Cyclopamine Inhibition of Sonic Hedgehog Signal Transduction Is Not Mediated through Effects on Cholesterol Transport

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Cyclopamine is a teratogenic steroidal alkaloid that causes cyclopia by blocking Sonic hedgehog (Shh) signal transduction. We have tested whether this activity of cyclopamine is related to disruption of cellular cholesterol transport and putative secondary effects on the Shh receptor, Patched (Ptc). First, we report that the potent antagonism of Shh signaling by cyclopamine is not a general property of steroidal alkaloids with similar structure. The structural features of steroidal alkaloids previously associated with the induction of holoprosencephaly in whole animals are also associated with inhibition of Shh signaling in vitro. Second, by comparing the effects of cyclopamine on Shh signaling with those of compounds known to block cholesterol transport, we show that the action of cyclopamine cannot be explained by inhibition of intracellular cholesterol transport. However, compounds that block cholesterol transport by affecting the vesicular trafficking of the Niemann–Pick C1 protein (NPC1), which is structurally similar to Ptc, are weak Shh antagonists. Rather than supporting a direct link between cholesterol homeostasis and Shh signaling, our findings suggest that the functions of both NPC1 and Ptc involve a common vesicular transport pathway. Consistent with this model, we find that Ptc and NPC1 colocalize extensively in a vesicular compartment in cotransfected cells.

Key Words: neural development; holoprosencephaly; teratogen; U18666A; progesterone; membrane trafficking.

INTRODUCTION

Hedgehog family members are extracellular signaling molecules involved in embryonic patterning in many animal phyla. In vertebrates, Sonic hedgehog (Shh) plays a critical role in patterning of the neural tube. Notochord-derived Shh mediates both the induction of ventral cell types in the neural tube and the ventral exclusion of dorsal cell types, and Shh released from the prechordal plate is required for the induction of ventral cell types in the forebrain (Tanabe and Jessell, 1996; Dale et al., 1997). Loss of normal Shh function leads to a loss of ventral cell types all along the neural tube. This has significant consequences, especially in the forebrain, which fails to divide into hemispheres (Chiang et al., 1996; Roessler et al., 1996). An undivided forebrain, or holoprosencephaly, in its most extreme form is associated with cyclopia (for review, see Siebert et al., 1990; Roessler and Muenke, 1998).

Cyclopamine is one of a small group of steroidal alkaloids, produced by Veratrum lily species, that induce holoprosencephaly and cyclopia in mammalian and avian embryos. Cyclopamine causes holoprosencephaly by blocking the response to Shh of cells normally sensitive to Shh.
loss of Shh responsiveness within the developing neural tube, due to early embryonic exposure to cyclopamine, results in a phenocopy of the Shh null mutant (Cooper et al., 1998; Incardona et al., 1998). Our previous studies demonstrated that a suite of Shh-dependent cell types in the neural tube was affected in embryos with cyclopamine-induced holoprosencephaly. Furthermore, cyclopamine inhibits multiple Shh-mediated signaling events in other tissues at very different times of development, including somites (Incardona et al., 1998), heart (J.P.I., unpublished observation), gut (Kim and Melton, 1998), limb/fin (Neu- 

Two decades of work by Keeler and colleagues demonstrated that unique structural features of cyclopamine and the related teratogenic compound jervine (Fig. 1) are required for the induction of holoprosencephaly in whole animal studies (reviewed in Gaffield and Keeler, 1996a,b). Subtle changes in the structures of cyclopamine and jervine dramatically affect their teratogenic potency, suggesting that a precise interaction with a target is required for their inhibitory action. Since cyclopamine blocks the response to Shh, the cyclopamine target is likely to be involved in Shh signal transduction. Given the extreme hydrophobicity of cyclopamine and related compounds, we expect that their target is a membrane-associated protein, possibly an element of the Shh receptor complex. Elements of the Shh receptor are the multiple membrane spanning proteins Patched (Ptc) and Smoothened (Smo), which act in concert to mediate the Shh signal (Marigo et al., 1996; Stone et al., 1996; Chen and Struhl, 1998; for review, see Ingham, 1998; Murone et al., 1999). Shh binds to Ptc, which in turn releases the inhibition of Smo by Ptc, thus activating the Shh response.

