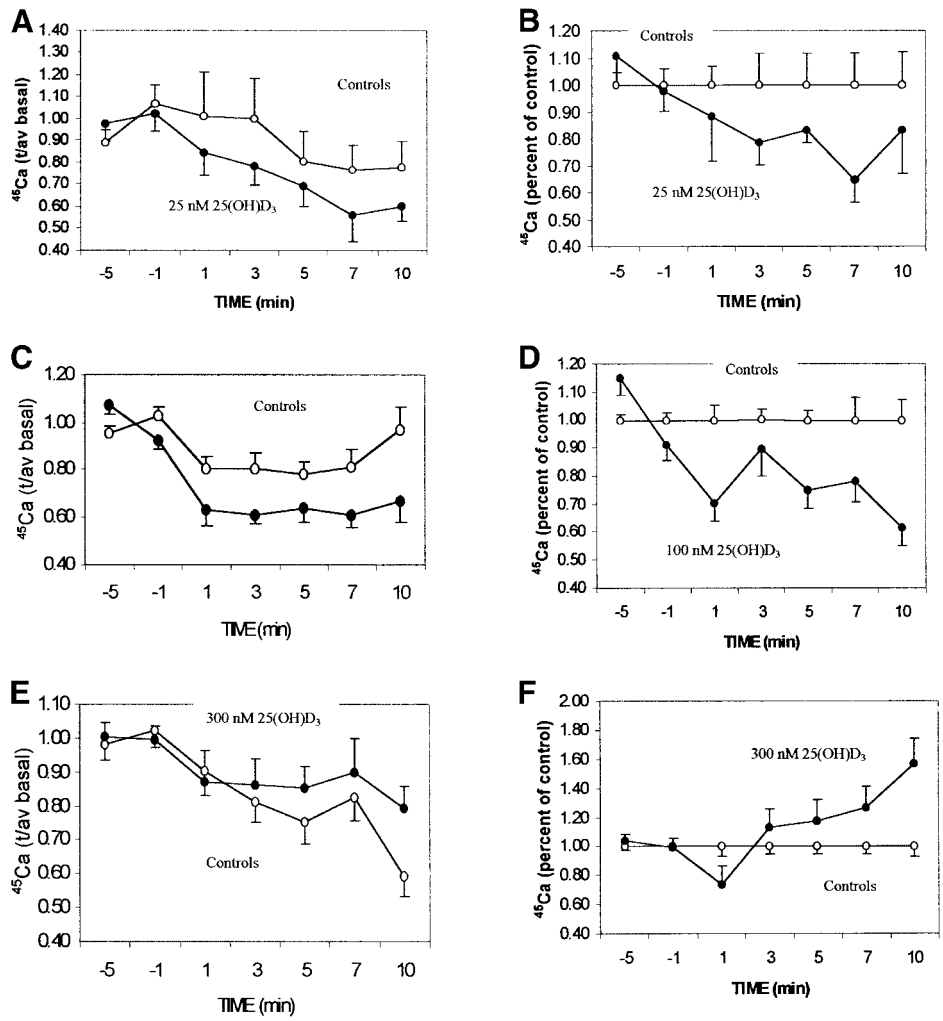

Fig. 2. Effect of a range of 25(OH)D_3 concentrations on isolated intestinal epithelial cells. Cells from two duodena per experiment were isolated by citrate chelation, collected by centrifugation, and resuspended in GBSS containing 0.1% BSA. Five millilitres of cell suspension were combined with 5 μCi of $^{45}CaCl_2$ and then 2-ml aliquots removed to separate 12 ml polyethylene test tubes. After removal of duplicate 100 μl basal period samples, test substances were added at T = 0 and sampling continued. All samples were pipetted into 900 μl of ice cold GBSS to dilute isotope and inhibit the uptake process. After collecting the cells by centrifugation, the pellets were disrupted in 1 ml of double-distilled, deionized water, and protein and radioactivity determined. **A**, **B**: Twenty-five nanomoles of 25(OH)D_3 or 0.01% ethanol, final concentration (n = 4) independent experiments, $P < 0.05$ at T = 7 min; (**C**, **D**) 100 nM 25(OH)D_3 or vehicle controls (n = 10), $P < 0.05$ at T = 1, 5, and 10 min; (**E**, **F**) 300 nM 25(OH)D_3 or vehicle (n = 10), $P < 0.05$ at T = 10 min.

