INHIBITION OF CHOLINE UPTAKE IN SYNCTIAL MICROVILLUS MEMBRANE VESICLES OF HUMAN TERM PLACENTA

SPECIFICITY AND NATURE OF INTERACTION

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Abstract—The potency and nature of the inhibitory effect of various cationic drugs on the transport of choline across the placental syncytiotrophoblastic membrane was investigated. Tetraethylammonium, a model substrate for organic cation transport, was a poor inhibitor. Enlarging the degree of alkylation of the quaternary ammonium increased the inhibitory effect, in proportion with increasing lipophilicity. Log concentration vs % control uptake curves showed marked differences in inhibitory potency for the different cationic drugs. Hemicholinium-3 inhibited mediated choline uptake in the micromolar range, whereas aretopine and mepiperphenidol were less potent. The H₂-receptor antagonists cimetidine, ranitidine, and famotidine inhibited choline uptake in the millimolar range. Dixon analysis revealed a competitive nature of inhibition for hemicholinium-3 and aretopine (Kᵢ = 40 μM and 1.2 mM, respectively). Cimetidine interacted noncompetitively (Kᵢ = 3.4 mM). Since relatively high concentrations were needed to reach half maximal inhibition, impairment of fetal choline supply due to maternal drug use during pregnancy is not to be expected.

Key words: choline transport; inhibition; cationic drugs; human placenta; syncytiotrophoblastic membrane vesicles

Choline, a cationic quaternary ammonium compound, is an essential substrate for adequate growth and development of the fetus. Choline serves as a substrate for the synthesis of phospholipids and acetylcholine. Since the human placenta and fetus do not synthesize choline, fetal supply is highly dependent on the proper transfer of this nutrient from maternal to fetal circulation [1]. The initial step in placental transfer involves uptake across the syncytiotrophoblastic membrane of the syncytiotrophoblast. In the perfused human placental cotyledon, trans-placental choline transfer was slow in contrast to the fast and high placental uptake [2]. Since there was a small excess trans-placental choline transfer in comparison with the extra-cellular marker mannitol, preferentially towards the fetal circulation, the human placenta possesses a unidirectional pathway for choline in the maternal to fetal direction. Placental choline uptake at the maternal side was inhibited by choline itself and at the fetal side by HC-3,‡ a well-known competitive inhibitor of choline transport, suggesting the existence of specific transport systems at both sides of the trophoblast. Evidence for mediated trophoblastic choline uptake was also found in human placental fragments, which accumulated choline against a concentration gradient, inhibitable by HC-3 with a Kᵢ of 0.45 mM. However, because uptake in fragments is the net result of transport across both the syncytiotrophoblastic and basal membranes, it is impossible to differentiate between inhibition of the choline transporter at both sides of the trophoblast. Isolated vesicles of these membranes are a more appropriate tool for investigating the location and nature of such interactions. Recently, we described the mechanisms of choline uptake into isolated SMMV of human term placenta [3]. Uptake was not sodium-dependent or coupled to proton transport. An inside negative membrane potential enhanced choline uptake, showing that a negatively charged inner membrane surface acts as a driving force for trophoblastic choline uptake. Mediated transport was confirmed by the trans-stimulatory effect of unlabeled choline and cis-inhibitory effects of HC-3, the organic cation transport inhibitor mepiperphenidol, and H₂-receptor antagonists. The model substrate for organic cation transport, TEA and NMN, did not inhibit choline transport. Furthermore, we found that uptake under trans-stimulation conditions was saturable with a Kᵢ of 550 μM. Our results were confirmed in a study by Grassl, who used the same approach [4]. In addition he showed, by studying the inhibitory potency of a large number of organic cations, that at least two sites of interaction with the placental choline transporter can be postulated: a negative site that binds with the positively charged nitrogen and a site of hydrogen bonding that interacts with the primary alcohol. Furthermore, the degree of nitrogen group alkylation appeared to be of importance.

Since trophoblastic uptake is the rate-limiting step in fetal choline supply, interaction of maternally adminis-
tered drugs with the choline transporter at the microvillus membrane could have clinical implications for fetal growth and development. This study was designed to investigate further the specificity of the choline transporter at the syncytiotrophoblast, by characterizing the inhibitory potency and nature of the interaction of various cationic drugs.

**MATERIALS AND METHODS**

**Chemicals**

\[^{3}H\]-Choline was obtained from Amersham (Buckinghamshire, U.K.). Cimetidine was kindly donated by Smith, Kline & French (Welwyn Garden City, Herts, U.K.), and mepipiperphendol and famotidine by Merck, Sharp & Dohme (Rahway, NJ, U.S.A.). All other chemicals were purchased from either Sigma (St. Louis, MO, U.S.A.), Merck (Darmstadt, Germany), or Boehringer Mannheim (Mannheim, Germany), and were of analytical grade. GF/FP filters were obtained from Whatman Int. Ltd. (Maidstone, U.K.).