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The structural similarity between cyclopamine and cholesterol suggests a tantalizing potential mode of action of cyclopamine, especially in light of the association of holoprosencephaly, and thus attenuation of Shh signaling, with genetic or pharmacologic impairment of enzymes involved in the terminal steps of cholesterol biosynthesis. These latter conditions produce both reduced levels of tissue cholesterol and high levels of sterol precursors (Tint et al., 1994; Kolf-Clauw et al., 1996). In particular, mutations in the gene encoding Δ7-dehydrocholesterol (7-DHC) reductase (Fitzky et al., 1998) or inhibition of 7-DHC reductase by the inhibitor AY-9944 promote mild forms of holoprosencephaly (Roux and Aubry, 1966; Kelley et al., 1996). The simple hypothesis that both cyclopamine and AY-9944 inhibit the response of cells in the developing neural tube to Shh through the inhibition of cholesterol synthesis was refuted, however, since AY-9944 is effective only when responding cells are deprived of exogenous cholesterol and therefore have to rely on endogenously synthesized cholesterol, while the action of cyclopamine is independent of the activity of the cholesterol synthetic pathway (Incardona et al., 1998). Accumulation of putatively teratogenic cholesterol precursors, rather than cholesterol depletion, may underlie Shh-response inhibition by AY-9944. The

Cyclopamine, Cholesterol Transport, and Shh Signaling

FIG. 1. The structures of steroidal alkaloids and steroids tested for inhibition of Shh signaling. The structure of cholesterol is shown at top left and the generic jervane-type steroidal alkaloid structure is at top right. Natural jervane alkaloids and synthetic derivatives are listed below the generic structure, with differences indicated for the functional groups R" and R", and for the type of C-C bond at three variant positions. Cycloposine has a single glucose (glc) residue in a glycosidic linkage to the 3-OH. Veratramine (middle, left) occurs naturally and also is derived from cyclopamine by electrophilic attack on the furan (E) ring under acidic conditions. The spirosolane-type steroidal alkaloids solasodine and tomatidine are represented by a generic structure (middle, right). These two alkaloids differ only in the position of the N in the F ring (R" and R") and in the level of saturation at C-5, C-6. Note the differences in the E-F ring attachment to ring D between jervanes and spirosolanes, which results in an orientation of the spirolanopterine moiety of cyclopamine perpendicular to the planar steroid framework. The glycoalkaloid saponins tomatine and solasonine possess 4- and 3-residue oligosaccharides attached to the 3-OH group of tomatidine and solasodine, respectively. The steroidal amine U 18666A [3β-(2-diethylaminoethoxy)androstenedione] is represented at bottom left and progesterone at bottom right.

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In addition to cyclopamine and AY-9944, other compounds disturbing cholesterol homeostasis reportedly inhibit the response to Shh (Cooper et al., 1998). These compounds include other hydrophobic amines and steroids that interfere with intracellular cholesterol trafficking, such as imipramine, progesterone, and U18666A (Liscum and Munn, 1999). This suggested the possibility that Shh signaling through Ptc is linked to cellular cholesterol homeostasis, in particular to intracellular cholesterol transport (Cooper et al., 1998). A target for the hydrophobic amines and steroids that block intracellular cholesterol transport may be the Niemann–Pick C1 (NPC1) protein (Neufeld et al., 1999). Treatment of cells with these compounds induces a cellular cholesterol phenotype indistinguishable from that of cells from Niemann–Pick type C (NP-C) patients, which lack the NPC1 protein (Liscum and Faust, 1989; Rodriguez-Lafresse et al., 1990; Roff et al., 1991; Yoshikawa, 1993; Butler et al., 1992; Lange and Steck, 1994). NPC1 disease is a neurodegenerative lysosomal storage disorder, with an underlying and severe defect in intracellular transport of cholesterol and sphingolipids. Yet, loss of NPC1 function by mutation is not associated with effects on Shh signaling; NPC1 and NPC1-null mice do not have congenital malformations. This suggests that the inhibition of Shh signaling by cyclopamine, hydrophobic amines, and steroids is not related to their effects on intracellular cholesterol transport. Comparison of Ptc and NPC1 protein sequences demonstrates a significant similarity, largely, but not completely, confined to the membrane-spanning regions (Carstea et al., 1997; Loftus et al., 1997). This similarity includes a so-called “sterol-sensing domain,” a pattern of conserved transmembrane regions present in several other proteins involved in cholesterol homeostasis (Kumagai et al., 1995; Hua et al., 1996; Carstea et al., 1997; Loftus et al., 1997; Bae et al., 1999).

The similarity between Ptc and NPC1 suggests another mechanism by which hydrophobic amines and steroids interfere with Shh reception: not through their documented action on NPC1, but rather by acting on the same target as cyclopamine, a critical element of the Shh signal transduction cascade. It remains a good possibility that this target is Ptc, given its homology to NPC1. A converse prediction of this model is that cyclopamine and related compounds have an effect on cholesterol homeostasis through their interaction with NPC1 comparable to that of progesterone or U18666A.

