Biochimica et Biophysica Acta, 770 (1984) 15–21
Elsevier

BBA 71963

25-HYDROXYSTEROLS INCREASE THE PERMEABILITY OF LIPOSOMES TO Ca^{2+} AND OTHER CATIONS

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(Received June 13th, 1983)

Key words: Hydroxycholesterol; Hydroxy vitamin D-3; Liposome; Cation permeability; Membrane permeability

25-Hydroxycholesterol and 25-hydroxy vitamin D-3 increased the permeability of liposomes to Ca^{2+} measured by the arsenazo III encapsulation technique. This effect was sensitive to the lipid composition of the membrane, with changes that decreased the motional freedom of phospholipid acyl chains decreasing Ca^{2+} permeability. The greatest permeability was observed with the zwitter-ionic phospholipids, phosphatidylcholine and phosphatidylethanolamine, whereas the acidic phospholipids, phosphatidylinositol and phosphatidylserine, depressed Ca^{2+} permeability. The effect was not specific for Ca^{2+}. Other divalent cations were translocated in the order $Mn^{2+} > Mg^{2+} = Ca^{2+} \gg Sr^{2+} = Ba^{2+}$. The permeability of liposomes to the monovalent cation, Na^+, was also substantially increased. The effect did not appear to be due to ionophoretic properties of the sterols, and it is suggested that perturbation of the membranes by the polar 25-hydroxyl group may play a role in increasing membrane permeability.

Introduction

Hydroxylated sterols are potent inhibitors of cholesterol biosynthesis. 25(OH)Chol has been shown to inhibit 3-hydroxy-3-methylglutaryl-CoA reductase activity in cultured cells [1], isolated cells [2] and tissue cells in vivo [2]. The secosterol, $25(OH)D_3$, also inhibits reductase activity in cultured L_2C cells [3]. Hydroxylated sterols produce other effects on cells as well, including an inhibition of DNA synthesis [4,5], an altered membrane permeability [6], a decrease in the rate of endocytosis [7], a change in cell shape [8,9], an increase in the fragility of cultured L cells [5], and a decrease in the fragility of erythrocytes [10]. $1,25(OH)_2D_3$ also inhibits 3-hydroxy-3-methylglutaryl-CoA reductase activity and DNA synthesis [11], and at much lower concentrations stimulates messenger RNA synthesis [12], phosphatidylcholine synthesis [13], and phospholipid acylation and deacylation in intestinal cells [13,14]. It has been proposed that many of the divergent effects of hydroxylated sterols are a consequence of blocked sterol synthesis [15]. When the inhibitory effects of a range of different hydroxylated sterols were compared, however, several were found to effectively block sterol synthesis, while having no effect on DNA synthesis [11]. Also, in a mutant line of Chinese hamster ovary cells, manipulating the cholesterol content of membranes by varying the extent of medium supplementation, had no effect on membrane permeability [16]. This suggests that mechanisms apart from blocked sterol synthesis must be invoked to explain the pleiotropic effects of hydroxylated sterols.

The amphiphilic nature of these sterols suggests that they should be incorporated into membranes.

Abbreviations: $25(OH)D_3$, 25-hydroxy vitamin D-3; 25(OH)Chol, 25-hydroxycholesterol; $1,25(OH)_2D_3$, 1,25-dihydroxy vitamin D-3; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine.

Confirmation of this has been obtained by Hsu et al. [9] who observed that hydroxylated sterols can be readily incorporated into erythrocyte membranes. Weissmann and co-workers [17,18] have developed a sensitive technique for monitoring the Ca^{2+} permeability of liposomal membranes, in which the Ca^{2+}-sensitive metallochrome dye, arsenazo III, is encapsulated in multilamellar vesicles. In this report, we have utilized this technique to examine the effect that the incorporation of 25(OH)D_3 and 25(OH)Chol into liposomes has on their permeability to Ca^{2+} and other cations. Our results suggest that the incorporation of 25-hydroxylated sterols into membranes and the resultant permeability changes should be considered as contributing to the pleiotropic effects of these sterols, including pathological changes that occur when mammals ingest large amounts of these sterols.

