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DEPENDENCE OF MEMBRANE POTENTIAL ON Ca^{2+} TRANSPORT IN CULTURED CYTOTROPHOBLASTS OF HUMAN IMMATURE PLACENTAS

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The electrical membrane properties of cultured human cytotrophoblast were examined by means of a standard electrophysiological technique. The mean values of the membrane potential (E_m) and the membrane resistance in a physiological medium were around −49 mV and 12 MΩ, respectively. The membrane potential was dependent, to a large extent, on the external Ca^{2+} concentration ($[Ca^{2+}]_0$). Deprivation of external Ca^{2+} reduced membrane potential to about −20 mV, and an increase in $[Ca^{2+}]_0$ caused a hyperpolarization in a saturable manner. The Ca^{2+}-dependency of membrane potential was affected remarkably by $[K^+]_0$, but not by $[Na^+]_0$ or $[Cl^-]_0$. The intracellular Ca^{2+} injection hyperpolarized the membrane in a Ca^{2+}-free medium. A Ca^{2+} channel blocker, verapamil, completely abolished the Ca^{2+}-dependent E_m. The Ca^{2+}-dependent E_m was also suppressed by cooling or by the application of metabolic inhibitors. It is suggested that the Ca^{2+}-dependent E_m in cultured human cytotrophoblast is caused by a Ca^{2+} influx which, in turn, increases the K^+ conductance of the cell membrane, presumably due to stimulation of Ca^{2+}-activated K^+ channel.

Introduction

The trophoblast exhibits a variety of physiological activities, including the transfer of oxygen, ions and nutrients to the fetus, and the synthesis of steroid and polypeptide hormones [1]. However, the mechanisms by which trophoblasts regulate such diverse biological functions remain unknown. As an initial approach toward this problem, we attempted to examine the electrical membrane properties of trophoblasts which are at the background of such diverse functions. A few reports [2–4] are available concerning the membrane potential in trophoblasts. In these studies, however, no detailed ionic mechanisms were analyzed. In the present study, the ionic mechanisms of membrane potential in cytotrophoblast were characterized in the explant culture of human placenta. The results show that the membrane potential of these cells depends significantly on the Ca^{2+} transport across the cell membrane.

Materials and Methods

Cell culture

Normal human placental chorionic villi in early gestational ages (5–8 weeks) were obtained from 41 young pregnant women under aseptic conditions at the time of artificial interruption of pregnancy for socioeconomic reasons. The chorionic villi were rinsed several times in Hanks' balanced salt solution and immediately cut into small fragments by fine curved scissors. These fragments were inoculated onto gelatin-coated glass coverslips [5] placed in short cubic tubes and were incubated in a closed system at 37°C. Medium 199 supplemented with 20% fetal calf serum (plus 100 units of penicillin and 100 μg of streptomycin per

ml) was used as a culture medium. Culture media were replaced every day, and also 5 h prior to the morphological or electrophysiological study. The mononuclear cytotrophoblasts which migrated from fragmented villi were employed in the present study.

Electrophysiology

The microelectrode preparation and electronic instruments employed in the present electrophysiological study were identical to those described previously [6]. The method of intracellular Ca^{2+} injection has also been described previously [7]. Data were accepted only when the transmembrane potential was stable for at least 30 s and returned to the original zero level upon withdrawal of the electrode from the cell. When a test solution was applied, it was replaced by the control solution within 15 min. The temperature of the cell chamber was usually maintained at 37°C by circulating warm water. If necessary, the bath temperature was altered by changing the temperature of the circulating water. All the data presented herein are the mean ± S.E., with the number of observations, n, in parentheses.

