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Properties of Two Multisubstate Cl^- Channels from Human Syncytiotrophoblast Reconstituted on Planar Lipid Bilayers

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Abstract. We describe the first successful reconstitution of placental ionic channels on planar lipid bilayers. An apical plasma membrane-enriched vesicle fraction from human syncytiotrophoblast at term was prepared by following isotonic agitation, differential centrifugation, and Mg^{2+}-induced selective precipitation of nonapical membranes, and its purity was assessed by biochemical and morphological marker analysis. We have already reported that, unlike previous patch-clamp studies, nonselective cation channels were incorporated in most cases, a result consistent with the higher permeability for cations as compared with Cl^- and with the low apical membrane potential difference at term revealed by fluorescent probe partition studies, and microelectrode techniques. In this paper, we report that Cl^--selective channels were incorporated in 4% of successful reconstitutions (14 out of 353) and that their analysis revealed two types of activity. One of them was consistent with a voltage-dependent, 100-pS channel while the other was consistent with the lateral association of 47-pS conductive units, giving rise to multibarrelled, DIDS-sensitive channels of variable conductance (300 to 650 pS). The latter displayed a very complex behavior which included cooperative gating of conductive units, long-lived substates, voltage-dependent entry into an apparent inactivated state, and flickering activity. The role of the reported Cl^- channels in transplacental ion transport and/or syncytium homeostasis remains to be determined.

Key words: Placenta — Apical plasma membrane vesicles — Single-channel recording — Cl^- channels — Multiple conductance levels — Cooperativity

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Introduction

In humans, a successful gestation depends on the activity of a transient organ of fetal origin: the placenta. This organ acts as intestine, kidney, lung, liver, and endocrine gland to support prenatal life. Moreover, some gestation-associated diseases, such as intrauterine growth retardation, are thought to arise from placental defects.

This fetal organ is composed, mostly, of villous tissue (chorionic villi). These villi, in the human, hemomonochorial placenta at term, are formed by a single epithelial layer (syncytiotrophoblast) enclosing a core of mesenchimal tissue where fetal capillaries are embedded along with fibroblasts, macrophages, and a few cytotrophoblast cells. The syncytiotrophoblast is a morphological syncytium (Gaunt & Ockleford, 1986), namely, a continuous, multinucleated structure which results from the fusion of the underlying cytotrophoblast cells thus lacking intercellular membranes. This way, the transport of substances between maternal and fetal blood should be exclusively transcellular across both the apical and basal membranes. Moreover, as in other transport epithelia such as kidney and intestine, the rate-limiting step for transport of small solutes is thought to be associated with the epithelium rather than with the vascular endothelium.

The syncytial nature of the human syncytiotrophoblast at term is unique among epithelia. In this respect, this atypical feature is only shared by the epithelium that covers the outer surface of parasitic platyhelminthes like *Echinococcus granulosus* (Morseth, 1967; Chappell, 1980), studied by us earlier (Reisin & Pavisic de Falá, 1984; Ibarra & Reisin, 1994; Grosman & Reisin, 1995). In addition, unlike orthodox epithelia, both syncytiotrophoblast faces are bathed directly (apical) or indirectly (basal) by blood.

With respect to ion transport, the information available on ion conductances has arisen, for the most part, from radiotracer-flux (Byrne et al., 1993; Faller, O'Reilly & Ryan, 1994), microelectrode (Greenwood, Boyd & Sibley, 1993; Greenwood et al., 1996), and fluorescent probe partition (Illsley & Sellers, 1992) studies while direct measurements of ion currents are strikingly scarce. These indirect studies suggested the presence of K^+, Na^+, and Cl^- channels on apical membranes (Shennan & Boyd, 1987; Illsley & Sellers, 1992; Byrne et al., 1993; Greenwood et al., 1993; Faller et al., 1994; Greenwood et al., 1996). Nevertheless, mostly anion channels were reported from direct, electrophysiological measurements on that surface by Brown et al. (1993) (patch-clamp of intact villi) and Riquelme et al. (1995) (patch clamp of giant liposomes reconstituted with apical membrane fragments) even when, as reported by Illsley and Sellers (1992), the permeability of apical plasma membrane vesicles to Cl^- is lower than that to K^+ or Na^+.

We report here the first successful reconstitution of apical plasma membrane vesicles from human syncytiotrophoblast at term on planar lipid bilayers. Unlike previous studies (Brown et al., 1993; Riquelme et al., 1995) we reconstituted, in most cases, nonselective cation channels (Grosman & Reisin, 1996), the anionic channels being only seldom observed. The present paper, a part of which has already been published in abstract form (Grosman & Reisin, 1996), will deal with the anionic conductances.

Materials and Methods

Apical Plasma Membrane Vesicle Preparation

The method outlined by Booth, Olaniyan and Vanderpuye (1980) was followed with some minor modifications. Full term, normal human placentae were obtained within 20 min of vaginal delivery and immediately processed. All steps were carried out at 0–4°C and all centrifugations were made on a SS-34 rotor, unless otherwise indicated. Villous tissue was fragmented, washed with unbuffered 150 mM NaCl, and cut into small pieces. A given amount of tissue was agitated with a magnetic stirrer for 1 hr in 1.5 volumes of a 10-mM N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES)-KOH, pH 7.4 buffer containing 0.1 mM ethylene glicol-bis (β-amino ethyl ether) N, N, N′, N′-tetraacetic acid (EGTA), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and either 150 mM NaCl, 250 mM sucrose or 150 mM KI as homogenization buffer osmolytes. The agitated tissue was poured through several layers of cheesecloth and the filtrate was centrifuged for 10 min at 3,100 rpm. The supernatants were centrifuged for 10 min at 11,000 rpm and the corresponding supernatants were centrifuged for 90 min at 14,000 rpm. The pellets were resuspended in a 10-mM HEPES-KOH, pH 7.4 buffer containing 250 mM sucrose and, then, solid $MgCl_2 \cdot 6H_2O$ was added to a final concentration of 10 mM to selectively precipitate nonapical membranes. The suspension was incubated for 10 min with constant stirring after which it was centrifuged for 15 min at 5,000 rpm. Finally, the supernatants were centrifuged for 90 min at 14,000 rpm and the brush border-enriched pellets were resuspended in a 10-mM HEPES-KOH, pH 7.4 buffer containing 250 mM sucrose and 20 mM KCl. The membrane suspension was then aliquoted and stored at −20°C. These purified fractions were assayed for biochemical and morphological markers, and reconstituted on planar lipid bilayers.

Enzyme Assays

Alkaline phosphatase, acid phosphatase, and succinate dehydrogenase activities were assayed as described earlier (Grosman & Reisin, 1995). NADH dehydrogenase activity was measured in a reaction mixture consisting of a 10-mM 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.4 buffer containing 0.66 mM $K_3Fe(CN)_6$ and 113 μM NADH. The oxidation of NADH was followed at 340 nm. All enzymatic assays were done at room temperature (20–25°C).

Proteins

Membrane fraction protein content was determined by using a Lowry modified method for samples containing sucrose, chelating agents, and membranes (Markwell et al., 1978). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) was conducted according to the method of Schägger and von Jagow (Schägger & von Jagow, 1987). 20 μg of each sample were applied in volumes of 4 μL and electrophoresed into the stacking gel at 50 V and, into the running, at 100 V. Gels were stained for protein detection with Coomassie Brilliant Blue R-250.

