Ca²⁺-binding protein of the human placenta

Characterization, immunohistochemical localization and functional involvement in Ca²⁺ transport

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The Ca²⁺-binding protein (HCaBP) of the human placenta was studied with respect to its biochemical properties, tissue and cellular distribution, and possible involvement in placental Ca²⁺ transport. Optimal Ca²⁺ binding by the HCaBP occurs at pH 7–8 and in 100 mM-Na⁺ and 3 mM-Ca²⁺. The HCaBP possesses at least 10 Ca²⁺-binding sites with a K_d of 5×10^{-6} M ([Ca²⁺]). Highly specific rabbit-derived anti-HCaBP antibodies were used for HCaBP immunoquantification and immunohistochemistry, which revealed that the HCaBP is localized in the chorionic villi and is primarily associated with the trophoblastic cells of the placenta. In addition, an *in vitro* cell-free assay system for Ca²⁺ uptake was constructed with microsomal membranes isolated from term placental tissues. Ca²⁺ uptake by the placental microsomal fraction exhibited characteristics indicative of active Ca²⁺ transport such as temperature-dependence, saturability and energetic requirement. In this system, preincubation of microsomal membranes with anti-HCaBP antibodies inhibited Ca²⁺ uptake, suggesting that the HCaBP is functionally involved in placental membrane Ca²⁺ uptake.

The chorioallantoic placental membrane of the human conceptus is the embryonic organ responsible for the translocation of nutrients from the maternal circulation to the developing foetus. Among these nutrients calcium is particularly vital and is needed in the formation of the skeletal elements, the maintenance of neuromuscular functions and the homeostasis of blood coagulation mechanisms. Perfusion and tracer studies (Twardock & Austin, 1970; McIntosh & Lutwak-Mann, 1974) have strongly suggested that the transplacental uptake of Ca²⁺ by the conceptus is an active-transport process carried out up a concentration gradient, since the free Ca²⁺ ion concentration is higher in the foetal blood than in the maternal blood (Delivoria-Papadopoulos *et al.*, 1967; Tan & Raman, 1972). However, the structural complexity of the placenta, a result of the intimate interaction between maternal and foetal tissues, has severely limited the understanding of the mechanism of transplacental Ca²⁺ transport (Truman & Ford, 1984).

Abbreviations used: CAM, chorioallantoic membrane; HCaBP, Ca²⁺-binding protein of human placenta.

We have used extensively the chorioallantoic membrane (CAM) of the chick embryo as an experimental model system to understand Ca²⁺ transport across placental membranes. During development, the CAM is responsible for the translocation of over 120 mg of calcium from the calcareous eggshell into the embryonic circulation. We have chosen to study the CAM owing to its relative structural simplicity, resulting from the absence of any maternal tissues. The major finding in our studies is the discovery of a high- M_r specific Ca²⁺-binding protein (CaBP) in the CAM that is functionally involved in the CAM Ca²⁺-transport function. Many aspects of the CAM CaBP, including its developmental expression (Tuan & Scott, 1977), physicochemical properties (Tuan *et al.*, 1978a; Tuan, 1979), cellular localization (Tuan *et al.*, 1978b), mode of biosynthesis (Tuan *et al.*, 1978b; Tuan, 1980a) and regulation of expression (Tuan *et al.*, 1978c; Tuan, 1980b, 1983), have been studied. More recently we have also identified a membrane-bound Ca²⁺-activated ATPase as a near-neighbour protein of the CaBP on the CAM ectodermal surface (Tuan & Knowles, 1984). On the basis of these observations in the chick CAM, we have embarked on a study of the human

placenta (Tuan, 1982). Interestingly, our findings have revealed that the human placenta also contains a high- M_r specific Ca^{2+} -binding protein (HCaBP), which increases in amount as a function of gestation. The HCaBP has been purified to homogeneity and appears to have an M_r value of 150 000, a pI of 4.6 and a high content of acidic amino acids. We have also raised in rabbits a specific antiserum against the HCaBP.

In view of our observations on the chick CAM CaBP, it is tempting to speculate on the possible participation of the HCaBP in the Ca^{2+} -transport function of the human placenta. For this purpose, I report here further investigations on the HCaBP with respect to its physicochemical properties, its cellular distribution within the placental structure and its involvement in placental Ca^{2+} uptake.