Methods

Materials. All phospholipids, vitamin D-3 and cholesterol were purchased from Sigma Chemical Co., St. Louis, MO, and found to migrate as a single spot by thin-layer chromatography. 25(OH)Chol was obtained from Steraloids Inc., 25(OH)D_3 was generously supplied by UpJohn Co., and 1,25$(OH)_2D_3$ by Hoffmann-La Roche. $^{45}CaCl_2$, algal [^{14}C] PC, [26,27-^{3}H]25(OH)D_3, and [26,27-^{3}H]25(OH)Chol were obtained from New England Nuclear, Boston, MA, whereas ^{22}NaCl and (1,2-^{3}H)-labelled vitamin D-3 were obtained from Amersham, Arlington Heights, IL.

Liposome preparation. Liposomes were prepared, unless stated otherwise, by mixing lipids in the proportion 70 mol% egg yolk PC, 20 mol% DCP and 10 mol% sterol. Multilamellar vesicles were prepared in 145 mM KCl, 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (pH 7.45), 3.1 mM arsenazo III (Aldrich) to give 10 μmol total lipid per ml of buffer, as described by Weissmann et al. [17]. They were swollen overnight at room temperature and sized by filtration through a 0.4 μm polycarbonate filter [19]. With this method, the encapsulation efficiency for all liposomal preparations was normally greater than 4 l/mol of lipid. Arsenazo III, not incorporated by liposomes, was removed by chromatography on a 25×1.6 cm Sephadex G-50 column in buffer without arsenazo III. The concentration of lipid in the final liposome fraction was estimated by measuring the A_{750} and relating this to a standard curve prepared by correlating absorbance with the phospholipid phosphorus content of liposome preparations [20]. To confirm that the final liposome sterol composition reflected that in the initial lipid mixture, sterols in liposomes were estimated by either HPLC using 2.5% propan-2-ol in hexane as the elution solvent on a Waters μ-Porasil column, or by following the concentration of a radioactive tracer.

Ca^{2+} permeability. The Ca^{2+} permeability of liposomes at 23°C was monitored at 650 nm following the addition of 1 mM $CaCl_2$ to the sample cuvette and reading this against a reference cuvette containing only liposomes. The uptake rate was determined between 2 and 12 min using a differential molar extinction coefficient of $1.89 \cdot 10^4\ M^{-1} \cdot cm^{-1}$, which was determined for these incubation conditions using the method described by Bauer [21]. This value is in agreement with other reported values [21,22]. Permeability to other divalent cations was measured using the following measured molar extinction coefficients at the optimal wavelengths for the cation-arsenazo III complexes: Mn^{2+}, $1.16 \cdot 10^4$ at 620 nm; Sr^{2+}, $1.92 \cdot 10^4$ at 650 nm; Mg^{2+}, $2.2 \cdot 10^3$ at 625 nm; and Ba^{2+}, $1.77 \cdot 10^4$ at 648 nm.

Na^+ permeability. Na^+ diffusion was monitored at 23°C using $^{22}Na^+$ and the dialysis technique described by Papahadjopoulos et al. [23]. It was measured as a self-diffusion rate in the absence of a Na^+ gradient across the membrane.

Ionophoretic properties. The translocation of $^{45}Ca^{2+}$ across an organic phase separating two aqueous compartments was measured in a Pressman cell [24] using the conditions outlined by Tyson et al. [25]. To conserve lipids, miniature cells were constructed out of teflon, and were sealed with tightly-fitting teflon caps. Donor and receiver compartments contained 0.3 ml, separated by 1 ml of chloroform presaturated with methanol and water.

Results

The effect of increasing concentrations of 25(OH)D_3 and 25(OH)Chol on the permeability of