To test these hypotheses we evaluated whether the relative potencies of cyclopamine, structurally related steroidal alkaloids that do not cause holoprosencephaly, U18666A, and progesterone to inhibit Shh signaling correlate with their effects on cholesterol transport. The results show a correlation between the attenuation of the Shh response and the disruption of NPC1-mediated vesicular trafficking. However, the relative concentrations of these compounds sufficient to perturb cholesterol trafficking and concentrations sufficient to block Shh signaling are very different. This suggests that the compounds act independently on targets within the Shh signaling pathway and the NPC1-mediated cholesterol transport pathway with varying efficiency. Our data suggest that the remarkable sensitivity of Shh signal transduction to cyclopamine and the less potent effects of other compounds we tested reflect a primary effect on Ptc function. The structural similarities of Ptc and NPC1 lead us to predict that Ptc mediates a vesicular trafficking process that is differentially sensitive to many of the same pharmacological manipulations and is crucial for Shh signal transduction.

**MATERIALS AND METHODS**

**Materials.** Veratrum steroidal alkaloids and their synthetic derivatives were obtained as described previously (Brown and Keeler, 1978; Gaffield et al., 1986). Progesterone was obtained from Sigma (St. Louis, MO) and U18666A was obtained from BioMol (Plymouth Meeting, PA). Steroidal alkaloids and progesterone were dissolved in 95% ethanol; U18666A was dissolved in water.

**Intermediate neural plate explants.** Explants of intermediate neural plate regions were isolated from Stage 9–10 chick embryos and cultured in collagen gel as previously described (Yamada et al., 1993; Incardona et al., 1998). Explants were distributed in batches of 10 onto pregelled collagen pillows and cultured at 37°C in Neurobasal medium supplemented with N3 mixture (N2 plus 10 mM hydrocortisone (Bottenstein, 1992)), MEM nonessential amino acids, penicillin/streptomycin, and 10 mM glucose. Test compounds were diluted in Neurobasal medium. In general compounds were titrated to determine the highest concentration in which explants maintained viability for 12–14 h. Shh signaling was then assayed at the concentrations just below those causing complete cell death within 12–14 h, allowing viability to 30 h. Control explants were cultured with an equivalent concentration of solvent (0.1% or less). For assessment of the Shh response, explants were harvested after 30 h of incubation, fixed in 4% paraformaldehyde, and processed for HN-F-3β, Isl1/2, and Pax7 immunofluorescence as described previously (Incardona et al., 1998). The response to Shh was quantified by tallying positive nuclei with a hand counter for at least 8 explants for each individual treatment.

For 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) assays, 15 explants per well were tested in duplicate or triplicate. So that more cellular material was assayed, slightly larger explants which included some dorsal and ventral regions were excised. After 24 h of incubation with the test compounds in the same medium as above, explants were washed with PBS, then quickly frozen in a dry ice/ethanol bath and stored at −80°C until assayed.

**Shh-N preparation.** Assays utilized recombinant Shh-N, either obtained as conditioned medium from human 293T cells transiently transfected with pShh-N as described previously (Roelink et al., 1995) or partially purified from High-Five (Invitrogen, Carlsbad, CA) cells infected with a recombinant baculovirus expressing Shh-N (H.R., unpublished). The specific activity of each preparation was determined by titration in the explant assay, and the Shh-N protein content was estimated by comparison to a standard after SDS-PAGE and Coomassie blue staining. Some preparations of Shh-N were shown to contain a fraction of material acylated at an N-terminal Cys, which has a potency 30-fold higher than unmodified Shh-N (Pepinsky et al., 1998). Our preparations most likely consisted of such a mixture, as suggested by the appearance of a doublet band on Western blots (data not shown). Also consis-
tent with this, the baculovirus preparation used for most of the experiments described here proved to be 10-fold more potent than the bacterially expressed, unmodified protein originally used to establish the Shh-N dose-response curve (Roelink et al., 1995; Ericson et al., 1997). However, neither the N-terminal acylation nor the C-terminal cholesterol modification of wild-type Shh is required to assess the inhibitory activity of the compounds tested, as similar results were obtained using bacterially expressed Shh-N (Cooper et al., 1998).

**RESULTS**

**The Neural Plate Explant Assay**

Chick embryo neural plate explants were cultured in the presence of Shh-N, which leads to the induction of markers for floor plate (HNF3β) and motor neuron (Isl1/2) differentiation, as well as repression of a marker for dorsal precursor cells (Pax7) (Roelink et al., 1995; Ericson et al., 1997; Briscoe and Ericson, 1999). In a typical Shh-N dose response, HNF-3β+ floor plate cells are maximally induced by 1–2 nM Shh-N, Isl1/2+ motor neurons are maximally induced by 0.3–0.5 nM Shh-N, and Pax7+ dorsal precursor cells are repressed by 0.05–0.1 nM Shh-N. Thus, at the highest Shh-N concentrations, HNF-3β+ induction predominates, although this is at the expense of Isl1/2+ cells (typically 60–100 Isl1/2+ cells and 1000–1200 HNF-3β+ cells). Isl1/2+ cells are maximally induced (typically 200–300 cells in a 1500-cell explant) at concentrations of Shh-N that induce few or no HNF-3β+ cells. Even lower concentrations of Shh-N repress Pax7 in the absence of Isl1/2 or HNF-3β induction. Therefore, the number of HNF3β cells is a straightforward, but somewhat less sensitive indicator of Shh signaling. The Shh-dependent induction of Isl1/2 is more sensitive, but maximum response occurs in a relatively narrow dose range, with the number of Isl1/2+ cells per explant decreasing both at higher and at lower Shh concentrations and can thus be used as a reliable quantitative indicator of the Shh response only in conjunction with Pax7 or HNF3β induction.