Materials and methods

Human placental tissues

Human term placentae were obtained shortly after uncomplicated full-term pregnancy from the Maternal Labor Unit of the Hospital of the University of Pennsylvania. After being rinsed with 0.9% NaCl, the tissue samples were either processed immediately or stored frozen at -70°C until use.

Purification of HCaBP

The procedure used was as previously reported (Tuan, 1982). Briefly, soluble extracts of placental tissues were prepared in a Tris buffer (13.3 mM-Tris/HCl, pH 7.4, containing 0.12 M-NaCl, 4.74 mM-KCl, 98.5 μM -glucose, 0.71 mM-dithiothreitol and 0.02% NaN_3) and fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (30% saturation). Further purification steps involved gel filtration (Sephacryl S-200) and preparative isoelectric focusing in Sephadex G-75 over a pH gradient of 3–6. Homogeneity of the purified HCaBP was based on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli & Favre, 1973).

Ca^{2+} -binding assay

Ca^{2+} -binding activity was determined as previously described (Tuan & Scott, 1977) by the Chelex 100 (Bio-Rad) ion-exchange competition method, with $^{45}\text{CaCl}_2$ (Amersham) as a tracer. Units of Ca^{2+} -binding activity were calculated as (radioactivity in supernatant)/(radioactivity retained by resin) and expressed as a percentage value. All samples were extensively dialysed against Tris buffer before assay for Ca^{2+} -binding activity in duplicates.

Determination of binding constants of HCaBP

The Ca^{2+} -binding constants of the HCaBP in Tris buffer were determined with a Ca^{2+} -specific electrode (Calcium Selectrode: Radiometer) (Tuan *et al.*, 1978a). The response (mV) of the Ca^{2+} -specific electrode was calibrated with standard CaCl_2 solutions in Tris buffer and was linear with respect to Ca^{2+} concentrations from 1 μM to 100 mM.

For measurements of Ca^{2+} binding, 3.0 ml of an approx. 3–5 mM standard CaCl_2 solution in Tris buffer was titrated with a solution of purified HCaBP of known concentration (approx. 0.5 mg/ml in Tris buffer) at 25°C . After each addition of CaBP, the solution was allowed to equilibrate for at least 3–5 min with slow stirring before the reading (mV) was recorded from the pH-meter. By using the calibrated curve, the mV readings were converted into the corresponding Ca^{2+} concentrations in the solution. The data were plotted in accordance with Scatchard (1949).

Spectroscopic measurements

The effect of Ca^{2+} on the conformation of HCaBP was analysed spectroscopically by using u.v.-absorption and intrinsic-fluorescence measurements. (a) The u.v.-absorption spectrum of the HCaBP in Tris buffer at 25°C was determined with a Perkin-Elmer scanning spectrophotometer. (b) The intrinsic-fluorescence spectrum of the HCaBP in Tris buffer at 25°C was determined with an Aminco Bowman spectrofluorometer (excitation at 280 nm). Ca^{2+} concentration in the sample was sequentially varied from 1 μM to 1 mM.

Immunochemical procedures

Specific anti-HCaBP antibodies were prepared from the anti-HCaBP antiserum reported previously (Tuan, 1982) by means of a standard procedure involving the use of $(\text{NH}_4)_2\text{SO}_4$ precipitation and ion-exchange chromatography (Tuan *et al.*, 1978b). These antibodies were used to investigate (a) the effect of antibody binding on the Ca^{2+} -binding activity of the HCaBP by using the Chelex 100 assay method (see Tuan, 1980b) and (b) the relative HCaBP content in various regions of the placental unit and associated structures by means of single-radial-immunodiffusion assay (Tuan, 1980b).

Fluorescence immunohistochemistry of HCaBP

The cellular localization of the HCaBP in the foetoplacental structures was carried out by means of indirect immunofluorescence histochemistry. The procedure used was essentially similar to that used for the chick CAM CaBP (Tuan *et al.*, 1978b).