Microscopy

Pelleted membrane fractions (33,100 rpm for 15 min in a 90 Ti rotor) and a small (~2 cm^3) piece of villous tissue from a freshly delivered placenta were fixed with a 0.1-M sodium cacodylate-HCl, pH 7.2 buffer (cacodylate buffer) containing 3% (v/v) glutaraldehyde and 1% (w/v) formaldehyde for 2 hr at 0–4°C. The samples were, then, washed with the cacodylate buffer, postfixed with the same buffer containing 1% (v/v) osmium tetroxide for 1 hr at room temperature, dehydrated through graded alcohols and propylene oxide, and embedded in Epon Araldite. Thick sections, stained with Methylene Blue-Azur II, and ultrathin sections, stained with uranyl acetate and lead citrate, were examined under light and electron microscope (Zeiss EM 109), respectively.

Channel Reconstitution

Lipid bilayers were formed by using either one of three mixtures of synthetic phospholipids (Avanti Polar Lipids, Birmingham, AL) and cholesterol (recrystallized from ethanol) in *n*-decane. All the phospholipids used were 1-palmitoyl-2-oleoyl-based, the polar head group being either choline (POPC), ethanolamine (POPE) or serine (POPS). The lipid solution (~20–25 mg/mL) was spread over a 250-μm diameter aperture in a polystyrene cuvette by means of a thin glass or plastic rod. The cuvette was inserted into one of a pair of pits machined on a polyvinyl chloride holder thus defining two aqueous compartments (800 and 1600 μL) separated by a planar lipid film as described by Alvarez, Benos and Latorre (1985). Both sides of the lipid bilayer were bathed by a 10–15 μM Ca^{2+} solution which was H^+-buffered (pH 7.4) with 10 mM MOPS-KOH ($[K^+] \cong 5$ mM). At the beginning of every experiment the *trans* side of the bilayer was bathed only by this solution while that bathing the *cis* side also contained 145 or 295 mM KCl or NaCl so as to generate a transbilayer osmotic gradient that promoted the vesicle-planar bilayer fusion. This way, the *cis* compartment (the

one into which membrane vesicles were added) turned out to be 150 or 300 mM in cations and 145 or 295 mM in Cl^-, whereas the *trans* side was just ~5 mM in K^+. After 145 or 295 mM KCl or NaCl were added to the *trans* solution, once a successful channel insertion took place, the ionic composition of both compartments was the same.

Electrical Recordings

Voltage was applied to the *trans* solution with either a DC voltage source or a function generator, the opposite side, *cis*, having been defined as virtual ground. Nevertheless, throughout the paper, the *cis* minus *trans* voltage was indicated. Electrical contacts were made through Ag/AgCl electrodes and 200 mM KCl, 2% (w/v) agar bridges. The current across the bilayer was recorded using a current-to-voltage converter with a 10 Gohm feedback resistor. The output voltage was low-pass filtered at 1 kHz (−3 dB) with an eight pole, Bessel type filter (Frequency Devices, Haverhill, MA) and displayed on an oscilloscope. Channel recordings were simultaneously digitized with a pulse code modulator, stored on videotapes with a VCR, and, later, acquired at 4 kHz (unless otherwise specified) with an AT computer for subsequent analysis. Some current traces shown in the Results were further filtered for display purposes. In all cases overall cutoff frequency values (fc) were calculated (Colquhoun & Sigworth, 1983) and are expressed as the −3 dB values. Bilayer formation was monitored by applying a 2.5 mV peak-to-peak, 20 Hz, triangular wave. Typically, 100–200 pF bilayers were obtained. All the experiments were performed at room temperature (20–25°C).

Data Acquisition and Analysis

pCLAMP Version 5.5 (Axon Instruments, Foster City, CA) and Sigmaplot Version 4.02 (Jandel Scientific, Corte Madera, CA) softwares were used. Occupancy probabilities (the fraction of time the current signal dwelled in each current level) were estimated by either one of two methods. One of them consisted of fitting the all-points current-amplitude histograms of the digitized current signal to Gaussian curves. The fractional area of each Gaussian component gives the corresponding estimation of the occupancy probability. In symbols, for a record containing k open levels:

$$n = \frac{N_T b}{\sqrt{2\pi}} \sum_{j=0}^{k} \frac{P_j}{\sigma_j} exp - \frac{(i-\mu_j)^2}{2\sigma_j^2} \quad (1)$$

where n is the number of digitized points at each i (current) value, N_T is the total number of digitized points, b is the histogram bin width, σ_j and μ_j are the standard deviation and the mean of the jth current level, respectively, π is the geometric constant, and P_j is the fractional area of each Gaussian component. The second method consisted of idealizing the filtered data by following a half-amplitude threshold-crossing algorithm. This way, the total number of digitized points becomes sorted among the different current levels. The number of points assigned to each level divided by their total number gives an alternative estimation of the occupancy probabilities. Both methods yielded nearly identical results. In multichannel bilayers, single-channel open probabilities (P_O) were estimated by assuming that, for an ensemble of n identical (in terms of conductance and P_O) and independent channels not exhibiting modal gating activity, the discrete random variable *number of open channels* must be binomially distributed. Nevertheless, when their P_O values are different, this variable is distributed according to a related distribution whose probability function, for the simplest case of two channels, is:

$$P_c = (1 - P_{OA})(1 - P_{OB}) \quad (2a)$$

$$P_{O1} = (1 - P_{OA})P_{OB} + (1 - P_{OB})P_{OA} \quad (2b)$$

$$P_{O2} = P_{OA}P_{OB} \quad (2c)$$

where P_c, P_{O1}, and P_{O2} are the probabilities of zero, one, and two open channels, and P_{OA} and P_{OB} are the individual open probabilities. Thus, P_{OA} and P_{OB} values can be calculated either by constraining the P_j values in the Gaussian fits (*see* above) to obey the binomial law according to Eqs. 2 or by arithmetically solving these simultaneous equations. When more than two channels co-reconstituted these expressions were adequately adapted. The finding of a solution to these equations by following either procedure was taken as an evidence for the independent activity of the co-reconstituted ion channels. Both methods yielded nearly identical results. The voltage-dependence of the P_O was investigated by fitting P_O *vs.* membrane potential data points to the following equation (Boltzmann equation):

$$P_O = \frac{1}{1 + K_0 exp(z\delta FV/RT)} \quad (3)$$

where K_0 is the equilibrium constant for channel closing at 0 mV, z and δ are, respectively, the charge of, and the fractional voltage drop sensed by the gating particle, V is the applied voltage, and F, R and T have their usual meanings. I-V plot data points were fitted by either straight lines or second-order polynomials. Permeability ratios were calculated from the extrapolated reversal potentials by applying the Goldman-Hodgkin-Katz equation.

Materials

All reagents were purchased from Sigma Chemical (Saint Louis, MO) unless otherwise stated.