At a constant high Shh-N concentration (2.5 nM), the cyclopamine dose response mimics a reverse of the Shh-N dose response (Roelink et al., 1995; Ericson et al., 1997). Induction of HNF-3β+ cells is reduced and more Isl1/2+ cells appear at low concentrations of cyclopamine (12–24 nM); at intermediate cyclopamine concentrations HNF-3β induction is completely blocked (24–48 nM); while Isl1/2 induction persists; at higher cyclopamine concentrations both HNF-3β and Isl1/2 induction is blocked, while some Pax7 repression persists (60 nM); all signaling is blocked by 120 nM cyclopamine (Fig. 3, data not shown and Incardona et al., 1998). Because each marker (HNF3β, Isl1/2, and Pax7) responds maximally to a different threshold Shh-N concentration, comparisons of the inhibitory activity of the different compounds are most straightforward using a single marker. Although in general we tested the effects of most of the compounds on all three markers, only data for HNF3β are shown.

**Steroidal Alkaloids Have Specific Structural Requirements for Inhibition of Shh Signaling**

To determine if Keeler and colleagues’ results reflected an interaction with a proximate target within the Shh signaling pathway, or differences in whole-animal pharmacokinetics, we tested many of their same collection of steroidal alkaloids both in the neural plate explant assay and by direct application to chick embryos (data not shown). To
alkaloids thus affect Shh signaling at a concentration 100-fold above that sufficient to obtain comparable inhibition by cyclopamine, but are unable to completely block signaling. Similarly, application of 5 \( \mu \)g tomatidine or solasodine to chick embryos in ovo resulted in 1 or 2 of 60 embryos with mild holoprosencephaly and minor defects in the floor plate at cervical levels (data not shown), suggesting that teratogenic concentrations are achieved in embryos only rarely. In contrast, malformations in cyclopamine-treated embryos are much more common and show greater severity both grossly and at the molecular level (Incardona et al., 1998). Therefore, the in vitro and in ovo effects on Shh signaling for steroidal alkaloids with E–F ring structures different from cyclopamine are consistent with their weaker teratogenicity.

In addition to the E–F ring system, changes in both polarity and steric bulk of the planar steroid ring system also affect teratogenic potency. When administered by gavage to hamsters, jervine was consistently more potent than cyclopamine (Gaffield and Keeler, 1996b). This is likely due to the greater resistance of jervine to stomach acid-catalyzed opening of the furan ring than cyclopamine (Gaffield and Keeler, 1996a), resulting in a higher effective dose in whole animals (Fig. 1). However, jervine is a weaker teratogen in chick embryos, producing fewer holoprosencephalic embryos (data not shown), and 5- to 10-fold less potent than cyclopamine in the explant assay, producing an incomplete (90%) block of HNF-3\( \beta \) induction (Fig. 2) at 240 nM. In contrast, oxidation of the 3-OH in cyclopamine to produce cyclopamine-4-en-3-one (Fig. 1) resulted in enhanced potency in the hamster assay (Brown and Keeler, 1978), and this derivative produced a near-complete inhibition of Shh signaling in explants at 48 nM (Fig. 2). Cyclopamine-4-en-3-one appears to be at least 2-fold more potent than cyclopamine in the explant assay.

Other structural changes of the planar ring system also produced a loss of potency in the hamster assay (Brown and Keeler, 1978; Gaffield and Keeler, 1993). Reduction of the C-5, C-6 and C-12, C-13 double bonds of jervine produced the weaker teratogen, tetrahydrojervine (Fig. 1). Tetrahydrojervine is about threefold weaker than jervine at blocking HNF-3\( \beta \) induction in explants (Fig. 2), producing 43% inhibition at 240 nM. Finally, cycloposine, a glycoalkaloid analog of cyclopamine bearing a single glucose bonded to the 3-OH (Fig. 1), was found to be considerably weaker than cyclopamine in the explant assay (Fig. 2), producing 34% inhibition at 120 nM. Similarly, application of 5 \( \mu \)g tomatidine or solasodine to chick embryos in ovo resulted in 1 or 2 of 60 embryos with mild holoprosencephaly and minor defects in the floor plate at cervical levels (data not shown), suggesting that teratogenic concentrations are achieved in embryos only rarely. In contrast, malformations in cyclopamine-treated embryos are much more common and show greater severity both grossly and at the molecular level (Incardona et al., 1998). Therefore, the in vitro and in ovo effects on Shh signaling for steroidal alkaloids with E–F ring structures different from cyclopamine are consistent with their weaker teratogenicity.