Solutions and chemicals

As the control medium, a Tris-buffered saline was used. The medium was composed of 129.3 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$ and 0.9 mM $MgCl_2$, and was buffered to pH 7.3 ± 0.1 with 13 mM Tris-HCl. Changes in K^+ concentrations were accomplished by replacing part of the NaCl with an equimolar amount of KCl, keeping the total K^+ and Na^+ concentration (134.7 mM) constant. Low Na^+ solutions were prepared by replacing appropriate amounts of NaCl in the control medium with equimolar amounts of Tris-HCl or choline chloride. Low Cl^- solutions were prepared by replacing an appropriate amount of NaCl with Na_2SO_4, and mannitol was added to adjust the osmolarity of this solution to the same as that of control medium. In some experiments, $CaCl_2$ or $MgCl_2$ in the control medium was deleted to obtain a Ca^{2+}- or Mg^{2+}-free solution. When the effect of divalent cations was examined, various amounts of $CaCl_2$, $MnCl_2$, or $SrCl_2$ were added to a Ca^{2+}-free solution in the presence of 0.9 mM Mg^{2+}, and $BaCl_2$ or $MgCl_2$ were added to a Mg^{2+}-free solution in the presence of 1.8 mM Ca^{2+}. EDTA (disodium salt) was employed as a chelating agent. Ruthenium red was purchased from Chroma-Gesellshaft Schmidt and Co. Verapamil was a gift from Eisai Co. (Tokyo). Ethanol was used as a vehicle for verapamil. The addition of up to 2% ethanol did not affect significantly the electrical properties of the cell membrane. Ouabain, KCN, NaN_3 and NaF were purchased from Nakarai Chemical Co.

Microscopy

Morphological studies were made on the cells cultured for 30–40 h. For phase-contrast microscopy, the cells were washed in a control medium and observed in a living state under a Nikon microscope (Optiphoto NT). For scanning electron microscopy, the cells were rinsed three times with warm Hanks' salt solution (37°C) and then fixed with 2% glutaraldehyde in a phosphate-buffered saline at pH 7.4 for 1 h at 37°C. After rinsing with a phosphate-buffered saline, the cells were post-fixed with 1% osmium tetraoxide in the saline for 1 h at room temperature and dehydrated in a graded series of ethanol. After replacing ethanol with isoamil acetoacetic acid, the cells were dried by the CO_2 critical-point procedure. After coating with gold in a sputter coater (Shimadzu IC-50) the specimen was examined in a Shimadzu ASM-SX scanning electron microscope at 15 kV.

Results

Morphology

On the second day of culture, human cytotrophoblasts started to migrate from the fragments toward the substrate surface. As shown in Fig. 1a, these cells were mononuclear and epithelial-like [5,8]. Examination by scanning electron microscopy revealed that the cell surface is covered with short microvilli and that there are prominent fine filopodia at the margin of the cell (Fig. 1b).

Membrane potential in physiological media

When a microelectrode was inserted into a cell, a stable membrane potential of around −50 mV was obtained in a culture medium. The average membrane potential recorded from 913 cells in the control Tris-buffered saline was −48.6 ± 0.3 mV

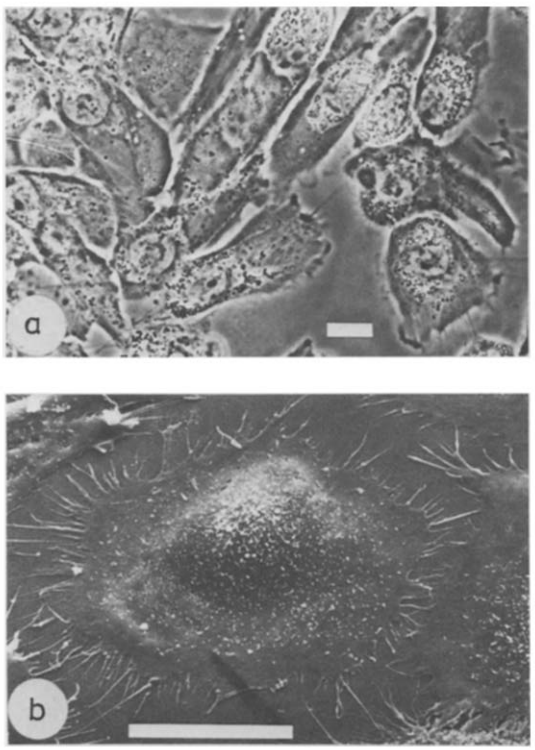


Fig. 1. Micrographs of cultured human cytotrophoblast. (a) Phase-contrast micrograph. Bar, 20 μm. (b) Scanning electron micrograph. Bar, 20 μm.