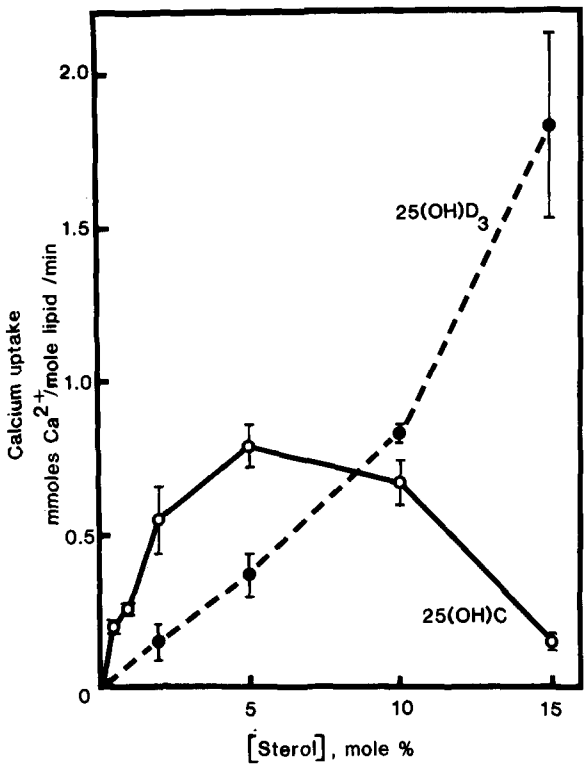

Fig. 1. The effect of sterol concentration on the permeability of liposomes to Ca^{2+}. The concentration of dicetyl phosphate was kept constant at 20 mol% and that of PC and sterol varied. Vertical bars denote S.E. of the mean for at least three different liposome preparations. 25(OH)C, 25(OH)Chol.

liposomes to Ca^{2+} is illustrated in Fig. 1. Both sterols substantially increased liposome permeability at concentrations above 1 mol%. 25(OH)Chol had a greater effect at concentrations up to 5 mol%, but above this level $25(OH)D_3$ was more potent in stimulating a Ca^{2+} influx. Rechromatography of liposomes after 72 h of incubation, by which time they were distinctly blue, verified that the dye remained completely entrapped in the liposomes and that no lysis had occurred. Permeability decreased with molar concentrations of 25(OH)Chol above 10 mol% and with molar concentrations of $25(OH)D_3$ above 15 mol% where highly variable results were obtained. The amount of dye encapsulated by these liposomes was 4.3 ± 0.5 l/mol ($n = 5$) and was similar to that encapsulated by liposomes containing lower levels of 25-hydroxy sterols. An analysis of the lipid composition indicated that the sterols had been effectively incorporated into the liposomes.

TABLE I

COMPARATIVE EFFECT OF STEROLS ON LIPOSOME PERMEABILITY TO Ca^{2+}

Liposomes consisted to 70 mol% egg PC, 20 mol% dicetyl phosphate, and 10 mol% sterol. Data represent the mean ± S.E. for three liposome preparations. n.d., not detectable.

Sterol	Ca^{2+} uptake rate (mmol Ca^{2+}/mol lipid per min)
Cholesterol	n.d.
25(OH)Chol	0.67 ± 0.05
Vitamin D-3	n.d.
$25(OH)D_3$	0.83 ± 0.03
$1,25(OH)_2D_3$	0.70 ± 0.06

This effect of increasing liposome permeability to Ca^{2+} depended on the presence of a 25-hydroxyl group on the isoprenoid side chain. As shown in Table I, the parent sterols (cholesterol and vitamin D-3) at 10 mol% had no effect on liposome permeability to Ca^{2+}. The effect of the more polar vitamin D metabolite, $1,25(OH)_2D_3$,

TABLE II

EFFECT OF LIPID COMPOSITION ON THE Ca^{2+} PERMEABILITY OF LIPOSOMES CONTAINING $25(OH)D_3$

Unless indicated the liposome composition consisted of 70 mol% PC, 20 mol% dicetyl phosphate, and 10 mol% $25(OH)D_3$. Liposomes with phosphatidylinositol and phosphatidylserine contained no dicetylphosphate. Data represent the mean ± S.E. for three liposome preparations.

Liposome composition	Ca^{2+} uptake rate (mmol Ca^{2+}/mol lipid per min)
Dimyristoylphosphatidylcholine	0.56 ± 0.05
Dielaidoylphosphatidylcholine	0.31 ± 0.03
POPC	1.36 ± 0.02
Dioleoylphosphatidylcholine (DOPC)	0.87 ± 0.04
PC (45%), cholesterol (25%)	0.47 ± 0.15
PC (60%), phosphatidylserine (30%)	0.11 ± 0.01
PC (60%), phosphatidylinositol (30%)	0.07 ± 0.01
PC (40%), phosphatidylethanolamine (30%)	0.56 ± 0.03

TABLE III

DIVALENT CATION SPECIFICITY OF LIPOSOME PERMEABILITY

Liposomes consisted of 70 mol% egg PC, 20 mol% DCP, and 10 mol% 25(OH)D_3. Cations were added to produce a concentration of 1 mM. Data represent the mean ± S.E. of at least three liposome preparations.

