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EARLY STIMULATION OF ALKALINE PHOSPHATASE ACTIVITY IN RESPONSE TO 1α,25-DIHYDROXYCHOLECALCIFEROL

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SUMMARY : Alkaline phosphatase activity (APA) stimulation in response to 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) has been studied in vitamin-D-deficient rat intestinal brush borders prepared from ex-vivo-perfused duodeno-jejunal segments. Basal APA in intestines perfused with ethanol remained constant throughout the experiments. APA was significantly increased when intestines were perfused with $1,25(OH)_2D_3$ (3 nM) for 30, 45 or 60 min. A dose-effect response was observed when $1,25(OH)_2D_3$ increased in the perfusion medium. The maximal alkaline phosphatase activity after a 45-min perfusion (2404 ± 379 mU/mg prot.) was observed when $1,25(OH)_2D_3$ concentration was 6 nM. Cholecalciferol had no effect in this system.

Current data suggest that the renal active metabolite of vitamin D3, $1,25(OH)_2D_3$[x], acts through a nuclear mechanism similar to that described for other steroid hormones (1, 2). This nuclear action results in de novo protein synthesis. Among the newly synthetized proteins is the intestinal CaBP (3). Vitamin D metabolites also stimulate intestinal brush border alkaline phosphatase (4, 5, 9) and ATPase activities (4, 6, 9). Both the protein synthesis and the enzymatic activation require a several-hour lag-time after $1,25(OH)_2D_3$ administration (7, 8, 5). However, several reports indicate that the increase in intestinal calcium absorption in response to $1,25(OH)_2D_3$ takes place earlier than the CaBP synthesis or the APA stimulation (7, 8). Such discrepancy led some workers to look for earlier effects of $1,25(OH)_2D_3$. A stimulation of specific membrane protein has thus been reported as early as 4 hours after $1,25(OH)_2D_3$ administra-

[x] Abbreviations : $1,25(OH)_2D_3$: 1α,25-dihydroxycholecalciferol ; CaBP : calcium-binding protein ; APA : alkaline phosphatase (EC.3.1.3.1.) activity.

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tion (10). In the present study, we looked for a possible early mucosal brush border alkaline phosphatase activation in response to $1,25(OH)_2D_3$ infused via the superior mesenteric artery in an ex-vivo isolated intestinal segment.

MATERIALS AND METHODS : Animals used in all experiments were 6 week-old male C.D. Cobs rats (Charles Rivers, France) raised on a vitamin D-deficient diet, weighing between 100 and 150 g. They were anesthetized with intra-peritoneal sodium pentobarbital (50 mg/kg). The ex-vivo perfusion system that has been used is close to that described by Windmueller et al. (11) and by Eloy et al. (12). The 10-to 20-cm long duodeno-jejunal segment to be perfused was gently exteriorised and kept at 37°C throughout the experiment. The segment was limited by two ligatures, the upper ligature being done immediately distal to the biliary duct confluent. A polyethylene aortic catheter (Clay-Adams P.E. 50) was inserted in the aorta with its tip facing the origin of the superior mesenteric artery. The renal arteries, the middle and right colic arteries, the coeliac artery and the lower aorta were successively ligated in order to isolate the intestinal segment to be perfused from the systemic circulation. A polyethylene catheter (Clay-Adams P.E. 160) was then inserted in the portal vein, and the affluents to the portal vein other than the superior mesenteric veins were ligated. When surgery was completed, the aorta was tightly ligated above the tip of the aortic catheter, and the perfusion started immediately. The vascular perfusion medium consisted in a modified Krebs-Henseleit solution (NaCl : 118 mM, KCl : 4.75 mM, $CaCl_2$: 2.5 mM, KH_2PO_4 : 1.2 mM, $MgSO_4$: 1.18 mM, $NaHCo_3$: 25 mM), containing 5.5 mM of glucose, 2.5 per cent (w/v) of bovine serum albumin (Fraction V powder, Sigma) and 2.5 per cent (w/v) of Dextran (M.W. : 70 000, Pharmacia). The medium was adjusted to pH 7.5 with sodium bicarbonate. It was oxygenated in a rotative oxygenator with O_2 and CO_2 (95 and 5 per cent respectively), yielding a partial oxygen pressure of 250 Torr. The medium did not contain hemoglobin or erythrocytes. Figure 1 gives a schematic representation of the perfusion system. The medium was perfused in the isolated intestinal segment via the superior mesenteric artery with a peristaltic pump (Gilson, minipuls HP4) providing a constant input. The medium was not recycled from the portal catheter. As shown in Fig. 1, it was possible to perfuse two rat intestinal segments in parallel. $1,25(OH)_2D_3$ or cholecalciferol was dissolved in ethanol and diluted in Krebs-Henseleit solution (final concentration of ethanol : 1 per cent) in a syringe which was racorded to the main vascular tubing through a polyethylene catheter. The syringe content was infused at a constant rate (0.36 ml/min) with a perfusion pump (Braun, Melsungen). The final perfusion input was 1.56 ml/min providing a perfusion pression of 80 to 100 Torr which remained constant over a 90-min period after surgery. In control experiments, 1 per cent ethanol in Krebs Henseleit medium was infused. In the first 30 perfusion experiments each pair of perfused rats included one experimental and one control rat. Later on, one control out of three experimental perfusions were carried out each day. The experimental scheme was as follows : after surgery, the perfusion was run out for 20 minutes (equilibration period). The end of the equilibration period is referred as time zero. At time zero, the