Briefly, various regions and associated tissues of the placenta (amnion, chorionic plate, decidua basalis, decidua proper, decidua septum, uterine wall and umbilical cord) were dissected out and frozen-embedded at -70°C in Tissue-Tek O.T.C. compound (Lab-Tek). Cryosections of $6\text{--}8\text{ }\mu\text{m}$ thickness were cut and stained with anti-HCaBP serum (1:20 dilution), washed and stained with fluorescein-labelled goat anti-(rabbit IgG) antibodies (Behring Diagnostics, 1:20 dilution). The sections were mounted in buffered glycerol (pH 8.0) and examined under phase contrast, Nomarski differential interference contrast and fluorescence optics with an Olympus BH2 photomicroscope equipped with a 100 W high-pressure mercury-vapour lamp and 495 nm/530 nm excitation/barrier filters. Photomicrographs were taken with Kodak Tri-X film.

Preparation of placental microsomal fraction

Preparation of the microsomal fraction (referred to below simply as microsomes) from human placenta was carried out essentially as described for the chick CAM (Tuan, 1979). Briefly, an iso-osmotic homogenate of freshly obtained human placenta was fractionated by differential centrifugation and microsomes were obtained by centrifugation (80000 g for 80 min) of the post-mitochondrial fraction. Microsomal membrane vesicles were obtained by suspension in 10 mM-imidazole/HCl, pH 7.0, containing 5 mM- MgCl_2 and 0.1 M-KCl to a concentration of 5–10 mg of protein/ml.

Assay of microsomal Ca^{2+} uptake

Under standard assay conditions, microsomes were incubated in imidazole buffer at 37°C with or without 5 mM-ATP in the presence of 0.1 mM- CaCl_2 and a tracer quantity of $^{45}\text{Ca}^{2+}$ (approx. $0.5\text{ }\mu\text{Ci/ml}$). At the end of specific intervals of incubation, a $100\text{ }\mu\text{l}$ portion of the incubation mixture was layered on to a cushion of silicone oil (sp.gr. 1.041; Nye, New Bedford, MA, U.S.A.) contained in a $400\text{ }\mu\text{l}$ -capacity polypropylene microcentrifuge tube, and centrifugation was carried out at 13000 g for 1 min to pellet the microsomal vesicles. After aspiration of the aqueous phase containing free, unincorporated, Ca^{2+} and the bulk of the silicone oil, the membrane pellet was solubilized by sonication in a liquid-scintillation cocktail (ACS II; Amersham) and counted for radioactivity. Active microsomal Ca^{2+} uptake was defined as the difference between the rates of Ca^{2+} accumulation in the presence and in the absence of ATP. By using this assay, linear Ca^{2+} uptake was observed for up to 2 min of incubation (see the text below for further details).

Isotopic determination of Ca^{2+} /ATP ratio in microsomal Ca^{2+} uptake

This was carried out by assaying the release of the γ -phosphate group from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Amersham) during microsomal Ca^{2+} uptake. The procedure used was that of Seals *et al.* (1978), which employed sodium dodecyl sulphate to terminate microsomal Ca^{2+} uptake in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ followed by extraction of the phosphomolybdate complex into xylene/2-methylpropanol to separate $[\text{P}^{32}]\text{P}_i$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and quantification by liquid-scintillation counting. Ca^{2+} -specific ATP hydrolysis was based on the difference in hydrolysis in the presence and the absence of 0.1 mM- CaCl_2 in the assay mixture.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Ca^{2+} -binding activity

In initial experiments, characterization of HCaBP activity was carried out by using the Chelex 100 Ca^{2+} -binding assay. Since only relative parameters of the activity were being considered, an enriched preparation of the HCaBP (see the legend to Fig. 1) was used for these studies. Ca^{2+} -binding activity was studied with respect to pH, ionic strength and $[\text{Ca}^{2+}]$. The results in Figs. 1(a), 1(b) and 1(c) demonstrated that HCaBP activity is (1) pH-dependent with an optimum at pH 7–8, (2) sensitive to ionic strength with the optimum at 100 mM- Na^+ , and (3) saturable at 3–4 mM- Ca^{2+} .

The quantitative binding affinity of the HCaBP for Ca^{2+} was further studied titrimetrically by means of a Ca^{2+} -specific electrode (Selectrode; Radiometer) and with a purified electrophoretically homogeneous preparation of the CaBP. From the Scatchard plot of the binding data shown in Fig. 2, the HCaBP appears to possess a single set of approx. $10\text{ }\text{Ca}^{2+}$ -binding sites with an apparent K_d of $5 \times 10^{-6}\text{ M}$.