Results

Biochemical and Morphological Analysis

Early results from our laboratory indicated that an apical membrane-enriched fraction obtained by following the method of Booth et al. (1980) (a procedure involving the agitation of fragmented villous tissue in isotonic NaCl followed by differential centrifugation and Mg^{2+}-induced selective precipitation of nonapical membranes) fused infrequently with planar lipid bilayers and that channels were very rarely reconstituted as single entities. Thus, to test whether the use of NaCl (high ionic strength) as the homogenization buffer osmolyte was responsible for such a behavior, we designed two other membrane isolation procedures which replaced the 150 mM NaCl for either 250 mM sucrose (low ionic strength) or 150 mM KI (mild chaotropic conditions) in an attempt to mildly destabilize protein-protein and protein-lipid hydrophobic interactions. All three preparations were as-

sayed for marker enzymes, SDS-PAGE electrophoresis, electron microscopy, and ion channel reconstitution. Alkaline phosphatase, acid phosphatase, succinate dehydrogenase, and NADH dehydrogenase were used as enzymatic markers of apical plasma membranes, lysosomes, mitochondria, and endoplasmic reticulum, respectively. As peroxisomes were reported to be absent in the human trophoblast at term (Carlson, Wada & Sussman, 1976) their presence in the membrane fractions was not investigated. The Table indicates the enrichment factors (ratios of specific activities) of the fractions relative to the filtrate for the four marker enzymes. Clearly, all three procedures yielded membrane fractions well enriched in apical plasma membranes with no significant subcellular contaminants. Figure 1 shows the results of light and electron microscopy of the samples. Comparison of higher magnification micrographs of intact brush border membranes with those of purified membrane fractions indicates that the latter are enriched in both nearly intact and vesiculated microvilli. The micrograph shown in panel *F* illustrates the effect of replacing the automatic (with a magnetic stirrer) agitation of the fragmented tissue by the manual rotation of a glass flask containing the tissue suspension. The higher level of contamination revealed by this micrograph was confirmed by enzymatic analysis and led us to adopt the automatic procedure throughout. The electrophoretic pattern of the purified membrane fractions is shown in Fig. 2. The results of the biochemical and morphological analysis of the membranes demonstrated that they were indistinguishable.

Ion Channel Reconstitution

Only 4% of successful ion channel reconstitutions (14 out of 353) incorporated Cl^- conductances consisting of identifiable (at our time resolution) discrete current steps. In other cases, smaller Cl^- conductances were co-reconstituted along with nonselective cation channels but due to the reduced conductance of the former and/or to filtering effects no discrete current steps could be identified. These smaller currents were inhibited by 1 mM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and will not be dealt with in this paper.

The analysis of the higher conductance anion channels revealed two types of activity. Neither the specific osmolyte used during homogenization nor the lipid bilayer composition (either POPE-POPC, POPE-POPC-cholesterol or POPE-POPS-cholesterol) seemed to affect channel behavior or vesicle fusion rate. Figure 3 shows some features of a Cl^- channel that was reconstituted in <1% (3 out of 353) of successful channel reconstitutions. It displayed high selectivity for Cl^- over K^+ and, under symmetrical ionic conditions (295 mM Cl^-), exhibited a slight rectification averaging a conductance of 100 pS (Fig. 3*D*). In all cases, the presence of current sublevels was evident being clearer in the single-channel trace (Fig. 3*A*). On one hand, the fact that, in the presence of salt in the *cis* but not in the *trans* side, the current amplitude of such sublevels increased as the voltage was made more negative indicated that they were anionic conductances instead of cationic, co-reconstituted smaller channels. On the other hand, the frequent occurrence of concerted transitions spanning the main and the sublevels almost ruled out the possibility of independent, co-reconstituted smaller Cl^- channels. Instead, subconductances are, very likely, an integral part of the 100-pS channel. We also investigated whether the current signal arising from a two- and a three-channel containing bilayer can be accounted for by the independent activity of co-reconstituted channels or not. Independence was tested on the basis of the binomial law according to Eqs. 2 (*see* Materials and Methods), and the random variable *number of open channels* turned out to be distributed according to it as exemplified in Fig. 3*E*. Whether the differences recorded in the individual P_O values during this experiment reflect channel heterogeneity or they arose as a result of protein-protein interactions between, otherwise, identical channels remains to be determined. In contrast, the individual P_O values estimated for the two-channel containing bilayer were closer to each other; the plot of average P_O *vs.* membrane potential (Fig. 3*F*) revealed that the equilibrium constant for channel closing at 0 mV (K_0) is 0.765 and that $z\delta$ is 0.664. In other terms, $V_{1/2}$ [i.e., $(-RT/z\delta F)\ln K_0$] is 10 mV and the voltage change per *e*-fold change in K_0 (i.e., $RT/z\delta F$) is 38 mV.

Figure 4 shows some characteristics of a second type of Cl^- channel that was reconstituted in ~3% of successful channel incorporations. It displayed a high selectivity for Cl^- over K^+, and a very complex behavior in terms of conductance and gating. The inspection of the traces indicated that the size of the current steps (either openings or closings) was highly variable but, in all cases, it seemed to be an integer multiple of a smaller unit conforming a pattern of equally-spaced current sublevels which, in addition, shared the same ion selectivity. The systematic analysis of the traces enabled us to identify a "basic" conductive unit of 47 pS (symmetrical 145 mM Cl^-) whose *I-V* relationship is illustrated in Fig. 4*C*. For the estimation of the different current levels the FETCHAN program (pCLAMP software Version 5.5) was used. Current recordings were browsed in search of well-defined, low-noise current dwellings. Upon visual inspection of these segments at 450 Hz (−3 dB) and 512 milliseconds/trace, horizontal cursors were manually located at positions that fitted best (by eye) those clearer signals. These cursors served to trace the identified current levels along the whole recording, especially in those segments where their occurrence was less evident. Once the most easily detected levels were defined the rest was assigned by calculating, on their basis, a common el-