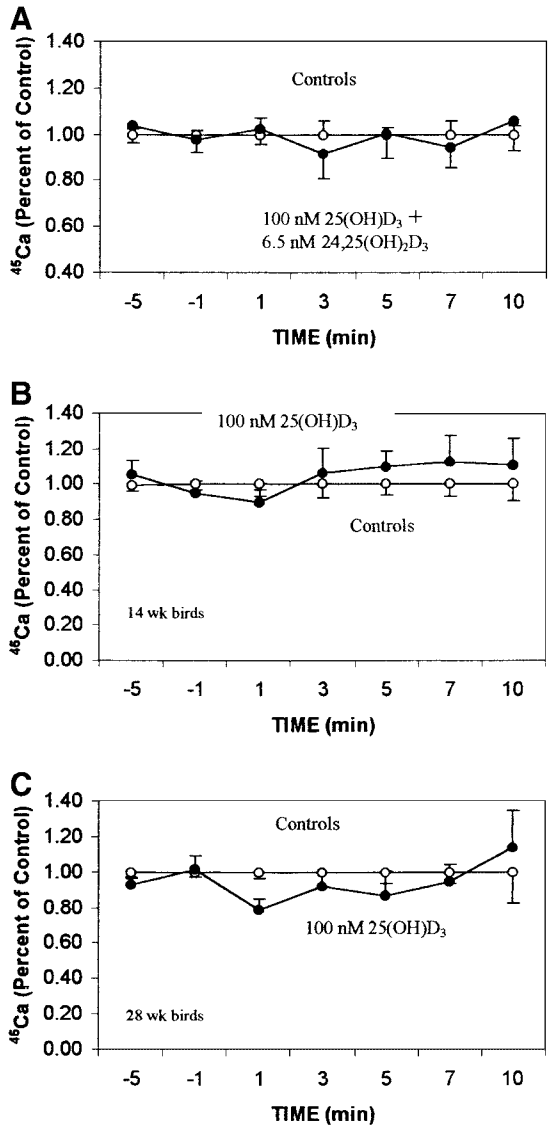

Fig. 3. Abolition of the effect of 100 nM $25(OH)D_3$ on calcium uptake by $24,25(OH)_2D_3$ and age. Cells were isolated and processed as described in the legend to Figure 2. **A**: 6.5 nM $24,25(OH)_2D_3$ plus 100 nM $25(OH)D_3$ vs vehicle controls in cells from 7-week old birds (n = 4); (**B**) 100 nM $25(OH)D_3$ vs vehicle controls in cells from 14-week old birds (n = 5); (**C**) 100 nM $25(OH)D_3$ vs vehicle controls in cells from 28-week old birds (n = 4).

in order to determine whether the metabolite had a direct effect on enterocytes. The results of these studies are depicted in Figure 2. Figure 2A, C,E illustrate the results from cells treated with 25, 100, or 300 nM $25(OH)D_3$ or the vehicle ethanol (0.1%, final concentration) expressed as ^{45}Ca treated/average basal cpm/mg protein. Regardless of treatment condition, radionuclide levels were found to remain steady for the basal phase and gradually decline over the following 10 min. This behavior has been reported previously [Nemere and Campbell, 2000; Zhao and Nemere, 2002] and has been found to occur even in cells cultured for 24 h [Sterling and Nemere, unpublished observations]. For a simpler comparison of treatment effects, the results are also displayed as percent of control. Figure 2B illustrates that treatment of isolated intestinal cells with 25 nM $25(OH)D_3$ resulted in lower ^{45}Ca levels, relative to controls, with a significant ($P < 0.05$) decline to 70% of controls at 7 min. At 100 nM $25(OH)D_3$, cells had significantly less radioactivity at 1, 5, and 10 min after metabolite ($P < 0.05$, relative to corresponding controls), reaching 60% of control levels at the end of the incubation period (Fig. 2D). Surprisingly, 300 nM metabolite (Fig. 2F) promoted an increase in ^{45}Ca levels that was noticeable at T = 3 min and which increased to 150% of controls after 10 min of incubation ($P < 0.05$, relative to controls).