Ca^{2+} -binding and spectral properties

To examine possible Ca^{2+} -mediated conformational effects on the HCaBP, the u.v.-absorption and intrinsic-fluorescence spectra of purified HCaBP were studied as a function of $[\text{Ca}^{2+}]$. As shown in Fig. 3(a), in addition to the high peptide-amide-bond absorption maximum at 225 nm, the HCaBP exhibited an additional absorption maximum at 275 nm with a shoulder at 285 nm, characteristic of contribution from the 3% of tyrosine residues in the HCaBP (Tuan, 1982). No apparent

Fig. 1. Ca^{2+} -binding activity of HCaBP as a function of (a) pH, (b) ionic strength, and (c) Ca^{2+} concentration. Activity was determined by the Chelex assay as described in the Materials and methods section. The HCaBP sample was obtained from human term placenta after $(\text{NH}_4)_2\text{SO}_4$ precipitation and Sephacryl S-200 gel filtration. The enriched HCaBP preparation contained only one Ca^{2+} -binding species, as revealed by chromatography and isoelectric focusing (Tuan, 1982). In (a), the buffers (13.7 mM) used were sodium acetate, pH 4.5-6 (■), imidazole/HCl, pH 6-7 (△), and Tris/HCl, pH 7-9 (●), each containing 120 mM-NaCl, 4.74 mM-KCl, 0.98 μM -glucose, 0.71 mM-dithiothreitol and 0.02% NaN_3 . In (b), Tris/HCl buffer, pH 7.4, containing various concentrations of NaCl was used. In (c), CaCl_2 was included at various concentrations in Tris/HCl buffer, pH 7.4, containing 120 mM-NaCl.

Fig. 2. Scatchard plot of Ca^{2+} binding by HCaBP. The binding data were obtained titrimetrically with purified HCaBP as described in the Materials and methods section. $[\text{Ca}]_b$, concentration of bound Ca^{2+} ; $[\text{Ca}]_f$, concentration of free ionic Ca^{2+} ; $[\text{HCaBP}]$, concentration of HCaBP. From the Scatchard plot, the dissociation constant was determined from the slope of the line ($-1/K_d$) and the number of binding sites from the intercept on the abscissa.

differences with respect to ϵ and λ_{max} , were observed between the absorption spectra obtained in the absence and in the presence of various Ca^{2+} concentrations (20 μM - Ca^{2+} shown here).

Upon excitation at 280 nm, the HCaBP was intrinsically fluorescent with an emission λ_{max} of 310-315 nm (Fig. 3b), presumably due to its tyrosine residues. When the intrinsic fluorescence spectrum was examined as a function of $[\text{Ca}^{2+}]$, it was found that, although quantum yield was not affected and λ_{max} remained at 310-315 nm, considerable sharpening of the emission peak resulted as a function of increasing $[\text{Ca}^{2+}]$. The data in Fig. 3(c) show that the peak widths at half peak height decrease gradually as a function of total $[\text{Ca}^{2+}]$ so that from 1 μM - to 1 mM- Ca^{2+} the fluorescence-emission peak sharpened by over 20 nm.

Effect of anti-HCaBP antibodies on HCaBP activity

The immunospecificity of the anti-HCaBP serum (and purified IgG) was further confirmed in the present work by incubating placental extract with the anti-HCaBP preparations. The results (Fig. 4a) showed that Ca^{2+} -binding activity (or HCaBP) was specifically removed from the placental ex-

Fig. 3. *U.v.-absorption and intrinsic-fluorescence-emission spectra of HCaBP*

(a) U.v.-absorption spectrum of HCaBP in Tris buffer at 25°C. Inset: absorption spectra (250–300 nm) of purified HCaBP in the absence and in the presence of 20 μM - CaCl_2 . The concentration of HCaBP was 0.5 mg/ml. (b) Intrinsic-fluorescence-emission spectrum of purified HCaBP in Tris buffer at 25°C with excitation at 280 nm in the presence of 1 μM - and 0.38 mM- CaCl_2 . The concentration of HCaBP was 0.5 mg/ml. (c) Intrinsic-fluorescence-emission peak analysed by peak width at half peak height as a function of total $[\text{Ca}^{2+}]$ in Tris buffer.