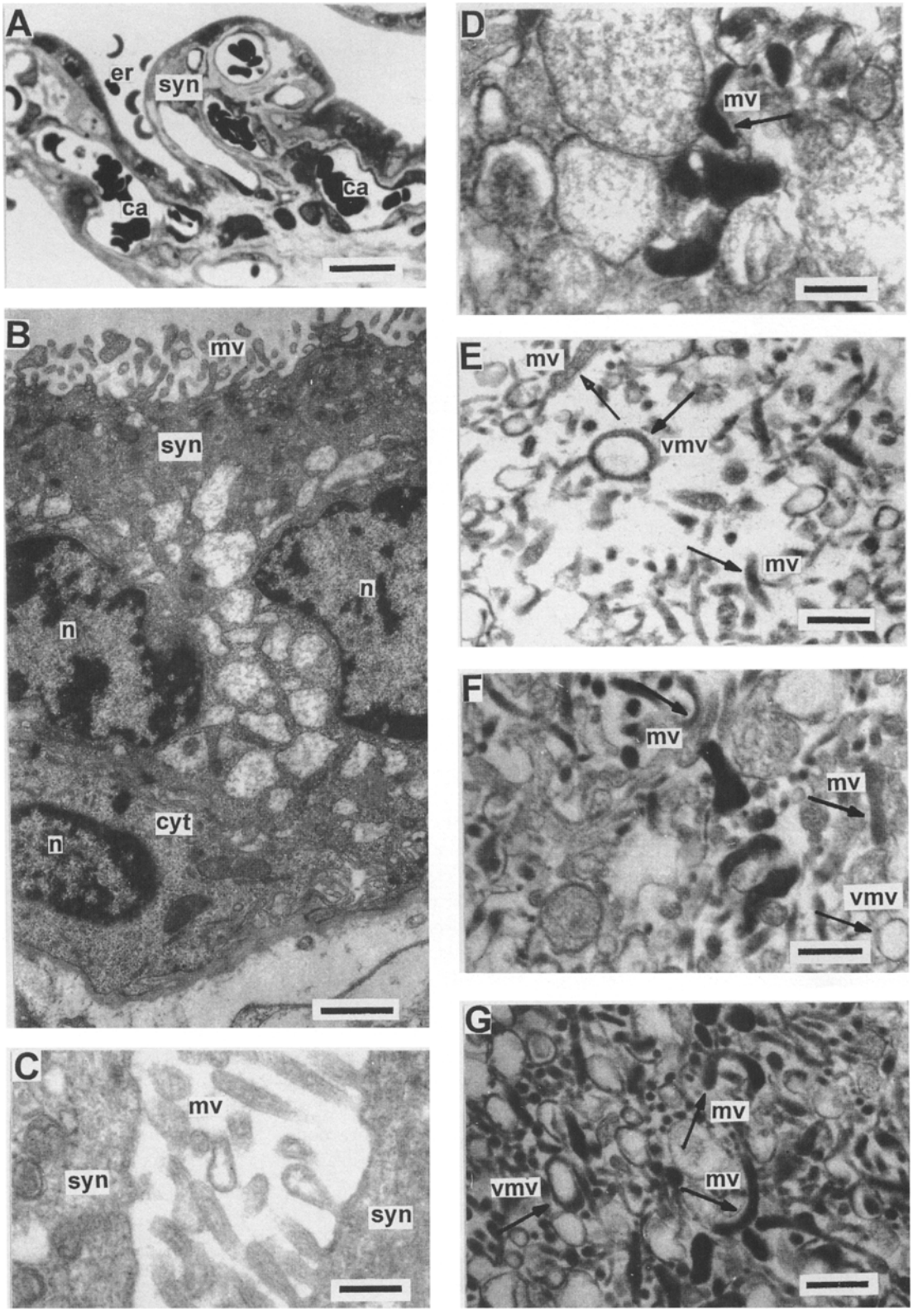

Fig. 1. (*A*) Light microscopy of intact placental villi. *er:* maternal erythrocytes. *ca:* fetal capillary containing fetal erythrocytes. *syn:* syncytiotrophoblast. Bar: 10 μm. (*B*) Electron micrograph of the outermost part of villi. *mv:* microvilli. *syn:* syncytiotrophoblast. *n:* nuclei. *cyt:* cytotrophoblast. Bar: 1 μm. (*C*) Electron micrograph of two appossing microvilli-containing apical membranes. *syn:* syncytiotrophoblast. *mv:* microvilli. Bar: 250 nm. (*D*) Electron micrograph of the suspension obtained following the isotonic agitation, the filtration step, and the centrifugation at 3100 rpm. *mv:* microvilli. Bar: 250 nm. (*E*) (*F*) and (*G*) Electron micrographs of apical membrane-enriched fractions obtained using either 150 mM NaCl, 250 mM sucrose or 150 mM KI as homogenization buffer osmolytes, respectively. (*E*) and (*G*) automatic procedure. (*F*) manual procedure (*see* text). *mv:* microvilli. *vmv:* vesiculated microvilli. Bars: 250 nm.

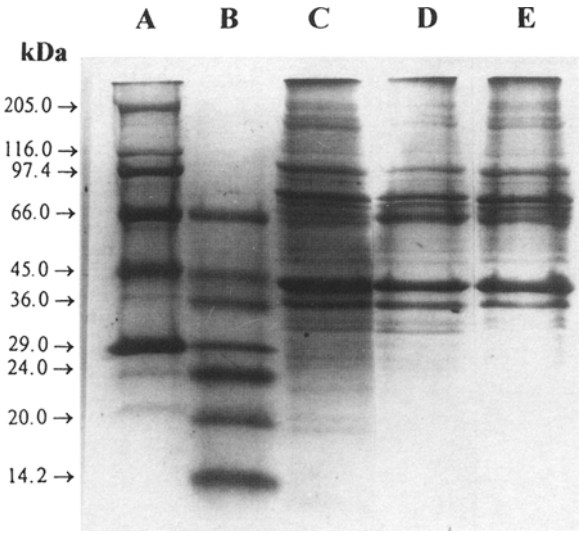

Fig. 2. SDS-PAGE. (*A*) and (*B*) Molecular weight markers. (*C*) (*D*) and (*E*) Brush border-enriched membrane fractions obtained using either 150 mM NaCl, 250 mM sucrose or 150 mM KI as homogenization buffer osmolytes, respectively. The main protein bands in lanes *C*, *D*, and *E* correspond to: (kDa) 39, 44, 66, 77, and 91.

mentary conductance and, then, by simply multiplying this basic unit by integer numbers. The occupancy probabilities were time variable, differed from bilayer to bilayer and, most frequently, not all the indicated current levels were occupied in the tracings. In this respect, the current records in Fig. 4 are particularly interesting as they show the occupancy of almost all of the indicated conductance levels. As it may be clearly seen in the figures, some current sojourns fell out the indicated dashed lines, a fact that could be attributed to rapid, unresolved flickering transitions between the identified sublevels and/or to even smaller currents steps. Nevertheless, regarding this latter possibility, we present here a plausible sublevel organization pattern, based on an equally spaced model, that is capable to predict the most salient conductance levels recorded at our resolution bandwidth. As discussed by Fox (1987) and Meves and Nagy (1989), this behavior is highly suggestive of a parallel array of identical (in terms of conductance and selectivity) ion-conducting units gating cooperatively thus giving rise to channels often referred to as "multibarrelled" (Miller & White, 1984; Hunter & Giebisch, 1987). However, the fact that the simultaneous, concerted closings and openings did not necessarily reach (when closing), or come from (when opening) the zero current level, as is evident from Figs. 4 and 6, indicating that the common gate mechanism (a common, general gate superimposed over independent, individual ones) proposed to account for the activity of some multibarrelled channels (Miller & White, 1984; Hunter & Giebisch, 1987), fails to underlie the behavior recorded by us. In addition, the fact that *complete* channel closures and openings could occur frequently (Figs. 4 and 6) and even consecutively (Fig. 5) as concerted, single-current steps, makes the possibility of several independent "common gate-having" channels in the bilayer, absolutely unlikely.