Effect of $24,25(OH)_2D_3$ or Age

Additional experiments with $25(OH)D_3$ were conducted with 100 nM metabolite, a concentration that is equivalent to circulating levels in chickens [Horst et al., 1981]. Incubation of cells from young chicks with vehicle or a combination of 6.5 nM $24R,25(OH)_2D_3$ and 100 nM $25(OH)D_3$ resulted in equivalent levels of radionuclide (Fig. 3A), indicating an inhibitory effect of $24,25(OH)_2D_3$. Thus, physiological levels of $24,25(OH)_2D_3$ appear to negatively regulate both $1,25(OH)_2D_3$ [Zhao and Nemere, 2002; Farach-Carson and Nemere, 2003] and $25(OH)D_3$, as observed in the present study.

An equivalent level of $25(OH)D_3$ was tested in cells isolated from 14- and 28-week old chickens. As indicated in Figure 3B,C, cells from these older age groups (14 and 28 week, respectively) were unresponsive to 100 nM $25(OH)D_3$.

Effect of Signal Transduction Activators

Since it was evident that $25(OH)D_3$ elicited rapid effects with regards to ^{45}Ca handling in isolated intestinal epithelial cells, we tested selected signal transduction activators at concentrations known to promote calcium transport [de Boland and Norman, 1990; de Boland et al., 1990] to determine if they could mimic the effects of the vitamin D metabolite. Figure 4A,B shows the effects of 2 μM BAY K8644, a calcium channel activator, on ^{45}Ca handling by enterocytes. A rapid increase in radionuclide uptake was observed, which became significant at

