INHIBITION OF CHOLINE UPTAKE IN SYNCYTIAL 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 microvillus 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 atropine 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 atropine (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 microvillus 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 choline uptake was also found in human placental fragments, which accumulated choline against a concentration gradient, inhibitable by HC-3 with a Ks 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 substrates 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 Ks 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 microvil-
sus membrane could have clinical implications for fetal
growth and development. This study was designed to
investigate further the specificity of the choline trans-
porter at the syncytiotrophoblast, by characterizing the inhibi-
tory potency and nature of the interaction of various cationic
drugs.

MATERIALS AND METHODS

Chemicals

\(^3\text{H}\)-Choline was obtained from Amersham (Buck-
inghamshire, U.K.). Cimetidine was kindly donated by
Smith, Kline & French (Welwyn Garden City, Herts,
U.K.), and mepiperphenidol and famotidine by Merck,
Sharp & Dohme (Rahway, NJ, U.S.A.). All other chem-
icals were purchased from either Sigma (St. Louis, MO,
U.S.A.), Merck (Darmstadt, Germany), or Boehringer
Mannheim (Mannheim, Germany), and were of analyt-
ical grade. GF/F filters were obtained from Whatman
Int. Ltd. (Maidstone, U.K.).

Preparation of SMMV

SMMV were prepared from fresh human term placen-
tae 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 MgCl\(_2\) 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
compared to starting mince, measured according to
Mircheff and Wright [7], was 24-fold (M\(_c\) = 70 ± 15 and
SMMV = 1690 ± 310 µmol/hr/mg, N = 14). Protein was
assayed with a Coomassie blue kit (Biorad, Munich,
Germany).

Uptake studies

Uptake of \(^3\text{H}\)-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 ex-
periments with different placenta.

Data analysis

From concentration vs % uptake curves of three con-
centrations of choline below \(K_m\) (50, 125, and 250 µM),
the concentration required to reach half maximal inhibi-
tion (IC\(_{50}\)) of several cationic drugs was estimated by
least-squares nonlinear regression analysis, using the
computer program GraphPad Inplot 4.0 (GraphPad Soft-
ware Inc., San Diego, CA, U.S.A.). The weighted resid-
ual 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 val-
ues 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 sta-
tistical 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 al-
kyla\(\)tion of TEA influences inhibitory potency. Enlarg-
ing the alkyl chains at the quaternary ammonium am-
rion resulted in a higher percentage of inhibition (Fig. 1). The

<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>
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<tr>
<td>HC-3</td>
<td>0.006 ± 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>Mepiperphenidol</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>3.80 (N = 1)</td>
<td>-</td>
</tr>
<tr>
<td>TEA</td>
<td>&gt;50 (N = 1)</td>
<td>-</td>
</tr>
</tbody>
</table>

IC\(_{50}\) values determined from inhibition curves at a choline
concentration of 250 µM and inhibitory constants \(K_i\) (competitive;
† 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.
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 μM 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 μM 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 mepiperphenidol and the anticholinergic drug atropine were less potent inhibitors. The H₂-receptor antagonists cimetidine, ranitidine, and famotidine showed IC₅₀ values only in the mM ranges. For TEA only a rough estimate of the IC₅₀ 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 IC₅₀ 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 μM. Increasing the substrate concentration decreased the inhibitory effectiveness of HC-3 and atropine, resulting in a lower IC₅₀ 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). IC₅₀ values for competitive inhibition, calculated from the Cheng-Prusoff equation for the three choline concentrations, were 40 μM for HC-3 and 1.2 mM for atropine (Table 1). In contrast, the IC₅₀ 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 IC₅₀ values 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 μM choline at 60 min significantly reduced as compared with control uptake. The other compounds did not interfere with the membrane integrity.

**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
Acknowledgements—

Inhibition of placental choline transport by cationic drugs

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