In most cases, the all-points current-amplitude histograms consisted of one main peak (close to the maximally open level) and some minor peaks as depicted in the histogram shown in Fig. 4*D*. The noise variance associated with the major peak (1 kHz filtering, 4 kHz sampling), in this representative histogram, was 4 pA^2 i.e., twice as high as that estimated for any peak in the amplitude histograms of the above reported Cl^- channel (Fig. 3*E*) or for the bare lipid bilayer (*not shown*) at the same bandwidth. This excess variance arose, at least partly, from the rapid, band-limited gating of several parallel 47-pS units which, this way, gave rise to a widened, unresolved common peak. Strikingly, for a given voltage, the mean of this main peak (apparent open channel conductance) varied from bilayer to bilayer as is shown in Fig. 5 for four different, representative experiments. These four "well-coupled" current pulses were selected from long "single-channel" traces that were subjected to the same current level analysis as any other tracing shown. This analysis suggested that this "variable conductance" was due to the activity of a variable number of conductive units whose own conductance, instead, remained constant from bilayer to bilayer (47 pS). This result is consistent with the recruitment of 47-pS units into a macromolecular complex of rather variable stoichiometry, 8 such units being the most common finding (mean number of units ± SD = 9.1 ± 2.5; mean conductance ± SD = 429 ± 116 pS; n = 8). The displayed pulses differ from other current signals in that they were unusually rectangular-shaped but they were preceded and followed by the more stepwise open-close activity that characterizes this maxi-Cl^- channel.

Figure 6 shows further evidences of the continuously changing behavior displayed by this maxi Cl^- channel. Part *A* shows a current pulse consisting of the concerted closing of eight 47-pS units followed by their stepwise opening, an additional indication of its multilevel and cooperative nature. Parts *B* (eleven units), and *C* (eight units) illustrate the entering of a flickery mode and the stability of some intermediate open levels besides of showing clearly the existence of equally-spaced substates and "well-coupled" current steps. Part *D* shows a channel containing, at least, eight 47-pS units; in the third trace, the gating of an apparently additional ninth conducting unit (not indicated by dashed lines) is shown. Shortly after, seven such units closed synchronously while one of them remained gating for a while before also "joining" the closed state. Part *E* illustrates the channel entering a long-lived, nonconducting state (a feature recorded in some but not in all of the experi-

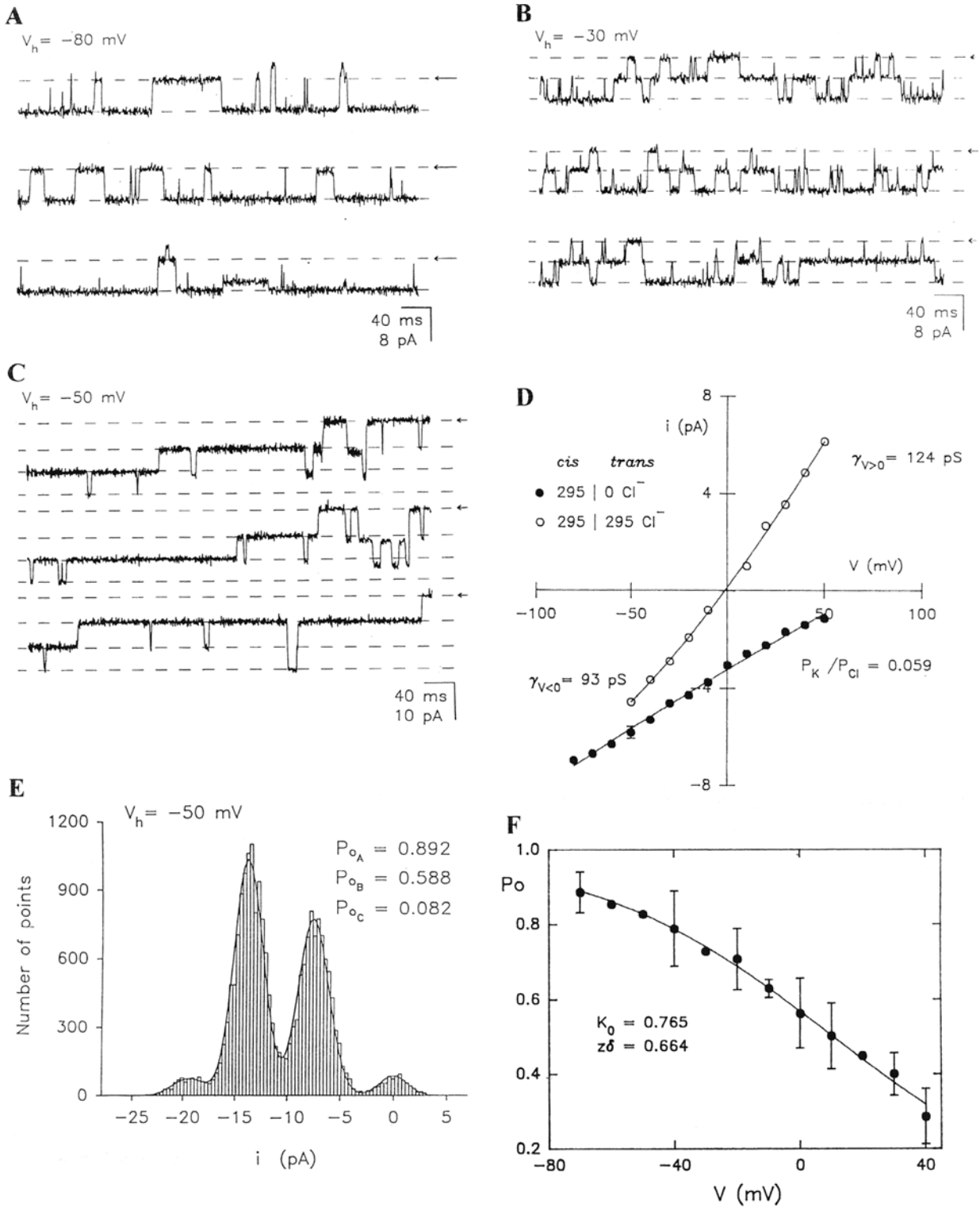

Fig. 3. Single-channel features (fc = 450 Hz) of the 100-pS Cl^--channel. (*A*) (*B*) and (*C*) Channel recordings from a single-, a two-, and a three-channel containing bilayer, respectively, with either 295 mM KCl (*A*) and (*B*) or 145 mM NaCl (*C*) added to the *cis* side. Arrowheads indicate the most populated low conductance state. Dashed lines enclose the main current step of each channel. The occurrence of substates is particularly clear in part *A*. (*D*) Single-channel *I-V* plots under symmetrical (n = 1) and asymmetrical (n = 2) KCl concentrations (n indicates the number of independent bilayers). (*E*) All-points current-amplitude histogram of the trace in part *C* (fc = 800 Hz) fitted by the sum of four Gaussian components corresponding to the alternative opening and closing of three coreconstituted channels. P_O values are the open probabilities of the co-reconstituted channels as estimated by following a binomial-based analysis. (*F*) Voltage-dependence of individual P_O values from the channels in part *B*. P_O values for both channels at each voltage were averaged. Data points were fitted by a Boltzmann equation.