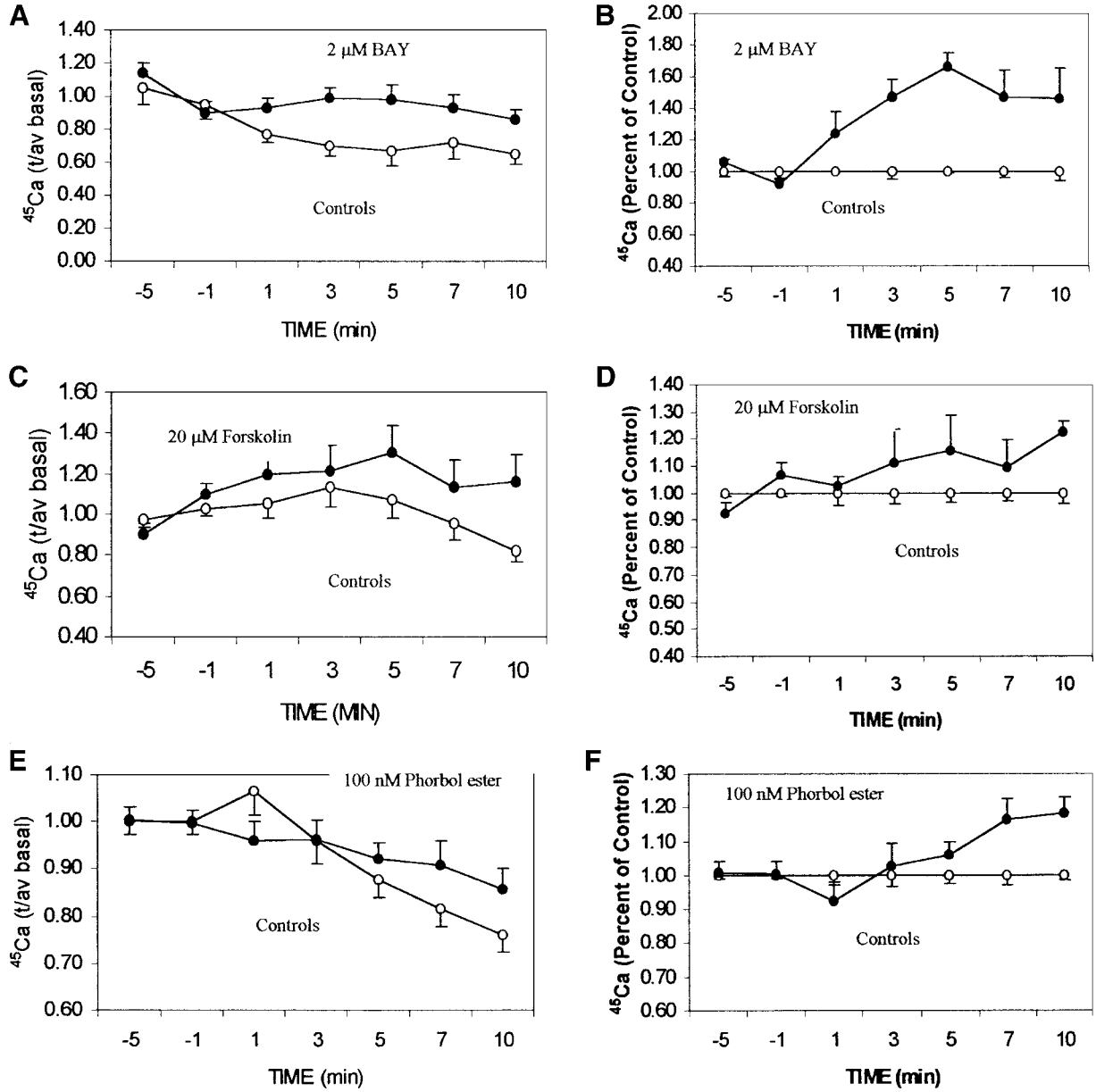

Fig. 4. Effect of signal transduction activators on calcium uptake by isolated intestinal cells of 7-week old birds. Cells were isolated and processed as described in the legend to Figure 2. **A**, **B**: Significant increases due to BAY K 8644 treatment were observed at T = 3–7 min, $P < 0.05$, relative to corresponding controls (n = 3); (**C**, **D**) significant increases due to forskolin treatment were observed at T = 10 min, $P < 0.05$, relative to corresponding controls (n = 6); (**E**, **F**) significant increases due to phorbol ester treatment were observed at T = 10 min, $P < 0.05$, relative to corresponding controls (n = 7).

3–10 min after addition ($P < 0.05$, relative to corresponding controls). Treatment of isolated intestinal cells with the adenylate cyclase activator, forskolin, resulted in a much slower increase in ^{45}Ca uptake, which became significant at 10 min after addition ($P < 0.05$, relative to controls, Fig. 4C,D). Finally, phorbol ester, an activator of protein kinase C, also enhanced radionuclide uptake to significant levels at 7–10 min of incubation ($P < 0.05$, relative to corresponding controls, Fig. 4E,F).

Effect of 25(OH)D_3 on Protein Kinase C and A Activities

Isolated intestinal epithelial cells were incubated in time course studies with 100 nM 25(OH)D_3 or vehicle, the cells collected by centrifugation, and the pellets extracted for analyses