**Preparation of SMMV**

SMMV were prepared from fresh human term placenta according to an established method [5], which was further improved upon in our laboratory [3, 6]. Briefly, tissue was minced in a Waring blender and stirred for 30 min to loosen the microvilli. After MgCl₂ aggregation and differential centrifugation, SMMV were harvested and suspended in the appropriate intravesicular buffer for uptake studies, to a final protein concentration of 10–15 mg/mL. Vesicles were frozen in liquid nitrogen and stored at -80° for four weeks at the maximum. This freezing and storage procedure did not influence choline uptake. The alkaline phosphatase enrichment of SMMV was further improved upon in our laboratory [3, 6].

**Uptake studies**

Uptake of \[^{3}H\]-choline into SMMV was measured in quadruplicate at 37° using a rapid filtration technique [8]. The samples were filtered through Whatman GF/F filters (average pore size 0.7 μm), and the radioactivity remaining on the filters counted in a Beckman LS 6000 LL liquid scintillation counter. Corrections were made for nonspecific filter binding. The exact conditions of the transport experiments are given in the legends. Uptake is expressed as pmol or nmol/mg protein or % of control uptake (mean ± SD), N representing the number of experiments with different placenta.

**Data analysis**

From concentration vs % uptake curves of three concentrations of choline below \(K_m\) (50, 125, and 250 μM), the concentration required to reach half maximal inhibition (IC\(_{50}\)) of several cationic drugs was estimated by least-squares nonlinear regression analysis, using the computer program GraphPad Inplot 4.0 (GraphPad Software Inc., San Diego, CA, U.S.A.). The weighted residual sums of squares of one- and two-site models were compared using the F-test. Transformation of the data according to Dixon revealed the nature of inhibition. The inhibitory constant (\(K_i\)) for a competitive inhibitor was estimated according to the equation of Cheng-Prusoff: 

\[ K_i = IC_{50}/(1 + S/K_m) \]

where \(S\) = choline concentration [9]. For \(K_m\) and \(V_{max}\) of choline, previously determined values were used, viz. 550 μM and 10 nmol/mg/10 sec, respectively [3]. In case of a noncompetitive inhibitor, IC\(_{50}\) is independent of \(S\), and consequently \(K_i\) equals IC\(_{50}\). Paired Student’s t-test was used to determine statistical significance (\(P < 0.05\)).

**RESULTS**

**Inhibitory potency of tetraalkylammonium compounds**

In our previous study it was found that 5 mM TEA did not inhibit choline (250 μM) uptake into SMMV [3]. We now investigated whether variation in the degree of alkylation of TEA influences inhibitory potency. Enlarging the alkyl chains at the quaternary ammonium resulted in a higher percentage of inhibition (Fig. 1). The IC\(_{50}\) values determined from inhibition curves at a choline concentration of 250 μM and inhibitory constants \(K_i\) (\(*\) competitive; \(\dagger\) noncompetitive) determined from inhibition curves at 50, 125, and 250 μM choline. Values are presented as means ± SD, \(N = 3\), except for famotidine and TEA.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC(_{50}) (mM)</th>
<th>(K_i) (mM)</th>
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<tbody>
<tr>
<td>Choline</td>
<td>0.55 ± 0.11</td>
<td>-</td>
</tr>
<tr>
<td>HC-3</td>
<td>0.069 ± 0.004</td>
<td>0.039 ± 0.009*</td>
</tr>
<tr>
<td>Atropine</td>
<td>1.45 ± 0.30</td>
<td>1.24 ± 0.10*</td>
</tr>
<tr>
<td>Mepipiperphendol</td>
<td>0.85 ± 0.55</td>
<td>-</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>3.39 ± 0.47</td>
<td>3.39 ± 0.47†</td>
</tr>
<tr>
<td>Famotidine</td>
<td>4.04 ± 0.62</td>
<td>-</td>
</tr>
<tr>
<td>TEA</td>
<td>&gt;50 (N = 1)</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 2. Inhibition of 250 μM [3H]-choline uptake into SMMV at 10 sec under trans-stimulation conditions by various organic cations. Experimental conditions were the same as described in the legend of Fig. 1. Values are expressed as % of representative control uptakes vs log concentration inhibitor (mM). Each point represents the mean ± SD of three experiments with three placentae, except for famotidine and TEA (N = 1).
increase in inhibitory potency of the compounds corresponded well with the increase in lipophilicity as given by their calculated log \( P \) values [10]. Because of the surface tension lowering properties of tetraalkylammonium compounds, we verified whether the vesicles stayed intact in the presence of the inhibitors. Only in the presence of THA was the equilibrium uptake of 250 \( \mu \text{M} \) choline at 60 min significantly reduced as compared with control uptake. The other compounds did not interfere with the membrane integrity.