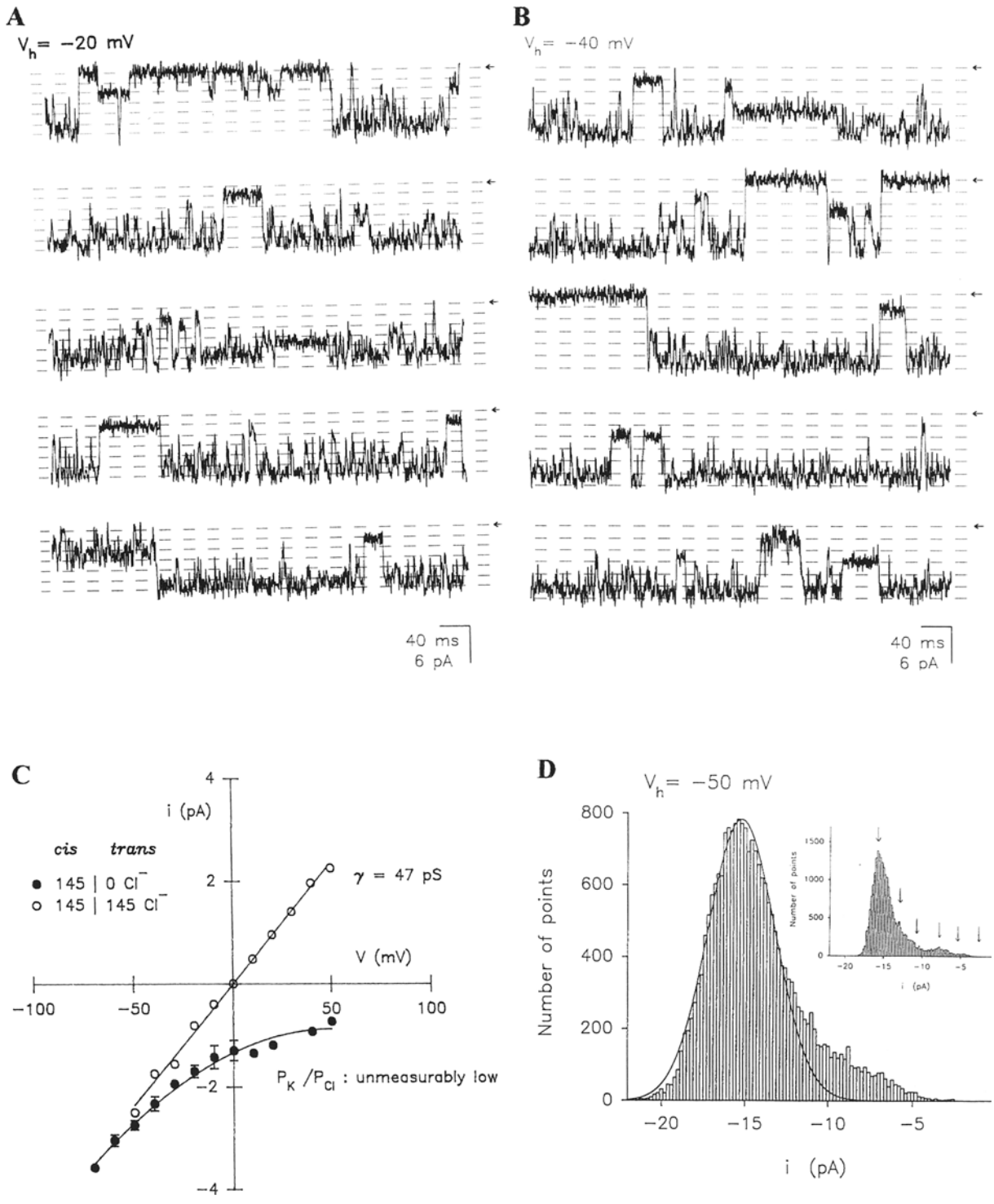

Fig. 4. Single-channel features (fc = 450 Hz) of the multibarrelled Cl^--channel. (*A*) and (*B*) Channel recordings from the same bilayer with 145 mM KCl added to the *cis* side. Arrowheads indicate the closed state. Dashed lines enclose the current passing through each putative elementary conductive unit. In this particular experiment, the "full" channel was formed by six such units. (*C*) *I-V* plots of the elementary conductive unit under symmetrical (n = 1) and asymmetrical (n = 7) KCl concentrations (n indicates the number of independent bilayers). (*D*) All-points current-amplitude histogram of a six-unit channel (fc = 1 kHz). The main peak was fitted by a Gaussian curve (σ = 2 pA). The *inset* shows the amplitude histogram constructed for the same, further filtered, data stretch (fc = 450 Hz). Arrowheads indicate the (hidden) position of the closed and the six open levels.

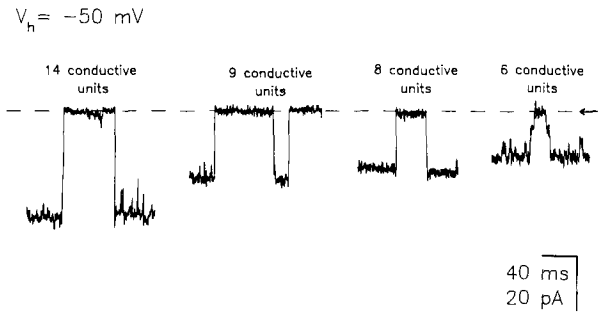

Fig. 5. Selected "well-coupled" current pulses recorded at −50 mV in four independent bilayers with 145 mM KCl added to the *cis* side. Each current pulse correspond to the largest current value observed in each case (fc = 450 Hz). Arrowhead indicates the closed state.

ments) by showing the current response to −50 ↔ +50 mV voltage steps. Both recovery from, and entry into this state were sensitive to voltage such that recovery was faster and entry ("inactivation," Blatz & Magleby, 1983; Kourie et al., 1996) was slower when the potential was stepped from −50 mV to +50 rather than from +50 to −50 mV. Voltage-dependent inactivation in epithelial Cl^- channels was also demonstrated to occur by means of patch-clamp, whole-cell recordings (Levitan & Garber, 1995).

Radiotracer-flux studies revealed that the Cl^- conductance of human placental microvillous membrane vesicles has two components one of them being sensitive to DIDS while the other is sensitive to diphenylamine 2-carboxylate (DPC) (Byrne et al., 1993). Consistently, Fig. 7 shows the inhibitory effect of 1 mM DIDS on this maxi Cl^- channel. In addition, channel behavior was unaffected by the presence of up to 1 mM Ca^{2+} on both sides of the bilayer.

Interestingly, about one half of the vesicle fusions leading to the maxi Cl^- channel insertion was also accompanied by the incorporation of the most abundant, nonselective cation channel, another strong hint of the colocalization of both types of channels on the same membrane vesicles.

Discussion

The paucity of direct information on human syncytiotrophoblast ionic channels is striking, especially considering the key role of this epithelium in placental physiology and the fact that some single channels from other brush border tissues, like the renal and intestinal epithelia, have already been studied. Though cytotrophoblast cells in culture can aggregate, fuse, and polarize to form syncytial units, they might not attain the same degree of differentiation as they do in the placenta at term (Hoshina et al., 1985). This fact, along with the difficulties encountered in patch-clamping the apical plasma membrane of intact villi (Brown et al., 1993), severely reduces the repertoire of suitable techniques for the direct study of the ionic channels present in the mature syncytiotrophoblast. Thus, the reconstitution of plasma membrane-enriched fractions on planar lipid bilayers appears as a valuable alternative technique, and attempts to obtain improved membrane preparations seem worthwhile.