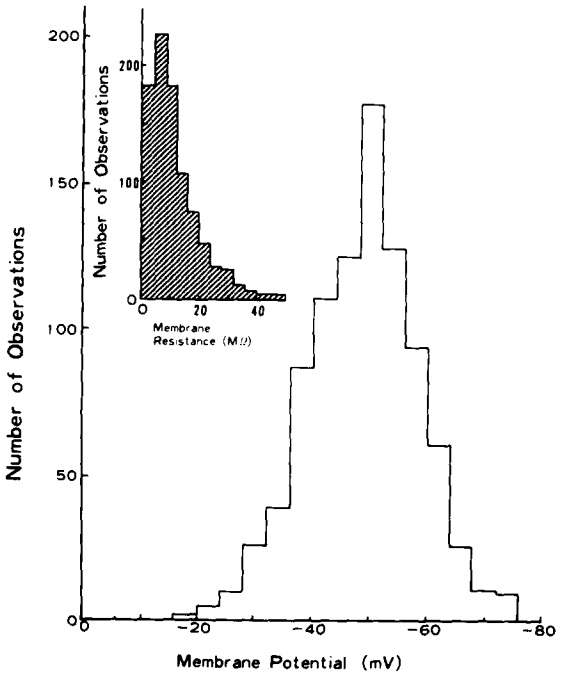


Fig. 2. Distribution of membrane potentials in cultured human cytotrophoblasts in the control medium. Insert: Distribution of membrane resistances.

with a range of -16 to -76 mV (Fig. 2). The average membrane resistance was 11.7 ± 0.3 MΩ with a range of 0.3 to 50 MΩ (Fig. 2, insert). During about 2 weeks in culture the membrane potential remained practically unchanged, but electrophysiological observations were made mainly on the second or third day of culture. Oscillations of the membrane potential found in cultured syncytiotrophoblasts [4] have never been encountered in cytotrophoblasts under the present culture condition.

Effect of monovalent ions

A reduction of the external concentration of Na^+ and Cl^- had little effects on the membrane potential. The mean membrane potentials were -50.3 ± 2.4 mV in 13 mM Na^+ solution and -52.9 ± 2.0 mV in 14 mM Cl^- solution, respectively. These values were not significantly different ($P > 0.25$) from the membrane potential observed in the control medium. In contrast, alterations in the external K^+ concentration showed a clear effect on the membrane potential. As shown in Fig. 3, the membrane potential was hyperpolarized by reducing $[K^+]_0$ and depolarized by increasing $[K^+]_0$ in the presence of 1.8 mM Ca^{2+}. The potential change against log $[K^+]_0$ was approximately linear, with a slope of 31 mV.

Effect of external divalent cations

The deprivation of Ca^{2+} from the external medium caused a remarkable depolarization. The mean membrane potential in Ca^{2+}-free media was -19.5 ± 1.1 mV ($n = 25$). The addition of EDTA (1 mM) to the Ca^{2+}-free solution induced no further depolarization. Also, in the Ca^{2+}-free solution, the dependence of the membrane potential on $[K^+]_0$ virtually disappeared (Fig. 3). These observations indicate that, while the membrane potential of cultured cytotrophoblasts is determined mainly by the K^+ concentration gradient, the K^+ conductance depends on the external Ca^{2+}.