**Inhibition of choline uptake by several organic cations**

Plots of log concentration inhibitor vs % of control uptake of 250 \( \mu \text{M} \) choline are shown in Fig. 2. The results of the nonlinear regression analysis of the typically sigmoid shaped curves are summarized in Table 1. Marked differences in inhibitory potency can be seen between the organic cations tested. HC-3 inhibited choline uptake for 50% at a relatively low concentration, whereas the organic cation transport inhibitor mepiphenidol and the anticholinergic drug atropine were less potent inhibitors. The \( \text{H}_2\)-receptor antagonists cimetidine, ranitidine, and famotidine showed IC\(_{50}\) values only in the mM ranges. For TEA only a rough estimate of the IC\(_{50}\) value could be made, because of the very high concentrations (>10 mM) necessary to achieve half maximal inhibition. In all cases a two-site model did not fit the data better than a one-site model (\( P > 0.2 \)).

**Nature of interaction with the choline transporter**

Although IC\(_{50}\) values provide a good measure of inhibitory potency, they cannot explain the nature of the interaction. We used the method of Dixon analysis to evaluate the type of choline transport inhibition by HC-3, atropine, and cimetidine. The concentration-dependent inhibition of these compounds was measured at three choline concentrations: 50, 125, and 250 \( \mu \text{M} \). Increasing the substrate concentration decreased the inhibitory effectiveness of HC-3 and atropine, resulting in a lower IC\(_{50}\) value at a lower choline concentration. Transformation of the data according to Dixon showed that the lines intersected above the \( X \) axis and to the left of the \( Y \) axis, indicating a competitive mode of interaction of HC-3 and atropine with the choline transporter (Fig. 3). IC\(_{50}\) values for competitive inhibition, calculated from the Cheng-Prusoff equation for the three choline concentrations, were 40 \( \mu \text{M} \) for HC-3 and 1.2 mM for atropine (Table 1). In contrast, the IC\(_{50}\) value for cimetidine was independent of the choline concentration. Dixon analysis resulted in an intersection of the lines on the \( X \) axis, consistent with a noncompetitive type of interaction (Fig. 3). Consequently, the IC\(_{50}\) value for cimetidine equaled the IC\(_{50}\) value of 3.4 mM (Table 1).

**DISCUSSION**

The present study demonstrates that several cationic drugs inhibited human placental choline transport across the syncytial microvillus membrane with different inhibitory potencies. HC-3 and atropine appeared to be competitive inhibitors, whereas cimetidine interacted noncompetitively. TEA, a model substrate for organic cation transport in various tissues, only inhibited choline transport at very high concentrations. Enlarging the degree of alkylation of this quaternary ammonium compound increased the inhibitory effect in proportion with increasing lipophilicity.

Wright et al. postulated a set of structural elements important for interaction with the choline transporter: (a) a terminal hydroxyl group; (b) the positive charge of the nitrogen; and (c) the presence of at least two free methyl...
groups at the positive nitrogen [11]. The placental choline transporter appeared to have a similar substrate specificity [4]. Small quaternary ammonium compounds such as TMA, acetycholine, and N-methyl-N-nicotinamide showed affinity for the rat intestinal choline carrier [12], whereas TMA showed no affinity for the human placent al and rabbit renal choline transporter [4, 11]. The inability of large molecular quaternary ammonium compounds to interact with the choline transporter was assumed to be due to steric hindrance, viz., the masking of the positive nitrogen [11, 12]. The failure of TEA, for instance, to interact with the choline transporter was further confirmed by the finding that TEA was not able to trans stimulate choline uptake into renal and intestinal brush-border membrane vesicles [11-13]. In the present study TMA, TEA, and TPrA did not inhibit or poorly inhibited placent al choline transport. In spite of a lack of determinants important for interaction with the choline transporter, further enlargement of the alky chains resulted in a significant inhibition of placent al choline transport by TBA, TPEA, and THA. For TPEA this was also observed in rabbit renal brush-border membranes [11], and in the study by Grassl [4] a higher degree of inhibition of choline uptake was found in the presence of trimethylphenylammonium, as compared to no significant inhibition with TMA. The reduced choline uptake in the presence of the more lipophilic quaternary ammonium compounds is likely to be of noncompetitive nature [14]. The higher degree of alkylation probably facilitates a nonspecific interaction (e.g., solubilization into the membrane or interaction with an allosteric binding site at maximal inhibition, impairment of fetal choline supply in vivo due to maternal drug use during pregnancy not to be expected. 

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REFERENCES