In spite of the indication of the occurrence of K^+, Na^+, and Cl^- conductances on human syncytiotrophoblast apical membranes (Shennan & Boyd, 1987; Illsley & Sellers, 1992; Byrne et al., 1993; Greenwood et al., 1993; Faller et al., 1994; Greenwood et al., 1996) previous direct electrophysiological measurements failed to identify the channels underlying all such activities. Although Brown et al. (1993) seemed to find the channel responsible for the DIDS-sensitive component of the Cl^- conductance, the channels accounting for the DPC-sensitive component, and the K^+ and Na^+ conductances were not identified thus far. In our case, the finding of an overwhelming majority of nonselective cation channels (Grosman & Reisin, 1996) might underlie, at least partly, the cation conductance (Shennan & Boyd, 1987), the higher permeability for cations as compared with Cl^- (Illsley & Sellers, 1992), and the low (−22 mV) apical membrane potential difference at term (Greenwood et al., 1993, 1996).

This paper confirms the previous finding of a DIDS-sensitive, maxi Cl^- channel in the apical surface of the human syncytiotrophoblast at term (Brown et al., 1993; Riquelme et al., 1995) and, in turn, gets deeper into its multilevel nature. It also describes some properties of a 100-pS Cl^- channel which was not reported before and which would be a candidate for the, thus far elusive, DPC-sensitive Cl^- channel.

The possibility of a contaminant, intracellular origin for these reconstituted Cl^- channels was taken into account given their low frequency of being observed. Nevertheless, it seemed very unlikely as, on one hand, neither reconstituted placental Cl^- channel resembles very closely any of the several organellar Cl^- channels known to date, and, on the other hand, both of them share many properties of other plasmalemmal Cl^- channels. For example, the outer mitochondrial membrane voltage-dependent anion channel (mi-VDAC) (Colombini, 1979) changes its ion selectivity with the applied potential being modestly anion selective (P_K/P_{Cl} ~0.5) at low potentials and slightly cation selective (P_K/P_{Cl} ~1.5) at higher voltages (Mirzabekov et al., 1993). In addition, its single-channel conductance changes linearly with ion concentration (0.45 nS in symmetrical 0.1 M KCl and 4.5 nS in symmetrical 1.0 M KCl) and has slow, in-the-second-range kinetics. The inner mitochondrial membrane has a 100-pS (symmetrical 150 mM KCl) channel with a P_K/P_{Cl} ~0.2 and voltage-dependent inactivation

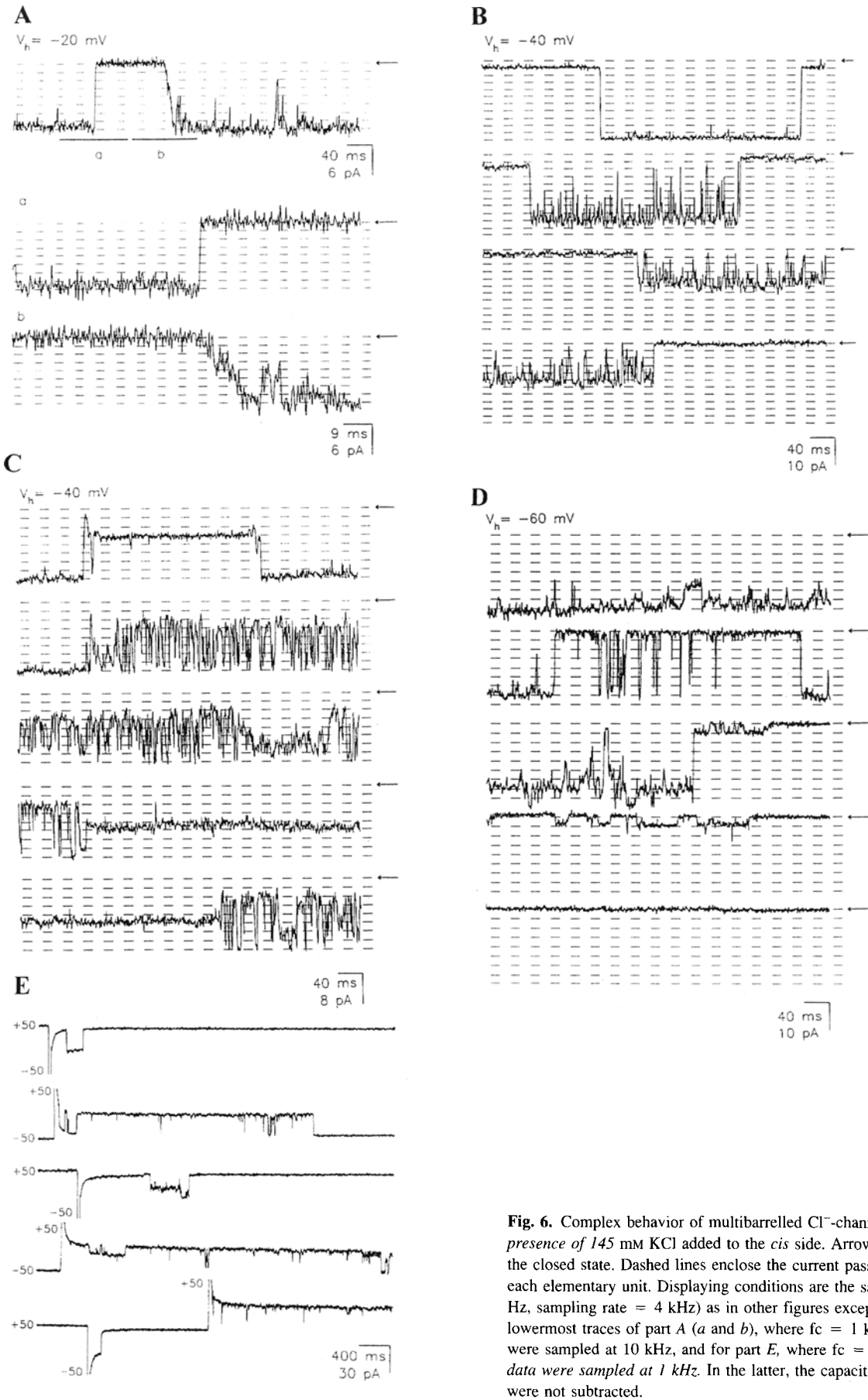

Fig. 6. Complex behavior of multibarrelled Cl^--channels *in the presence of 145* mM KCl added to the *cis* side. Arrowheads indicate the closed state. Dashed lines enclose the current passing through each elementary unit. Displaying conditions are the same (fc = 450 Hz, sampling rate = 4 kHz) as in other figures except for the lowermost traces of part *A* (*a* and *b*), where fc = 1 kHz and data were sampled at 10 kHz, and for part *E*, where fc = 240 Hz *and data were sampled at 1 kHz.* In the latter, the capacitive transients were not subtracted.