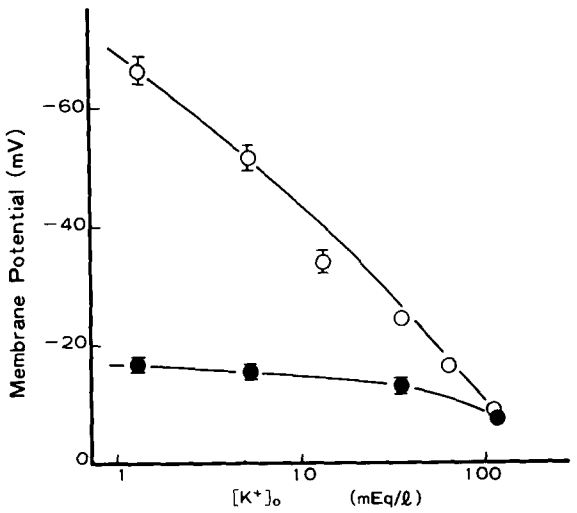


Fig. 3. Effects of $[K^+]_0$ changes on the membrane potential in cultured human cytotrophoblast. ○, $[Ca^{2+}]_0 = 1.8$ mM; ●, Ca^{2+}-free. Each point represents the mean of membrane potentials obtained in 6–26 cells. Vertical bar, S.E.

When the control medium was replaced by a high Ca^{2+} solution the membrane of cultured cytotrophoblasts was hyperpolarized strikingly. The greater the external Ca^{2+} concentration ($[Ca^{2+}]_0$), the more negative was the level of the membrane potential, and saturation of the hyperpolarization occurred at about 10 mM of $[Ca^{2+}]_0$. A certain amount of Sr^{2+} or Mn^{2+} added to the external solution in place of Ca^{2+} was also able to induce the hyperpolarization of membrane potential. Fig. 4a illustrates the hyperpolarizations thus induced by external Ca^{2+}, Sr^{2+} and Mn^{2+} at various concentrations.

In contrast, the externally applied Ba^{2+} or Mg^{2+} induced a depolarization of membrane potential in a dose-dependent manner (Fig. 4b) in spite of the presence of external Ca^{2+} (1.8 mM). 1 mM Ba^{2+} added to the Mg^{2+}-free solution that contained 1.8 mM Ca^{2+} caused the depolarization of membrane nearly to the E_m level obtained in the Ca^{2+}-free solution. The effects of Mg^{2+} were qualitatively similar to those of Ba^{2+}, but the former was less potent (Fig. 4b).

Effect of intracellular injection of divalent cations

In order to verify the role of the intracellular divalent cations in the membrane potential, the effect of intracellular injection of Ca ions was examined. When Ca^{2+} was injected into the cell bathed in a Ca^{2+}-free solution, the cell was hyperpolarized up to the saturation level (Fig. 4c). In response to a similar procedure, the cell was hyperpolarized by Mn^{2+} or Sr^{2+}, but not by Ba^{2+} or Mg^{2+} (Fig. 4c). These results would suggest membrane hyperpolarization was induced by a so-called Ca^{2+}-activated K^+ conductance [7,9–12].

Effect of Ca^{2+} transport inhibitors

In the light of the above observations, it is plausible that the K^+ conductance may be activated by Ca^{2+} transported from the outside of the cell. To test this hypothesis, the effects of several Ca^{2+} transport inhibitors were examined. Ruthenium red, an inhibitor of Ca^{2+}-binding to the plasma membrane [13], applied externally (0.5 –2 mM) caused a remarkable depolarization to about −22 mV (Fig. 2). Verapamil, a Ca^{2+} channel blocker [14], applied to the control medium also caused a marked depolarization, within a few minutes, to the level observed in a Ca^{2+}-free solution (Fig. 5). The membrane potential recovered to the original level when the cells were washed with the control medium 15 min after the application of these drugs. Verapamil and ruthenium red, however, affected little the membrane potential in a Ca^{2+}-free solution. Thus, the Ca^{2+} responsible for the maintenance of the membrane potential in cytotrophoblast is mainly, if not entirely, external in origin.