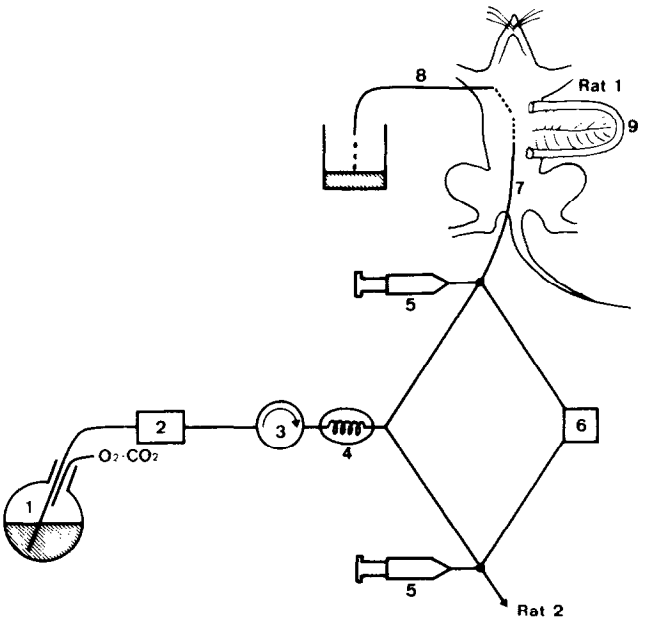
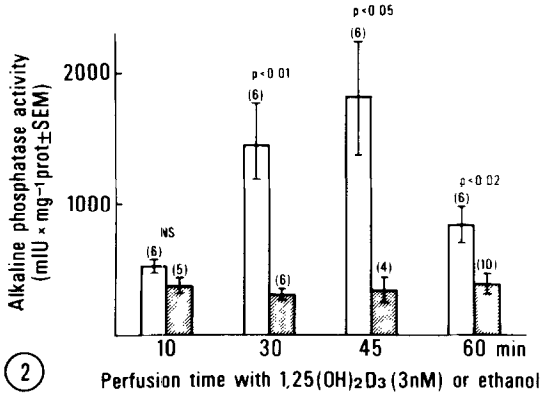


Figure 1 : Diagramatic representation of the perfusion system : 1) Rotative oxygenator ; 2) Nylon filter ; 3) Peristaltic pump ; 4) Heater ; 5) Syringe with $1,25(OH)_2D_3$, cholecalciferol or ethanol ; 6) Pressure recorder ; 7) Aortic catheter ; 8) Portal catheter ; 9) Perfused duodeno-jejunal segment.

infusion of $1,25(OH)_2D_3$, cholecalciferol or ethanol was started and lasted for various times. The perfused intestinal segment was then removed and the mucosa was scrapped with a microscope slide. Brush borders were then immediately prepared from the mucosa cells using the method described by Forstner et al. (13) with no modification. The preparation was carried on to the step referred as "purified brush borders". The brush borders were then resuspended in a Tris-HCl 10 mM, $MgCl_2$ 5 mM (pH 8.0) buffer, frozen and stored at - 60°C until their APA and protein content was measured. APA was determined using the method described by Bessey et al. (14) subsequently modified by Forstner et al. (13) with 10 mM p-nitrophenolphosphate (Merck) as substrate. APA is expressed as mIU (1 IU represents 1 μmole of p-nitrophenol released per minute) per mg of brush border protein determined simultaneously by the method of Lowry et al. (15) with bovine serum albumin (Sigma, RIA grade) as standard. Statistical calculations were done using Student's t test

RESULTS : Intestinal segments have been perfused for various times with either $1,25(OH)_2D_3$ (3 nM) or ethanol (control experiments) via the superior mesenteric artery. Figure 2 shows that the APA in brush borders from control animals remained constant for 60 min. $1,25(OH)_2D_3$ infusion for 30, 45 or 60 min resulted in a significant increase in APA (1454 $\pm$ 321, 1812 $\pm$ 463 and 815 $\pm$ 163 mIU/mg prot.