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Overall, these data show that the relative teratogenic potencies for steroidal alkaloids in the hamster assay (Gaffield and Keeler, 1996a,b) are very similar to their relative abilities to block Shh signaling in explants, with a trivial exception, the acid-catalyzed conversion of cyclopamine to veratramine in the nonruminant mammalian stomach. The
significant teratogenic potencies and precise structural requirements of certain steroidal alkaloids likely reflect a specific interaction with a component of the Shh signaling pathway.

To further exclude the possibility that the Shh-inhibiting actions of cyclopamine and related compounds are indirectly caused by interference with cholesterol homeostasis, we tested well-known cholesterol transport blockers for their capacity to inhibit the Shh response in neural plate explants in vitro.

**The Prototypical Cholesterol Transport Blockers U18666A and Progesterone Are Weak Shh-Response Inhibitors**

Using the explant assay, we compared the effects of cyclopamine to those of two compounds, U18666A and progesterone, with known effects on cholesterol transport, most likely as a consequence of their blocking of normal NPC1 function (Higgins et al., 1999; Neufeld et al., 1999; and Y. Lange, unpublished observations). U18666A and progesterone were reported to block the response to Shh-N at concentrations of 0.25 and 20 μM, respectively (Cooper et al., 1998). A more detailed analysis revealed that U18666A and progesterone inhibit Shh signaling only partially at those concentrations and cannot completely inhibit responses to Shh-N, even at higher concentrations near the toxic threshold (Fig. 3). Although inhibition of HNF3β induction is detected with low concentrations of U18666A (80% of control at 0.048 μM), HNF3β+ cells were detected in explants treated with 0.48 μM U18666A (19% of control), and Isl1/2 induction persisted at high levels (443% of control; data not shown). At U18666A concentrations of 2.4 μM HNF3β induction was completely blocked, but Isl1/2 induction still persisted at 33–66% of controls (data not shown). Concentrations higher than 2.4 μM were too toxic for explants.

Progesterone proved to be an even weaker inhibitor of Shh signaling at very high concentrations relative to cyclopamine (Fig. 3). At 10 μM progesterone had only a moderate inhibitory effect on HNF3β induction (68% of control), while increasing Isl1/2 induction (data not shown). Progesterone at 30 μM produced a more significant inhibition of HNF3β (5%), but significant Isl1/2 induction remained (67% of control; data not shown). Higher concentrations of progesterone killed the explants within 12 h. Progesterone and U18666A were, thus, unable to block all of the Shh responses without significant toxicity, and the concentrations of progesterone and U18666A that maximally inhibited Shh signaling (30 and 2.4 μM, respectively) produced considerable cell death at the periphery of the explants (data not shown). In contrast, the cell death observed in explants treated with 0.12 μM cyclopamine was comparable to that of explants cultured without Shh-N (data not shown), consistent with the complete block in Shh signaling. As summarized in Table 1, we estimated the IC₅₀ for inhibition of HNF3β induction by many of the compounds.

**TABLE 1**

Comparison of IC₅₀s for Inhibition of HNF3β Induction and Esterification of Cholesterol at the Endoplasmic Reticulum

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC₅₀ HNF3β inhibition</th>
<th>IC₅₀ esterification inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclopamine</td>
<td>0.01 μM</td>
<td>9 μM</td>
</tr>
<tr>
<td>Jervine</td>
<td>0.08 μM</td>
<td>7 μM</td>
</tr>
<tr>
<td>Veratramine</td>
<td>3 μM</td>
<td>3 μM</td>
</tr>
<tr>
<td>Tomatidine</td>
<td>2 μM</td>
<td>15 μM</td>
</tr>
<tr>
<td>Solasodine</td>
<td>1 μM</td>
<td>15 μM</td>
</tr>
<tr>
<td>U18666A</td>
<td>0.1 μM</td>
<td>0.1-0.5 μM</td>
</tr>
<tr>
<td>Progesterone</td>
<td>15 μM</td>
<td>3 μM</td>
</tr>
</tbody>
</table>

*IC₅₀s were estimated from plots similar to that shown in Fig. 3.

* Incorporation of [3H]cholesterol into esters was measured in rat hepatoma cells as described under Materials and Methods. IC₅₀s were estimated by plotting the percentage of esterified cholesterol in cells treated with concentrations of steroidal alkaloids ranging from 0.1 to 40 μM, relative to control cells incubated with ethanol solvent only. Values were obtained from triplicate measurements with SEM within 10%.

* Underwood et al., 1996.