Effect of temperature

The membrane potential was measured at various temperatures ranging from 9 to 40°C. A decrease in temperature caused a depolarization. However, the temperature effect was apparently dependent on the presence of Ca^{2+} in the medium (Fig. 6). This suggests that the Ca^{2+}-dependent component of the membrane potential is associated with cellular metabolism.

Effect of metabolic inhibitors

Several metabolic inhibitors were applied to test whether the Ca^{2+}-dependent component of the membrane potential is generated by an energy-consuming process. The application of an appropriate amount of KCN or NaN_3, a member of

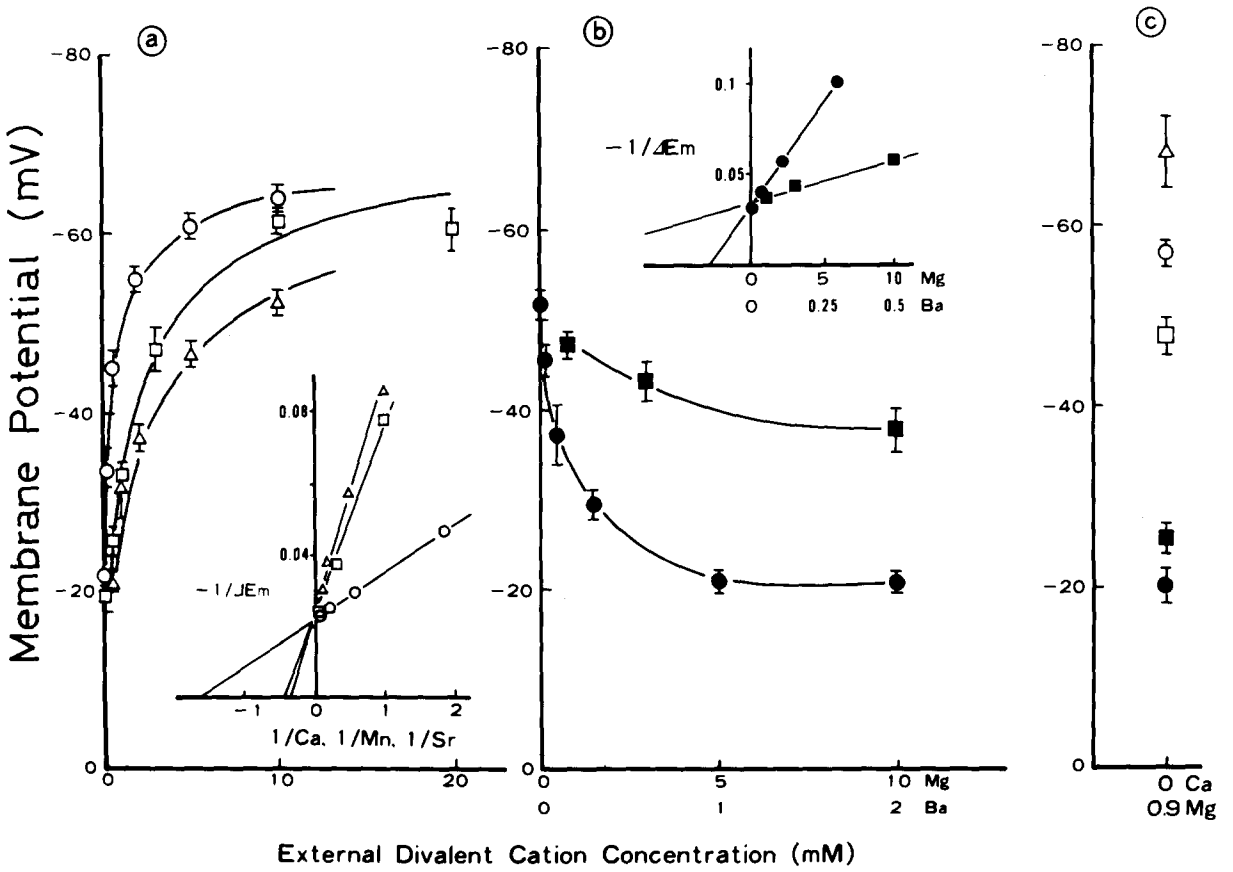
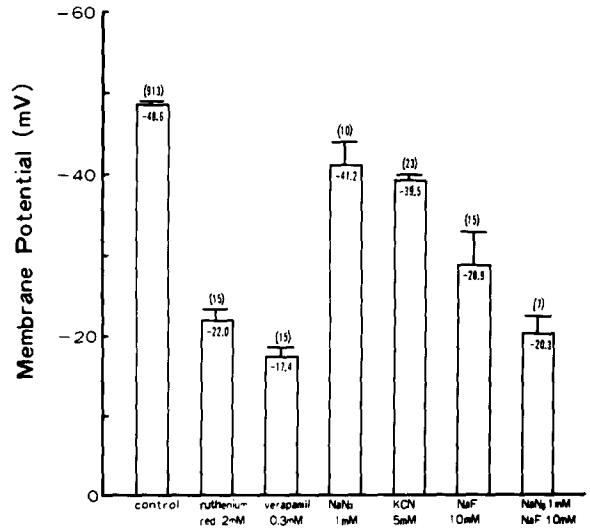