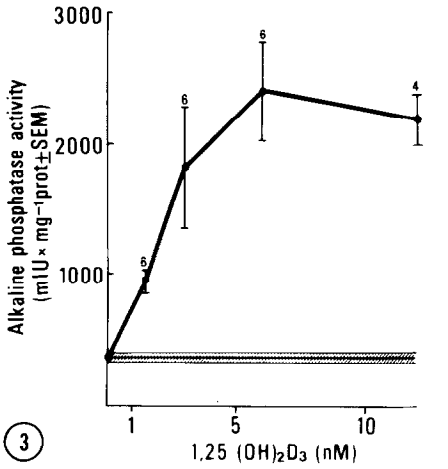


Figure 2 : APA in brush border membranes prepared from intestinal segments after various perfusion times. Open columns : APA from segments infused with 1,25(OH)$_2$D$_3$ (3 nM) ; dark columns: APA in segments infused with ethanol (vehicle) ; numbers in parentheses indicate the number of experiments.

Figure 3 : APA in brush border membranes from intestinal segments perfused for 45 min with increasing amounts of 1,25(OH)$_2$D$_3$. The dark area represents control values obtained with intestines perfused with ethanol. The numbers indicate the number of experiments.

± SEM respectively). However APA after a 60-min perfusion with 1,25(OH)$_2$D$_3$ was lower than APA after a 45-min perfusion with this sterol.

In figure 3 perfusion experiments have been carried out for 45 min with 1,25(OH)$_2$D$_3$ at various concentrations. Maximal APA has been found in intestines perfused with 6 nM of 1,25(OH)$_2$D$_3$. A dose-response effect was observed when 1,25(OH)$_2$D$_3$ concentration varied from 1.5 to 6 nM.

When intestinal segments have been perfused with high concentrations of cholecalciferol (115 nM), there was no significant APA increase above basal activities measured in control animals.

DISCUSSION : Our results clearly demonstrate an early and significant increase in mucosal brush border APA from ex-vivo perfused intestinal segments that have been infused with 1,25(OH)$_2$D$_3$ through the superior mesenteric artery. Such increase appears to be maximal 45 min after starting the infusion with 3 nM of 1,25(OH)$_2$D$_3$.

Maximal APA after a 45-min perfusion is found for a $1,25(OH)_2D_3$ concentration in the perfusion medium of 6 nM. Cholecalciferol (115 nM) does not increase APA above control values after a 45-min perfusion. The decrease in APA observed between a 45-min and a 60-min perfusion with $1,25(OH)_2D_3$ could be due to mucosa cell desquamation inside the intestinal lumen, a phenomenon that has already been encountered in this ex-vivo perfusion model (12).

In the chick, previous studies have demonstrated that vitamin D metabolites stimulate intestinal APA (4, 5, 8, 16). However time-course studies in the chick indicate that APA stimulation takes place at least 6 hours after oral or systemic administration of $1,25(OH)_2D_3$ or vitamin D (5, 8), but in these experiments no APA measurements have been reported as soon as 30 or 45 min after $1,25(OH)_2D_3$ administration. In the rat no time-course studies of APA in response to vitamin D metabolites have been reported. In our preliminary experiments, no increase in rat duodenal APA was observed 45 or 60 min after the intravenous injection of $1,25(OH)_2D_3$ (500 pmoles). It is therefore possible that the use of an ex-vivo perfused intestine has allowed us to detect the early phase of a biphasic APA stimulation in response to $1,25(OH)_2D_3$.

APA stimulation in response to $1,25(OH)_2D_3$ can be the result of three possible mechanisms. First, the sterol may directly modify the enzyme molecule as it does in the chick (17). Second it can alter the membrane composition resulting in enzyme activation. Indeed vitamin D is known to modify the lipidic composition of intestinal microvilli (18) where most of the intestinal alkaline phosphatase is located (19, 24). Third, APA stimulation may result form de novo synthesis of protein molecules with APA. However the early response to $1,25(OH)_2D_3$ rends this mechanism unlikely, although a $1,25(OH)_2D_3$ effect at the post-transcriptionnal level remains a possibility similar to that has been described for other steroid hormones (20). Present data do not permit to differenciate between these mechanisms.

Other $1,25(OH)_2D_3$ early effects have already been reported. $1,25(OH)_2D_3$ increases cAMP production from cultured chick embryo duodenal cells within 2 hours (21). Recently, Wilson and Lawson (10) have demonstrated that two chick intestinal brush-

border proteins are synthetized or modified 2 to 3 hours after intracardial administration of $1,25(OH)_2D_3$. Calcium intestinal transport in vitro is increased 1 to 2 hours after intracardial administration of $1,25(OH)_2D_3$ in the chick (7, 8). Birge and Miller (22) have reported that in cultured chick ileum explants a significant increase in phosphorus accumulation is demonstrable as early as 30 min after adding $1,25(OH)_2D_3$ to the culture medium. However, the role, if any, of the early APA increase in the calcium or phosphorus absorption stimulation in response to $1,25(OH)_2D_3$ remains unknown. Moog and Glazier (23) have shown that in mouse and chick intestine the amount of phosphate absorbed into the tissue is proportional to the APA. A possibility would be that could be studied using our ex-vivo perfusion system that the increase in phosphorus accumulation by cultured chick ileum explants in response to $1,25(OH)_2D_3$ reported by Birge and Miller (22) is somehow related to the early APA stimulation that is herein demonstrated.

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