Cation	Me^{2+} uptake rate (mmol Me^{2+}/mol lipid per min)
Ca^{2+}	0.77 ± 0.03
Mn^{2+}	1.27 ± 0.05
Sr^{2+}	0.08 ± 0.02
Mg^{2+}	0.81 ± 0.14
Ba^{2+}	0.07 ± 0.02

was similar to that of 25(OH)Chol and 25(OH)D_3.

Varying the fatty acid composition of the liposomes influenced the effect of 25-hydroxysterols on liposome Ca^{2+} permeability. Increasing the saturation of the fatty acids decreased Ca^{2+} permeability, as evident with dimyristoyl-PC and dielaidoyl-PC vesicles (Table II), although chain length may be a contributing factor. Elaidic acid, which has a *trans* double bond and an almost straight acyl chain configuration, could be expected to behave similarly to a saturated fatty acid. An interesting observation was that 1-palmitoyl-2-oleoyl PC (POPC) vesicles were much more permeable than those containing dioleoyl-PC (DOPC). Increasing the molecular order in the liposomes by adding 25 mol% cholesterol to vesicles containing 10 mol% 25(OH)D_3 also decreased vesicle permeability to Ca^{2+} (Table II). The phospholipid headgroup also had an effect. The presence of either 30 mol% phosphatidylserine or phosphatidylinositol substantially reduced the Ca^{2+} permeability of the liposomes, whereas phosphatidylethanolamine reduced liposome permeability only slightly.

TABLE IV

COMPARATIVE EFFECT OF STEROLS ON THE PERMEABILITY OF LIPOSOMES TO Na^+

Liposomes consisted of 70 mol% egg PC, 20 mol% DCP, and 10 mol% sterol. Data represent the mean ± S.E. of three liposome preparations.

Sterol	Na^+ uptake rate (mmol Na^+/mol lipid per h)
Cholesterol	1.03 ± 0.12
25(OH)Chol	20.7 ± 0.84
Vitamin D-3	3.90 ± 0.12
25(OH)D_3	13.2 ± 0.54

TABLE V

TRANSLOCATION OF Ca^{2+} IN A PRESSMAN CELL

The donor compartment contained 25 mM tetramethylammonium tris(hydroxymethyl)methylglycine (pH 8.3) and 10 mM $^{45}CaCl_2$ (2.1 μCi/ml). The receiver compartment contained 25 mM tetramethylammonium citrate (pH 5.4). The translocation of ^{45}Ca was measured from donor to receiver compartments through an organic phase consisting of chloroform presaturated with methanol and water. Without lipid no translocation was detected. The data represent the mean ± S.E. of at least three experiments. Values which do not share a common superscript are significantly different ($P < 0.05$) by the Student's *t*-test.

Lipid (0.42 mM)	mol Ca^{2+} translocated per h
Cholesterol	0.14 ± 0.07 [a]
25(OH)Chol	0.81 ± 0.16 [b]
Vitamin D-3	0.25 ± 0.08 [a,b]
25(OH)D_3	0.41 ± 0.17 [a,b,c]
Phosphatidic acid	4.21 ± 1.21 [d]
A23187 (10 μM)	29.0 ± 5.01 [e]

The specificity of the effect of 25-hydroxylated sterols towards divalent cations was examined (Table III). An influx was observed in the order $Mn^{2+} > Mg^{2+} = Ca^{2+} \gg Sr^{2+} = Ba^{2+}$.

The exogenous addition of valinomycin (1μM) to 25(OH)D_3-containing liposomes did not increase the permeability to Ca^{2+}, indicating that counter ion movements were occurring, not allowing a charge gradient to form across the membrane. The movement of Na^+ in response to the incorporation of 25-hydroxylated sterols is shown in Table IV. Vitamin D-3 itself increased the permeability of vesicles to Na^+ when compared with cholesterol. 25(OH)Chol was slightly more stimulatory than 25(OH)D_3.