Fig. 4. Effects of divalent cations on the membrane potential in cultured human cytotrophoblasts. ○, Ca^{2+}; □, Mn^{2+}; △, Sr^{2+}; ●, Ba^{2+}; ■, Mg^{2+}. Each point represents the mean value obtained from 4–47 cells. Vertical bar: S.E. (a, b) Mean membrane potentials (E_m) plotted against external divalent cation concentration. (c) Effects of intracellular injection of divalent cations on the membrane potential. Insert in (a): Double reciprocal plot for the amplitude of hyperpolarization against external Ca^{2+}, Mn^{2+} and Sr^{2+} concentrations. Insert in (b): Plot for the reciprocal of the amplitude of hyperpolarization against external Mg^{2+} and Ba^{2+} concentrations.



respiratory inhibitors, depolarized the membrane down to around −40 mV within 15–60 min (Fig. 5). NaF, one of the glycolysis inhibitors, also depolarized the membrane to about −30 mV (Fig. 5). When both inhibitors, NaN_3 and NaF, were applied simultaneously, the membrane potential was decreased further (Fig. 5), reaching the level (about −20 mV) observed in a Ca^{2+}-free solution or at low temperature. The effects of these chemicals were reversible even following a long

Fig. 5. Effects of inhibitors for Ca^{2+} transport or cell metabolism on the membrane potential in cultured human cytotrophoblasts. Number in parentheses: number of cells impaled. Vertical bar, S.E.

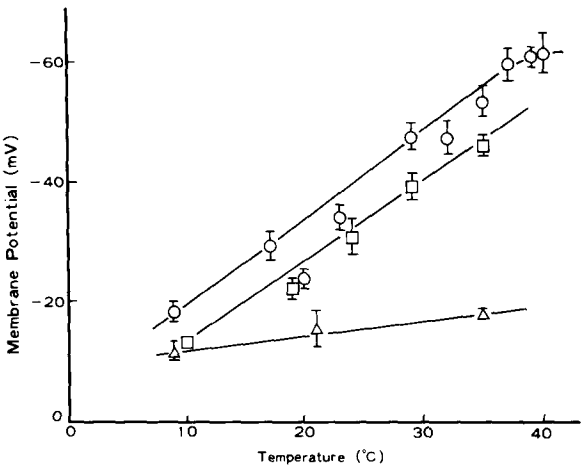


Fig. 6. Effect of temperature on the membrane potential in cultured human cytotrophoblasts. ○, $[Ca^{2+}]_0 = 10$ mM; □, $[Ca^{2+}]_0 = 1.8$ mM; △, Ca^{2+}-free. Each point represents the mean value obtained from 4–26 cells. Vertical bars, S.E.

exposure (up to 90 min). Since the membrane potential obtained in a Ca^{2+}-free solution was affected little by the application of these metabolic inhibitors (data not shown), it appears that the Ca^{2+}-dependent component of membrane potential in cytotrophoblast is associated with cellular metabolism, although the detailed mechanism remains to be elucidated.