Veratramine and the spirosolane steroidal alkaloids are roughly 100-fold weaker inhibitors than cyclopamine, U18666A is about 10-fold weaker, and progesterone is about 1000-fold weaker. If these compounds act indirectly on Shh signaling by interfering with cholesterol homeostasis in responding cells, there should be a similar correlation between the ability of each compound to inhibit the Shh response and the ability to block cholesterol transport.

The Ability of Steroidal Alkaloids and Steroids to Block Cholesterol Transport Does Not Correlate with Their Ability to Inhibit Shh Signaling

Under normal conditions, LDL-associated cholesterol is internalized by cells and subsequently distributed to various parts of the cell through an active process. Inhibition of intracellular cholesterol transport is manifested by several changes in cholesterol homeostasis that can be quantified: (1) increased activity of HMGR, the rate-limiting enzyme in cholesterol biosynthesis, due to reduced traffic of cholesterol to the ER, and thus ER cholesterol levels; (2) inhibition of esterification of cholesterol in the ER, also reflecting reduced cholesterol trafficking to the ER; and (3) accumulation of internalized LDL-cholesterol in lysosomes, due to the failure of normal redistribution of internalized LDL-cholesterol from late endosomes. Although the precise cholesterol transport pathway(s) measured by these assays are incompletely characterized, the assays provide complementary information and overall produce similar results. Using these three assays, we found that at relatively high concentrations, all steroidal alkaloids have similar effects on cholesterol homeostasis and that a concentration of cyclopamine sufficient to completely block Shh signaling does not disrupt cholesterol transport in neural plate cells. HMGR levels were measured in chick neural plate explants treated with 2.5 nM Shh-N and various compounds. Treatment with Shh-N alone (data not shown), plus 120 nM cyclopamine, sufficient to completely block Shh responsiveness, or 120 nM veratramine had no significant effect on explant HMGR levels (Fig. 4). Therefore, the complete inhibition of Shh signaling in explants is not coupled to a corresponding block in cholesterol transport to the ER. In contrast, treatment of explants with 120 nM U18666A resulted in a 4-fold increase in HMGR activity (Fig. 4), although this concentration of U18666A induces only a partial block of Shh signaling (Fig. 3). Progesterone at 30 μM produced a nearly 10-fold increase of HMGR activity (Fig. 4), but likewise, this concentration of progesterone produced only an incomplete block of Shh signaling (Fig. 3). Thus even when movement of cholesterol to the ER is significantly blocked by U18666A or progesterone, some Shh signaling still occurs. Overall, there is little correlation between a compound’s ability to inhibit cholesterol transport, as it relates to upregulation of HMGR activity in neural plate cells, and its ability to block Shh signaling, indicating that the inhibition of cholesterol transport and inhibition of Shh signal response are mediated via distinct targets.

To determine if the observed IC_{50}s for HNF3β induction correlated with IC_{50}s for inhibition of cholesterol transport, we utilized an assay of ER cholesterol esterification in a mammalian cell line (Table 1), since neural plate explants provide only very small amounts of tissue. At a cyclopamine concentration sufficient to completely block Shh response, there was no effect on esterification of plasma membrane-derived cholesterol (data not shown). At much higher concentrations cyclopamine and jervine had very similar effects on cholesterol esterification, with IC_{50}s of 9 and 7 μM, respectively. Veratramine, which does not induce holoprosencephaly, was slightly more potent, with an IC_{50} near 3 μM. All three jervane steroidal alkaloids produced an 80–90% block of cholesterol esterification at 12 μM (data not shown), which is near the toxic threshold for most cell types. The spirosolane steroidal alkaloids (tomatidine and solasodine) were slightly weaker inhibitors of cholesterol esterification, with virtually identical IC_{50}s of 15 μM. However, these compounds are orders of magnitude weaker than cyclopamine in Shh signaling inhibition. The significant differences in teratogenicity among the steroidal alkaloids are not reflected in their capacity to block esterification of cholesterol. However, the published IC_{50}s for inhibition of cholesterol esterification by U18666A and progesterone (Table 1) are relatively close to the concentrations at which they inhibit Shh signaling, although progesterone appears to be weaker at inhibition of Shh signaling than inhibition of cholesterol esterification.

Similar results were observed for accumulation of LDL-

![FIG. 4. Effects of steroidal alkaloids, U18666A, and progesterone compounds on HMGR activity in neural plate cells. Chick neural plate explants were cultured for 24 h in 2.5 nM Shh-N and the indicated concentrations of cyclopamine (CpA), veratramine (VER), U18666A (U), and progesterone (PG). HMGR activity was determined as described under Materials and Methods. The values represent the means ± SEM HMGR specific activity.](image-url)
cholesterol in lysosomes of cultured cells, assayed by filipin cytochemistry (Fig. 5 and Table 2). Cyclopamine and tomatidine were found to have very similar effects on trafficking of lysosomal LDL-cholesterol. A moderate accumulation of cholesterol was produced by 1.2 μM cyclopamine (Fig. 5C), observed in 61% of treated cells, and 1.2 μM tomatidine (Fig. 5G), in 58% of treated cells. Both compounds at 12 μM (Figs. 5D and 5H, respectively) produced a maximal effect comparable to 30 μM progesterone (Fig. 5B), with 100% of treated cells showing filipin 1 lysosomes. Veratramine was significantly more potent in this assay, producing a moderate cholesterol accumulation at 0.12 μM (Fig. 5E), with 67% of treated cells showing filipin 1 lysosomes.