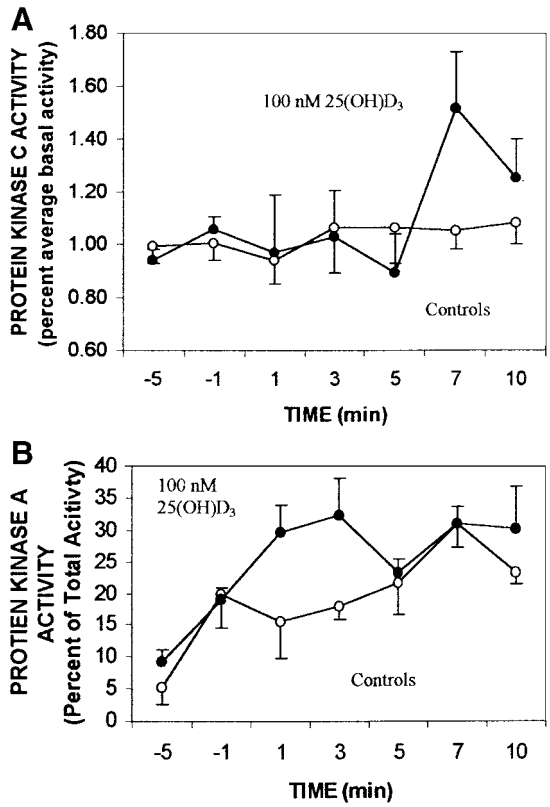

Fig. 5. Effect of 100 nM 25(OH)D_3 on protein kinase C and A activities. Cells were isolated and processed as described in the legend to Figure 2, with the exception that radionuclide was omitted from the time course incubation. Cell pellets from each of three independent experiments were stored at −20C until assayed. Pellets were then extracted with the appropriate buffer and incubated with the appropriate substrate plus gamma-^{32}P-ATP in the absence or presence of inhibitors. All samples were adjusted to either (**A**) 10 μg of protein (PK C) or (**B**) 5 μg of protein (PK A).

of protein kinases C and A. Figure 5 depicts the results of these experiments. Protein kinase C activity exhibited a small, statistically non-significant increase in activity 7 min after addition of the vitamin D metabolite (Fig. 5A). Protein kinase A activity increased to two-fold above controls 1 min after 25(OH)D_3 ($P < 0.05$), remained elevated at 3 min, and abruptly declined at 5 min (Fig. 5B).

DISCUSSION

Our studies indicate that physiological levels of the metabolite 25(OH)D_3 cause a direct and rapid effect on calcium handling by isolated intestinal epithelial cells. The rapidity of the response (1 min) suggests that 25(OH)D_3 is not metabolized to another form prior to cell stimulation. Indeed, the finding that 25(OH)D_3 decreases calcium levels in isolated cells is markedly different from the effect of 1,25(OH)$_2D_3$: this dihydroxylated metabolite produces no discernible effect in chicken enterocytes isolated from normal animals [Nemere and Campbell, 2000], while in rat enterocytes, it stimulates ^{45}Ca uptake [Nemere and Szego, 1981].

One interpretation of the 25(OH)D_3-mediated decrease in ^{45}Ca in isolated enterocytes is inhibition of uptake, although this is at odds with stimulation of net transport. Another interpretation is that 100 nM 25(OH)D_3 enhances extrusion in isolated intestinal cells, contributing to stimulated transport in perfused duodena. Unfortunately, it is difficult to test effects on uptake or extrusion due to the nature of the cell suspensions. Uptake of ^{45}Ca can only be demonstrated between 0.5–5 min after addition of radionuclide (I. Nemere, unpublished observations). At longer time points, the cells continually lose ^{45}Ca. It is possible that this represents a species difference since rat enterocytes do not exhibit the same behavior [Nemere and Szego, 1981]. Alternatively, the prolonged extrusion of label may be due to the absence of an extracellular matrix and would not be observed in adherent cells.

The observation that 300 nM 25(OH)D_3 stimulates ^{45}Ca uptake relative to controls presents a surprising inversion of effect. However, multiphasic dose-response effects have been reported for other vitamin D metabolites in mouse osteoblasts [Grosse et al., 1993]. The explanation may lie within the phenomenon of cross talk between signal transduction pathways. In the present work, we were unable to clearly elucidate the signal transduction pathway(s) involved in mediating the actions of 25(OH)D_3. Use of pharmacological agonists to stimulate calcium channels, adenylate cyclase (a proximal effector in the protein kinase A pathway), and protein kinase C produced increases in ^{45}Ca uptake relative to the vehicle controls. When 100 nM 25(OH)D_3 was tested for its effect on two selected pathways, only the protein kinase A pathway was stimulated in a sufficiently rapid manner (1 min) and to a significant extent. Comparing the results of forskolin with 25(OH)D_3 stimulation of protein kinase A activity suggests that (1) specificity of the pathway may require participation of an A kinase anchoring protein (AKAP) for both rapidity and targeting; and (2) the steroid or protein kinase A

may stimulate an as yet unknown signal transduction pathway to produce the observed effects on calcium handling.

Additional considerations for future work include whether the apparent membrane-initiated effects are mediated by a specific binding protein, and if so, whether the binding activity is the same as that reported by others [Gacad et al., 1997; Teegarden et al., 1997, 2000; Wu et al., 2000]. Finally, in order for 25(OH)D_3 to be considered a hormonally active metabolite, its activity will need to be determined in vivo, independent of further metabolism to 1,25$(OH)_2D_3$.

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