To determine whether the 25-hydroxylated sterols exert their effects on membrane permeability by acting as ionophores, their ionophoretic properties were examined in a Pressman cell (Table V). The ability of the 25-hydroxylated sterols to translocate Ca^{2+} was higher than that of the parent sterols but much less than that of the Ca^{2+}

ionophore, A23187, or phosphatidic acid, a phospholipid previously shown to translocate Ca^{2+} in this type of cell [25].

Discussion

25(OH)Chol and 25(OH)D_3 substantially increased the permeability of liposomes to Ca^{2+} at concentrations as low as 1 mol%. The extent of this permeability change at 10 mol% sterol was dependent on the lipid composition of the liposomes. An increase in fatty acid saturation or cholesterol concentration (changes which will decrease the motional freedom of phospholipid fatty acyl chains) decreased the effect of 25-hydroxysterols on permeability. The increased permeability observed with POPC, compared to egg yolk PC, suggests that the degree of membrane perturbation by 25-hydroxysterols is influenced by the positional distribution of fatty acyl chains in phospholipids. The nature of the phospholipid head group was also important. The interaction between hydroxysterols and phospholipids containing choline or ethanolamine resulted in the greatest permeability. The presence of the acidic phospholipids, phosphatidylserine and phosphatidylinositol, substantially decreased liposome permeability, presumably related to the binding of Ca^{2+} by the headgroups.

Apart from Mg^{2+}, the specificity of divalent cation uptake, $Mn^{2+} > Mg^{2+} = Ca^{2+} \gg Sr^{2+} = Ba^{2+}$, is in approximate agreement with their ionic radii where $Mg^{2+} < Mn^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$ [26]. The low permeability of the liposomes to Sr^{2+} and Ba^{2+} suggests that membranes containing 25-hydroxysterols are not very permeable to ions with an ionic radius $> Sr^{2+}$, 1.12 Å. The observed permeability rates may in part be influenced by differences in binding of these cations to the negatively charged membrane lipids. This may offer an explanation for the disparate results observed with Mg^{2+} but it may also reflect on the insensitivity of this technique for detecting Mg^{2+} permeability changes in view of the low molar extinction coefficient of Mg^{2+} for arsenazo III. The permeability of the membranes to Na^{+} confirmed that there was a general lack of specificity towards cations that could be translocated following 25-hydroxysterol incorporation into membranes.

The results with the Pressman cell suggest that 25-hydroxysterols do not act as ionophores translocating cations across membranes. Although their activity was higher than that of the parent sterols, it was still low. Their activity was lower than that of phosphatidic acid, which we have recently reported does not translocate Ca^{2+} across membranes despite its high translocation rate in a Pressman cell [27]. The likelihood that lipids form some type of micellar structure in organic solvents and the nonequivalence of the solvents used to the milieu of a lipid bilayer, suggest that Pressman cells are not good membrane models. The structures of 25-hydroxysterols are also inconsistent with their having ionophoretic properties.

The lack of specificity of the permeability change towards cations with these sterols and their low ionophoretic properties suggest that a general perturbance of membrane properties occurs rather than there being a specific sterol-cation interaction. Cholesterol is believed to be orientated with its 3β-OH group at the bilayer surface, with its steroid nucleus perpendicular to the plane of the membrane, and with the isoprenoid side chain deep in the hydrophobic interior of the membrane where it interacts with methylene groups of fatty acyl chains [28]. A side chain of precise length, saturation and bulk has been shown to be important in cholesterol/phospholipid interactions [29,30]. NMR studies have shown that this side-chain is very flexible [31] although methyl groups at C13 and C20 have been suggested to limit the motion of the portion of the chain proximal to the steroid nucleus [32]. The introduction of a polar hydroxyl group at the 25-position of the side-chain may severely limit sterol side-chain–fatty acyl chain interactions.