Table Marker-enzyme analysis

Enzymatic activity	Localization	Homogenization buffer osmolyte	Enrichment factor (fraction/filtrate)
Alkaline phosphatase	Apical membranes	150 mM NaCl	20.9
		250 mM sucrose	24.8
		150 mM KI	21.7
Acid phosphatase	Lysosomes	150 mM NaCl	1.1
		250 mM sucrose	1.3
		150 mM KI	0.5
Succinate dehydrogenase	Mitochondria	150 mM NaCl	0.3
		250 mM sucrose	0.4
		150 mM KI	0.3
NADH dehydrogenase	Endoplasmic reticulum	150 mM NaCl	0.7
		250 mM sucrose	1.1
		150 mM KI	0.7

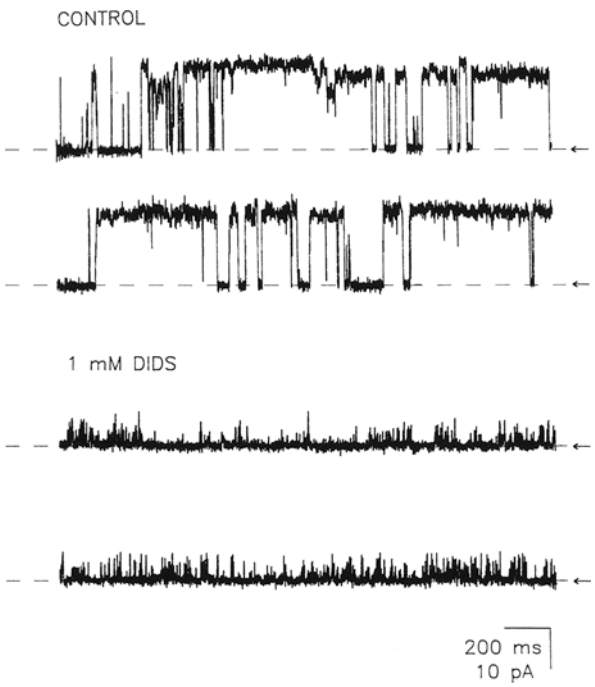

Fig. 7. Inhibitory effect of symmetrical 1 mM DIDS on the activity of the placental maxi-Cl^- channel (fc = 450 Hz) in the presence of 145 mM KCl added to both sides of the bilayer.

(Sorgato, Keller & Stühmer, 1987). Rough endoplasmic reticulum membranes from mammalian pancreas have high-conductance (250–300 pS in symmetrical 140–150 mM KCl) channels only slightly more selective for anions than cations (Schmid et al., 1988; Simon, Blobel & Zimmerberg, 1989) whereas those from rat liver have also a 320 pS (450/50 mM KCl, *cis/trans*) double-barrelled anion selective (P_K/P_{Cl} <0.14) channel (Morier & Sauvé, 1994). Finally, rat renal endocytotic vesicles have a 73 pS (symmetrical 140 mM KCl) channel highly selective for anions (Schmid, Burckhardt & Gögelein, 1989) which differs from placental Cl^- channels in kinetic and subconductance aspects. In addition, the reconstituted placental maxi Cl^- channel shares several distinctive properties of other plasmalemmal (as opposed to mitochondrial) high-conductance Cl^- channels: a high selectivity for anions over cations (P_K/P_{Cl} <0.1) which does not vary with the applied potential, and a fast, in-the-millisecond-range kinetics. Channels of this type were reported to occur in the plasma membrane of a variety of epithelia (Gögelein, 1988) (such as pulmonary alveolar cells, MDCK cells, trachea, urinary bladder, and A6 cells), skeletal muscle (Blatz & Magleby, 1983), neurones (Geletyuk & Kazachenko, 1985; Gage, Gibb & Krouse, 1986), and B lymphocytes (Bosma, 1989), among other tissues. An even more definitive support for the plasmalemmal, apical origin of the placental maxi Cl^- channel comes from its frequent, simultaneous co-reconstitution with nonselective cation channels, and from the direct observation of a similar DIDS-sensitive, maxi Cl^- channel on this placental surface by Brown et al. (1993), using the patch-clamp technique. On the other hand, the 100-pS channel, which is not clearly related to any intracellular channel listed, may have remained undetected in previous plasma membrane patch-clamp studies (Brown et al., 1993; Riquelme et al., 1995) in accordance with the general elusiveness of Cl^- channels in the microvillous syncytiotrophoblast membrane at term. In this respect, the low probability of reconstituting Cl^- channels is consistent with their low probability of being recorded in patch-clamp studies: Brown et al. (1993), in the only single-channel patch-clamp study of intact villi reported to date, found that less than 10 patches in 120 seals contained a Cl^- channel, a fact that might be associated with a low number of copies of anion-selective channels in this apical membrane. In addition, they found that the probability of recording Cl^- channels in trophoblast cells decreases from the cytotrophoblast cell-stage towards the fused and differentiated

mature syncytium, as gestation proceeds. Moreover, such a low channel density is not restricted to the syncytiotrophoblast but it was also the case of the sarcolemmal maxi Cl^- channel from embryonic rat cultured skeletal muscle, which was recorded in less than 5–10% of membrane patches examined (Blatz & Magleby, 1983), and of a 60-pS Cl^- channel from the sarcolemma of adult rat intact skeletal muscle recorded in 3 out of 139 patches (Chua & Betz, 1991).

The multisubstate nature of the placental maxi Cl^- channel reported here is not unique to it. Similar equally-spaced current levels have been described for a variety of cation- and anion-selective channels (Fox, 1987; Meves & Nagy, 1989). Particularly among the latter, the existence of channels with 6 (Krouse, Schneider & Gage, 1986), 12 (Gage et al., 1986), and 16 (Geletyuk & Kazachenko, 1985) open conductance levels or channel units was reported in the plasma membrane of mammalian alveolar cells, mammalian hippocampal neurones, and molluscan neurons, respectively. Nonetheless, unlike these channels, the channel we described did not display a fixed number of open current levels. Instead, this number ranged from 6 to 14, a fact we interpreted as the association of conductive units in the plane of the membrane with a variable stoichiometry though, alternatively, this finding could be interpreted as the variable extent of opening or closing of a fourteen- (or even more) barrelled channel. This multisubstate architecture of channels could also account for the high variation (between different patches) of the "single-channel conductance" reported for maxi Cl^--channels from rat muscle (430 ± 42 pS; mean ± SD) by Blatz and Magleby (1983), and from A6 epithelial cells (359 ± 45 pS; mean ± SD) by Nelson, Tang and Palmer (1984), among others, as we suggested for their placental counterpart (Fig. 5).

The method of visual inspection adopted in this work as a means of estimating signal amplitudes has been widely used previously (Krouse et al., 1986; Hunter & Giebisch, 1987; Bosma, 1989; Schreibmayer, Tritthart & Schindler, 1989; Ramanan & Brink, 1993). Though we are aware that this procedure may have introduced subjective elements and that the unfavorable signal-to-noise ratio (due to the combination of the relatively high background noise of the planar bilayer and the frequently fast gating activity of the presumed conductive units) impaired such an analysis, we found it to be the most adequate one in this first stage of channel characterization. An alternative method could have been to fit all-points current-amplitude histograms to Gaussian curves, an automated and objective procedure frequently used. Nevertheless, as shown in Fig. 4*D*, it is clear that those histograms failed even to display those peaks corresponding to the most evident current levels. More recently, Kourie et al. (1996), have resorted to the use of statistical techniques, such as those based on Hidden Markov Models (Chung et al., 1990), to estimate the current levels of two multisubstate Cl^- channels from skeletal muscle sarcoplasmic reticulum (SR). On the basis of such methods, they suggested the possibility that a high conductance SR Cl^- channel results from the cooperative gating of ~10 25 pS (250/50 mM Cl^-, *cis/trans*) "protochannels". These signal processing techniques may prove useful in our future way to the thorough understanding of current sublevel organization in this type of placental channels.

The role of the reported Cl^- channels in transplacental ion transport and/or syncytium homeostasis remains to be determined.

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