tract after incubation with the anti-HCaBP serum. The removal of HCaBP by anti-HCaBP serum was concentration-dependent and saturable, resembling standard immunoprecipitation reactions. Moreover, when a placental extract previously labelled with ^{45}Ca was incubated with anti-HCaBP serum, it was found that radioactivity was also immunoprecipitated (Fig. 4b). These results therefore clearly indicate that the antibodies were highly specific for the HCaBP, and furthermore demonstrate that antibody binding to the HCaBP does not displace Ca^{2+} already bound to the

HCaBP, suggesting that anti-HCaBP antibodies may recognize sites other than the Ca^{2+} -binding sites on the HCaBP. However, it needs to be pointed out that, in these immunotitrations, removal (or inactivation) of HCaBP activity (Fig. 4a) quantitatively precedes immunoprecipitation (Fig. 4b), suggesting that binding by anti-HCaBP antibodies might have diminished or inhibited the Ca^{2+} -binding activity of the HCaBP, although, once bound, Ca^{2+} was not displaced from the HCaBP by subsequent interaction with anti-HCaBP antibodies.

Fig. 4. Immunoprecipitation of HCaBP from total placental extract

(a) Removal of Ca^{2+} -binding activity from placental extract. (b) Immunoprecipitation of $^{45}\text{Ca}^{2+}$ -bound HCaBP from placental extract. In both cases an extract of term placenta (5.7 mg of protein/ml) enriched for HCaBP by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Tuan, 1982) was incubated with the indicated amount of anti-HCaBP serum at 4°C overnight. The immunoprecipitate was removed by centrifugation at $15000g$ for 10 min. In (a) the supernatant after centrifugation was assayed for Ca^{2+} -binding activity by the Chelex method (see the Materials and methods section). Percentage of activity remaining was calculated by comparison with the activity in the absence of the anti-HCaBP serum. In (b) the placental extract was prelabelled with $^{45}\text{Ca}^{2+}$ and the excess free label was removed by incubation with Chelex 100. The labelled placental extract was immunoprecipitated with anti-HCaBP serum, and the precipitated radioactivity was determined as a percentage of the total radioactivity.

Regional distribution and immunohistochemical localization of HCaBP

By means of single-radial-immunodiffusion assay (Tuan, 1980b) of tissue extracts, it was found that the HCaBP content per mg of total protein was highest in the chorionic villi and the decidua of the foetoplacental structure. Only a low HCaBP content was present in the amnionic membrane, and the umbilicus contained no detectable HCaBP.

Immunohistochemistry on cryosections of placenta revealed intense fluorescent staining in the chorionic villi (Fig. 5). The HCaBP appeared to be primarily localized to the peripheral cellular layer (syncytio- and cyto-trophoblasts) of the villus, whereas the central regions of loose connective tissue containing foetal capillaries were not stained with anti-HCaBP antibodies (Figs. 5a and 5b). Within the syncytiotrophoblasts, staining was localized mainly in the cellular cortex and clearly excluded the nucleus (Figs. 5a and 5c). In many instances, the staining appeared to be associated with the plasma membrane, since the cellular outline was traced by the fluorescence (Fig. 5c). The specificity of the immunohistochemical procedure was routinely tested by substituting the anti-HCaBP serum by pre-immune serum, which yielded no fluorescent staining (Figs. 5e and 5f).

Ca^{2+} uptake by placental microsomes *in vitro*

Microsomal membranes prepared from term-placental tissues were found to exhibit ATP-dependent uptake of Ca^{2+} (Fig. 6a) that was kinetically linear for up to 2 min of incubation at 37°C . To verify that the time-dependent accumulation of Ca^{2+} by the pelleted membranes was indeed a result of specific uptake rather than non-specific adsorption, initial experiments included first layering $25\mu\text{l}$ of 1 mM-EDTA on top of the silicone oil before centrifugation, so that, when the microsomal mixture was added to the tube and spun, any non-sequestered Ca^{2+} should be removed from the pelleted membranes by the EDTA. In these experiments it was found that the rate of time-dependent Ca^{2+} uptake was unaffected, although the absolute amount of Ca^{2+} associated with the membrane pellet was decreased as a result of removal of non-specifically adsorbed Ca^{2+} (results not shown). In all subsequent studies, microsomal Ca^{2+} -uptake activity was calculated on the basis of the kinetics of net uptake during the first 2 min of incubation. The temperature-dependence of microsomal Ca^{2+} uptake, as shown in Fig. 6(b), provided further confirmation of the active nature of the process.