A $(Na^+ + K^+)$-ATPase inhibitor, ouabain, affected little the membrane potential in the concentrations of 10^{-4}–10^{-3} M within 30 min, although the membrane depolarized gradually after more than 30 min. This result would indicate that the contribution of an electrogenic Na^+ pump to the membrane potential is negligible.

Discussion

A large part of the membrane potential in cultured human cytotrophoblast was found to be dependent on the external Ca^{2+}. Since the Ca^{2+}-dependendent component is affected by changes in the external K^+ concentration but not by Na^+ and Cl^-, the Ca^{2+}-dependent E_m is generated apparently by the K^+ conductance across the cell membrane. Even in the absence of external Ca^{2+}, the intracellular injection of Ca^{2+} restored the Ca^{2+}-dependent component. Therefore, it is concluded that the site of action of Ca^{2+} is the inside of the cell and that the effect is exerted by stimulation of the so-called Ca^{2+}-activated K^+ channel.

However, the origin of the intracellular Ca^{2+} responsible for the Ca^{2+}-dependent component must be the outside of the cell, since extracellular ruthenium red and verapamil abolished the Ca^{2+}-dependent component. A similar Ca^{2+}-dependent component of the membrane potential has been found in cultured intestinal epithelia [15] and in cultured L cells [7,16].

Assuming that the cell membrane is at equilibrium and that the individual ionic fluxes across the cell membrane obey an ohmic current-voltage relation, the following equation can be obtained for the change in membrane potential (ΔE_m) [7,17] when the ionic conductance change is confined to K^+.

$$\frac{-1}{\Delta E_m} = \frac{1}{E_m^0 - E_K} + \frac{G_m^0}{E_m^0 - E_K} \cdot \frac{1}{\Delta G_K} \qquad (1)$$

where $G_m = G_m^0 + \Delta G_m$, $E_m = E_m^0 + \Delta E_m$, and G_m^0 or E_m^0 represents the initial membrane conductance or potential, respectively; G_K or E_K refers to the conductance and the equilibrium potential for K^+. A similar equation has been given by Martin [18]. The plots of $-1/\Delta E_m$ against $1/\Delta G_K$ will give a straight line and the intercept on the ordinate will represent the value of $1/(E_m^0 - E_K)$. The insert of Fig. 4a shows that the relationship between the magnitude of the Ca^{2+}-dependent component of E_m ($-\Delta E_m$) and the concentration of Ca^{2+}, Mn^{2+} or Sr^{2+} obeyed the Michaelis-Menten type kinetics. Thus, G_K is related linearly to the external concentration of Ca^{2+}, Mn^{2+} or Sr^{2+}, to a first approximation. The E_K value estimated from the intercept on the ordinate is -65 mV. Since Ba^{2+} or Mg^+ applied to the external medium caused depolarizations in a dose-dependent manner, in spite of the presence of external Ca^{2+} (Fig. 4b), there is a possibility that these cations compete with Ca^{2+} for the site of Ca^{2+} transport on the membrane.

This study shows that cultured cytotrophoblasts have verapamil-sensitive Ca^{2+} channels on the membrane and that the membrane potential is dependent on Ca^{2+} and metabolic energy. There is a possibility that Ca^{2+} entry through the Ca^{2+}

channel which is responsible for the Ca^{2+}-dependent E_m plays an important role in the active Ca^{2+} transport across the placental epithelium [19–22].

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