In view of the precise interactions that occur between the steroid nucleus and phospholipids we believe that the most likely orientation of 25-hydroxysterols in membranes is one similar to that proposed for cholesterol, although other orientations are possible. We have found that in ESR studies with fatty acid spin label probes containing the spin label at various positions along the acyl chain, 25(OH)Chol is unable to increase the molecular order in egg yolk PC liposomes in a similar manner to cholesterol [33]. This is in contrast to the significant ordering effect of

cholesterol. This perturbance by the polar hydroxyl group at the 25-position apparently allows penetration of cations through the bilayer. Whilst no disordering of membrane lipids could be detected by this technique, the failure to order membrane lipids appears to be functionally expressed as a perturbance of membrane permeability. There is no apparent explanation for the lowered membrane permeability with high concentrations of 25-hydroxysterols in the membrane. It is possible that it is related to sterol-sterol interactions which may become prominent at such concentrations.

In cultured L cells, 25(OH)Chol has been observed to stimulate oubain-sensitive Rb^+ influx and oubain-insensitive Rb^+ efflux. No effect on oubain-insensitive Rb^+ influx was observed. This observation, together with relief of the inhibition by mevalonate (which circumvents the block in sterol synthesis and allows cholesterol levels to remain normal) suggested than an alteration in membrane sterol content produced the altered Rb^+ fluxes. However, the relief of inhibition by mevalonate was never complete, and no difference was observed on $(Na^+ + K^+)$-ATPase activities which is not compatible with the oubain inhibition of the measured Rb^+ influx. Furthermore, in a mutant cell line where the cholesterol content in membranes could be manipulated by varying the cholesterol content of the medium, membrane permeability to Na^+ was not effected by the sterol content of the membrane [16]. In contrast, K^+ transport in *Mycoplasma mycoides* was sensitive to the cholesterol content in its membrane which was varied by medium supplementation [34]. The possibility that 25-hydroxysterols induce permeability changes that affect DNA synthesis and cell shape should also be considered.

With the dye encapsulation technique used, permeability changes were detected with 25(OH)Chol levels as low as 0.5 mol%. The rate of Ca^{2+} translocation at this concentration, 0.2 mmol/mol lipid per min, and an encapsulation efficiency of 4 l/mol lipid, indicates that the intravesicular concentration of Ca^{2+} was raised to 50 μM within 1 min. As the most recent estimates of the cytoplasmic Ca^{2+} concentration within living cells are in the order of 100 nM [35,36], a concentration of 0.5 mol% of 25-hydroxysterols in the plasma membrane of a cell would have profound effects. Whilst the effects of the hydroxylated sterols may not be specific for Ca^{2+}, compared to other cations, a large concentration gradient exists for Ca^{2+} across the plasma membrane. Ca^{2+}-transporting systems in the plasma membrane of most cells may be able to pump much of this Ca^{2+} out of the cells. However, the long-term consequences of an enhanced Ca^{2+} uptake by cells is potentially detrimental in view of the Ca^{2+}-sequestering ability of sub-cellular organelles and the sensitivity of many intracellular enzymes to Ca^{2+} [37].

The physiological significance of these results is, we believe, related to the toxicity of vitamin D and oxidized derivatives of cholesterol. They are not apparently related to the mode of action of $1,25(OH)_2D_3$ as its concentration in serum is very low even during conditions of hypervitaminosis D [38]. Autoradiographic studies also indicate that in intestinal mucosal cells $1,25(OH)_2D_3$ is primarily localized in the nucleus, and there is no evidence of any preferential localization in brush border membranes where membrane permeability changes occur [39,40]. The toxicity of 25-hydroxysterols is evident through milligram quantities inducing severe atherosclerosis in rabbits [41]. During hypervitaminosis D in rabbits, arteries and other soft tissues calcify. Serum levels of $25(OH)D_3$ are much higher than those of the parent vitamin and both vitamin D and $25(OH)D_3$ are deposited in tissues that calcify [42]. The effects we have observed on liposome permeability are compatible with the hypothesis that the pathogenic consequences of ingestion of high levels of 25(OH)Chol and of vitamin D are due to the insertion of the 25-hydroxysterols in cellular membranes and a resultant change in their permeability to cations. It remains to be shown that significant amounts of 25-hydroxysterols are incorporated into cellular membranes during these pathological changes and the changes that occur in cultured cells upon exposure to 25-hydroxysterols.

Acknowledgements

This research was supported by the Burnsides Research Fund, funds solicited by F.A. Kummerow, whose encouragement was greatly appreciated.

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