Ca^{2+} uptake by placental microsomes was dependent on $[\text{Ca}^{2+}]$ up to 0.1 mM, above which saturation occurred (Fig. 6c). The energetic re-

Fig. 5. Fluorescence immunohistochemical localization of HCaBP in the chorionic villi of the human placenta. Cryosections ($6\text{--}8\text{ }\mu\text{m}$) of the frozen-embedded piece of placental cotyledon were stained as described in the Materials and methods section. (a), (c) and (e) Fluorescence optics; (b) and (f) phase contrast; (d) Nomarski differential interference contrast. (a) and (b) Chorionic villus bathed in the intervillous fluid of the maternal circulation (MC) showing the peripheral cellular layers (ST, syncytiotrophoblasts; CT, cytotrophoblasts) and the internal stromal loose connective tissue and foetal capillaries (FC). The fluorescent staining was predominantly associated with the ST and CT regions (arrows) and was absent from the interior of the villus, including the foetal vasculature. Within the ST and CT cells, the fluorescence was distributed in a cortical manner that excluded the nucleus (also see c and d). (c) and (d) Higher magnification of the ST and CT regions to show the exclusively extranuclear staining pattern of the HCaBP within the trophoblast cells. The fluorescence appeared to fill the cytoplasm in most cells, and in some it appeared merely to trace their outlines. (e) and (f) Chorionic villus treated with pre-immune serum instead of anti-HCaBP serum, showing the absence of specific fluorescent staining. Bar denotes $1\text{ }\mu\text{m}$.

Fig. 6. Ca^{2+} uptake by placental microsomes *in vitro*

(a) ATP-dependent Ca^{2+} uptake by human placental microsomal membranes at 37°C . Microsomes (10.5 mg of protein/ml) were isolated from human term placenta and assayed for Ca^{2+} uptake as described in the Materials and methods section. (b) Temperature-dependence of placental microsomal Ca^{2+} uptake. The assay was carried out as described above at the indicated temperatures. All activities are expressed as percentages of the highest value. (c) Microsomal Ca^{2+} uptake as a function of $[\text{Ca}^{2+}]$. Uptake activities were measured as described above in various total Ca^{2+} concentrations in the incubation mixture and expressed as percentages of that in $0.1 \text{ mM-}\text{Ca}^{2+}$.

quirement of the uptake process was specific for ATP, since substitution of ATP by GTP, ADP or β -glycerophosphate was ineffective in supporting Ca^{2+} uptake. On the basis of simultaneous analyses of Ca^{2+} uptake and ATP hydrolysis (see the Materials and methods section) the bioenergetic requirement of placental microsomal Ca^{2+} uptake was quantitatively determined. The results indicated an ATP/ Ca^{2+} ratio of approx. 50:1. This apparent inefficiency could be due in part to the heterogeneity of the microsomal preparations,

such that non-specific ATP hydrolysis was not corrected for completely. Taken together, these properties of placental microsomal Ca^{2+} uptake were indicative of an active process, which therefore represented a valid experimental system for the study of active membrane Ca^{2+} transport.

Effect of anti-HCaBP antibodies on placental microsomal Ca^{2+} uptake

The '*in vitro*' microsomal system was exploited to assess the functional involvement of the HCaBP in

Fig. 7. Effect of anti-HCaBP antibodies on placental microsomal Ca^{2+} uptake

Anti-HCaBP IgG was isolated by means of $(\text{NH}_4)_2\text{SO}_4$ precipitation and anion-exchange chromatography (Tuan *et al.*, 1978b), reconstituted to the original antiserum volume and dialysed exhaustively against the uptake buffer. Microsomes were preincubated with the antibodies at the indicated concentrations for 30 min at 4°C and assayed for Ca^{2+} uptake as described in the Materials and methods section. In control experiments, incubation with pre-immune rabbit IgG showed no effect on microsomal Ca^{2+} -uptake activity.

placental Ca^{2+} transport. For this purpose, Ca^{2+} uptake by microsomal vesicles pretreated with various concentrations of anti-HCaBP antibodies was determined. The results in Fig. 7 clearly demonstrate that pretreatment of microsomal vesicles with anti-HCaBP IgG affected Ca^{2+} uptake activity in a manner resembling standard immunotitration reactions with maximal inhibition at $1:10^5$ antibodies dilution. This finding is therefore consistent with the hypothesis that the HCaBP is functionally involved in placental Ca^{2+} uptake (or transport).