All the steroidal alkaloids tested thus have roughly similar cholesterol transport blocking activity, with veratramine more potent in some assays. These data are consistent with observations that most hydrophobic amines can block cholesterol transport, but clearly indicate that effects on intracellular cholesterol trafficking do not correlate with their teratogenic potency in blocking the Shh response. It is notable, however, that NPC1 appears to play a key role in the cholesterol trafficking pathway(s) measured by these three assays, as defects in each are observed in cells from N-P-C patients. Because some of these drugs may affect a vesicular compartment linked to NPC1 function (Higgins

**TABLE 2**

Quantification of the Accumulation of LDL-Cholesterol in Lysosomes Induced by Steroidal Alkaloids and Progesterone

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (μM)</th>
<th>Filipin+ cells/total (percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>N/A</td>
<td>2/27 (7)</td>
</tr>
<tr>
<td>Veratramine</td>
<td>0.12</td>
<td>18/27 (67)</td>
</tr>
<tr>
<td>12</td>
<td>25/25 (100)</td>
<td></td>
</tr>
<tr>
<td>Cyclopamine</td>
<td>1.2</td>
<td>17/28 (61)</td>
</tr>
<tr>
<td>12</td>
<td>27/27 (100)</td>
<td></td>
</tr>
<tr>
<td>Tomatidine</td>
<td>1.2</td>
<td>19/33 (58)</td>
</tr>
<tr>
<td>12</td>
<td>28/28 (100)</td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>30</td>
<td>29/29 (100)</td>
</tr>
</tbody>
</table>
et al., 1999; Kobayashi et al., 1999; Neufeld et al., 1999; Holtta-Vuori et al., 2000; Watari et al., 2000), it is possible that this same compartment is linked to Ptc function and Shh signaling.

**Ptc-1 and NPC1 Colocalize Extensively in Transfected Cells**

NPC1 is normally found on cytoplasmic vesicles with late endosomal characteristics (Higgins et al., 1999; Neufeld et al., 1999; Holtta-Vuori et al., 2000; Watari et al., 2000). In the Drosophila embryo Ptc is found on multivesicular bodies, indicating that it is present in a late endocytic compartment (Capdevila et al., 1994). Recent evidence suggests that Ptc-1, the vertebrate Ptc-family member expressed in neural plate cells, mediates the endocytosis of Shh during signaling in neural plate cells (Incardona et al., submitted for publication). In transfected cells, epitope-tagged Ptc-1 (PtcHA) is found predominantly intracellularly (Fig. 6A), with a significant component present in endocytic vesicles (Incardona et al., submitted for publication). Although PtcHA is essentially undetectable at the cell surface, PtcHA-expressing cells rapidly internalize Shh, which colocalizes with PtcHA in vesicles (Incardona et al., submitted for publication). The pattern of PtcHA immuno-fluorescence is virtually identical to that of NPC1 overexpressed in the same cell type (Fig. 6B and Higgins et al., 1999), with immunolabeled vesicles of 0.5–1 μm diameter appearing peripherally in filopodia, as well as a prominent perinuclear cluster of positive vesicles. In cells cotransfected with PtcHA and NPC1 constructs, both proteins colocalize extensively in peripheral vesicles and in the perinuclear region (Figs. 6C–6E), suggesting that Ptc-1 and NPC1 are targeted to the same compartment(s).

**DISCUSSION**

It was recently proposed that defects in intracellular cholesterol transport and homeostasis are the common basis for the induction of holoprosencephaly by cyclopamine and the 7-DHC reductase inhibitor AY-9944 (Cooper et al., 1998). This hypothesis was based on the findings that compounds known to block intracellular cholesterol transport also inhibited Shh signaling in vitro and that high concentrations of both AY-9944 and cyclopamine could also block cholesterol transport. Our data do not support this hypothesis for the following reasons: (1) Among the compounds tested, only cyclopamine completely blocked Shh signaling in the absence of cytotoxicity, and teratogenic concentrations did not block cholesterol transport in neural plate cells. (2) Cyclopamine and steroidal alkaloids with weak Shh inhibitory activity were generally equipotent in assays of cholesterol transport. (3) U18666A and progesterone are weak Shh antagonists, despite markedly affecting cholesterol homeostasis in neural plate explants.