Discussion

Our previous identification and purification of a high- M_r specific Ca^{2+} -binding protein (HCaBP) in the human placenta has provided a promising means for the biochemical characterization of the placental Ca^{2+} -transport function. In the present investigation I have examined the biochemical properties and cellular distribution of the HCaBP and have also obtained evidence suggesting its functional involvement in placental Ca^{2+} transport.

Ca^{2+} binding by the HCaBP is optimal at physiological pH and ionic strength, with an affinity ($K_d = 5 \times 10^{-6} \text{ M}$) that appears to be intermediate between those of extracellular

($K_d = \sim 10^{-4} \text{ M}$, e.g. prothrombin) and intracellular ($K_d = \sim 10^{-6}$ – 10^{-7} M , e.g. calmodulin) Ca^{2+} -binding proteins (Kretsinger, 1976; Van Eldick *et al.*, 1982). On the basis of the immunocytochemical localization reported in the present paper, the HCaBP is most probably a cytoplasmic protein, although definitive and exclusive subcellular localization was not possible owing to the limited resolution of the light-microscope. It needs to be pointed out that, although the HCaBP may be predominantly localized in the cytosol, it probably is also associated with membranes and/or membrane components, as suggested by the effect of anti-HCaBP antibodies on isolated subcellular membrane vesicles (see the discussion below).

My spectroscopic data indicate that Ca^{2+} binding significantly alters the intrinsic-fluorescence spectrum of the HCaBP. Since the emission λ_{max} of the Ca^{2+} -free HCaBP is not significantly different from that of free tyrosine, these residues on the average are probably not located in a highly non-polar region of the HCaBP. The significant sharpening of the emission peak observed in the presence of increasing $[\text{Ca}^{2+}]$ is most probably due to progressive clustering of the tyrosine residues into a more non-polar environment of the HCaBP (Edelhoch & Chen, 1980) as a result of Ca^{2+} binding. This interpretation thus suggests that Ca^{2+} binding induces conformational changes in the HCaBP.

The placental microsomal membranes described in the present paper represented a cell-free system that should be readily amenable to the experimental study of the mechanism of placental Ca^{2+} transport. In the present investigation, the apparent inhibition of placental microsomal Ca^{2+} uptake by anti-HCaBP IgG is a most significant finding, since it suggests that the HCaBP may indeed be a functional component of the Ca^{2+} -uptake (or -transport) mechanism of the placental membrane. The finding also suggests that the HCaBP is associated with and is accessible to the exterior of the microsomal membranes. A possible explanation of the immunoequivalent manner of the inhibition may be that efficient microsomal Ca^{2+} uptake normally requires the HCaBP to exist in a fluid mobile association with the microsomal membranes. Thus, at immunoequivalence, maximal immunocross-linking and immobilization of the HCaBP take place, thereby resulting in significant inhibition of uptake activity. It should be noted that, since biphasic components, i.e. soluble antibodies and membranous antigen-bearing vesicles, were involved, the exact nature of the immunointeraction and the mechanism of inhibition of uptake are necessarily complex and remain to be elucidated. The 'in vitro' cell-free microsomal membrane system used in the present work is modelled

after the assay system being used in our study of Ca^{2+} transport in the chick CAM (R. Tuan, M. Carson, J. Josefiak, K. Knowles & B. Shotwell, unpublished work). Preliminary results from our ongoing studies have shown that the placental microsomes prepared as described in the present paper contain plasma membranes, as indicated by the presence of marker molecules such as $\text{Na}^+ + \text{K}^+$ -dependent ATPase and 5'-mononucleotidase, as well as a Ca^{2+} -activated Mg^{2+} -dependent ATPase, in the microsomal preparations. Furthermore, gel-permeation studies on the chick CAM systems (R. Tuan, M. Carson, J. Josefiak, K. Knowles & B. Shotwell, unpublished work) show that the fractionation protocol described in the present paper yields primarily vesicular structures in the microsomal fraction prepared. Thus these cell-free membrane preparations of the placenta should provide a convenient Ca^{2+} -uptake assay system that is directly amenable to experimental manipulation.

Taken together, the results presented here have established means of studying the process of transplacental Ca^{2+} transport that may overcome some of the inherent complexities encountered in many previously published studies based on metabolite distribution and clearance *in vivo* (Delivoria-Papadopoulos *et al.*, 1967; Tan & Raman, 1972; McIntosh & Lutwak-Mann, 1974; Greizerstein & Abel, 1979; Delvin *et al.*, 1982; Durand *et al.*, 1982, 1983) and with the use of perfusion, tissue slices, and primary trophoblast cultures (Castaldo *et al.*, 1980; McKercher *et al.*, 1982; Yano *et al.*, 1982). For example, the identification of the HCaBP as a perspective functional transport component allows one to monitor its activity, regulation and biosynthesis to mark biochemically the transport functional status of the placenta. Secondly, the construction of '*in vitro*' cell-free membrane vesicles as an active Ca^{2+} uptake system should enable one to characterize directly the transport apparatus with respect to its stoichiometry and specificity, and also to identify or label functional groups by various biochemical and pharmacological modifications. Finally, functional reconstitution of transport-competent vesicles from fractionated membrane vesicles and purified components should be attempted to identify definitively and elucidate the functional roles of the putative transport component(s).

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References

- Castaldo, F., Cittadini, A., Romor, R., Paprella, P., Bompiani, A. & Terranova, T. (1980) *Acta Obstet. Gynecol.* **59**, 11–18
- Delivoria-Papadopoulos, M., Battaglia, F., Bruns, P. & Meschia, G. (1967) *Am. J. Physiol.* **213**, 363–366
- Delvin, E., Glorieux, F., Salle, B., David, L. & Varenne, J. (1982) *Arch. Dis. Child.* **57**, 754–757
- Durand, D., Dalle, M. & Barlet, J.-P. (1982) *Biol. Neonate* **42**, 120–126
- Durand, D., Braithwaite, G. & Barlet, J.-P. (1983) *Br. J. Nutr.* **49**, 475–480
- Edelhoch, H. & Chen, R. (1980) *Horm. Proteins Pept.* **9**, 109–173
- Greizerstein, H. & Abel, E. (1979) *Biol. Neonate* **36**, 251–254
- Kretsinger, R. (1976) *Annu. Rev. Biochem.* **45**, 239–266
- Laemmli, U. & Favre, M. (1973) *J. Mol. Biol.* **80**, 575–599
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- McIntosh, E. A. & Lutwak-Mann, C. (1974) *Biochem. J.* **138**, 97–105
- McKercher, H., Derewlany, L. & Radde, I. (1982) *Biochem. Biophys. Res. Commun.* **105**, 841–846
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Seals, J., McDonald, J., Bruns, D. & Jarett, L. (1978) *Anal. Biochem.* **90**, 785–795
- Tan, C. & Raman, A. (1972) *Q. J. Exp. Physiol.* **57**, 56–59
- Truman, P. & Ford, H. (1984) *Biochim. Biophys. Acta* **779**, 139–160
- Tuan, R. (1979) *J. Biol. Chem.* **254**, 1356–1364
- Tuan, R. (1980a) *Cell Calcium* **1**, 411–429
- Tuan, R. (1980b) *Dev. Biol.* **74**, 196–204
- Tuan, R. (1982) *Placenta* **3**, 145–158
- Tuan, R. (1983) *J. Embryol. Exp. Morphol.* **74**, 119–131
- Tuan, R. & Knowles, K. (1984) *J. Biol. Chem.* **259**, 2754–2763
- Tuan, R. & Scott, W. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1946–1949
- Tuan, R., Scott, W. & Cohn, Z. (1978a) *J. Biol. Chem.* **253**, 1011–1016
- Tuan, R., Scott, W. & Cohn, Z. (1978b) *J. Cell Biol.* **77**, 743–751
- Tuan, R., Scott, W. & Cohn, Z. (1978c) *J. Cell Biol.* **77**, 752–761
- Twardock, A. & Austin, M. (1970) *Am. J. Physiol.* **219**, 540–545
- Van Eldik, L., Zendegeui, J., Marshak, D. & Watterson, D. (1982) *Int. Rev. Cytol.* **77**, 1–61
- Yano, J., Okada, Y., Tsuchiya, W., Kinoshita, M. & Tominaga, T. (1982) *Biochim. Biophys. Acta* **685**, 162–168