The unique structure of cyclopamine is absolutely required for induction of holoprosencephaly in whole animals and to block Shh signaling in vitro. In contrast, the structural requirements associated with inhibition of intracellular cholesterol transport are different. Although the large majority of compounds with this activity are simply hydrophobic amines with very diverse structures (Lange and Steck, 1994), there is significant structural specificity for the inhibition of cholesterol transport by steroids, indicated by the potency of progesterone relative to other natural steroids (Butler et al., 1992). Moreover, U18666A, which is both a hydrophobic amine and a steroid, is one of the most potent blockers of cholesterol transport, with an IC₅₀ ranging from 100 to 800 nM depending on the assay (Underwood et al., 1996). Nevertheless, both progesterone and U18666A are relatively weak Shh antagonists, but U18666A is significantly more potent than progesterone. Although high concentrations of cyclopamine can inhibit cholesterol transport, we believe that this activity reflects a side effect unrelated to inhibition of Shh signaling.

The effects of cyclopamine on both embryos treated in ovo and neural plate explants must reflect inhibition of the signal transduction cascade at a very early point. Activation of Shh target genes in neural precursors occurs through either of at least two downstream branches: HNF3β expression in the floor plate is controlled by Gli transcription factors, whereas induction of motor neuron-specific genes is independent of Gli function (Ding et al., 1998; Matise et al., 1998; Park et al., 2000) and appears to involve another transcription factor (Krishnan et al., 1997a,b). Because floor plate and motor neuron inductions are both remarkably sensitive to inhibition by cyclopamine, the target of cyclopamine must be proximal to the bifurcation in the pathway leading to these distinct fates. Other elements of the Shh signaling cascade that are upstream of and influence Gli function include Fused and Suppressor of fused (Carpenter et al., 1998; Ding et al., 1999; Kogerman et al., 1999; Pearse et al., 1999; Stone et al., 1999). The fly homolog of Gli, Ci, also interacts with Costal2 (Robbins et al., 1997; Sisson et al., 1997), but no vertebrate Costal2 homolog has been identified. A role for any of these elements in motor neuron development has not been demonstrated, but it seems likely that cyclopamine acts upstream of their involvement. The only other upstream factors known to date are Ptc and Smo.

The simplest model explaining our data is a common mechanism of inhibition by cyclopamine, U18666A, and progesterone. It is unlikely that perturbation of Shh responsiveness is a direct or indirect consequence of NPC1 antagonism because Shh signaling is intact in embryos lacking NPC1. No malformations are associated with NPC1 mutations in humans or mice, although defects in cholesterol transport can be detected at fetal stages (de Winter et al., 1992; Vanier et al., 1992; Dumontel et al., 1993; Rodriguez-Lafraisse et al., 1994). Moreover, defects in intracellular cholesterol distribution similar to that observed in NP-C cells were recently associated with several other genetic lipid storage diseases (Puri et al., 1999), none of
which are known to produce defects in Shh signaling. Why would Shh signaling be inhibited as a consequence of pharmacologic disruption of intracellular cholesterol transport, but not genetic disruption of the same pathways? The relationship of Ptc to NPC1 and the known effects of U18666A and progesterone on NPC1 function suggest that Ptc function is the direct or indirect target of these compounds in the Shh signaling pathway.

We favor the hypothesis that a common vesicular trafficking pathway may underlie both cholesterol transport and the regulation of Shh signaling by Ptc. This might explain the disruption of Shh signaling by drugs that block cholesterol transport (U18666A, progesterone), as it is known that intracellular trafficking of NPC1 is dramatically affected by the same agents (Higgins et al., 1999; Neufeld et al., 1999). On the contrary, loss of one passenger on this pathway (e.g., NPC1) by mutation would not be expected to disrupt the trafficking of another (Ptc). Although the IC₅₀s for inhibition of both cholesterol transport and HNF3β induction are similar for both U18666A and...
progesterone, the idea that these two compounds affect Shh signaling via inhibition of cholesterol transport is not supported by the genetic data.

The extensive colocalization of PtcHA and NPC1 and the potential role of endocytic trafficking of Shh in signaling (Incardona et al., submitted for publication) suggest important functional ties to the structural similarity of Ptc and NPC1. It is possible that all the compounds indirectly affect both NPC1 and Ptc by affecting another protein or lipid with which they have a common functional interaction. On the other hand, the potency of cyclopamine could reflect an activity distinct from the other hydrophobic amines and steroids. Nevertheless, recent studies provided evidence for regional lipid specializations within endosomal compartments (Kobayashi et al., 1998; Mayor et al., 1998; Mukherjee et al., 1999), including a luminal region of late endosomes involved in cholesterol transport that is enriched with a unique lipid, lysosphosphatidic acid, and perturbed by U18666A (Kobayashi et al., 1999). We speculate that the function of both NPC1 and Ptc may be related to such intracellular lipid specializations and that the regulation of Smo activity by Ptc involves vesicular trafficking.

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