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Oscillating Activity of a Calcium-Activated K^+ Channel in Normal and Cancerous Mammary Cells in Culture

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Summary. Calcium-activated potassium channels were the channels most frequently observed in primary cultured normal mammary cell and in the established mammary tumor cell, MMT060562. In both cells, single-channel and whole-cell clamp recordings sometimes showed slow oscillations of the Ca^{2+}-gated K^+ current. The characteristics of the Ca^{2+}-activated K^+ channels in normal and cancerous mammary cells were quite similar. The slope conductances changed from 8 to 70 pS depending on the mode of recording and the ionic composition in the patch electrode. The open probability of this channel increased between 0.1 to 1 μM of the intracellular Ca^{2+}, but it was independent of the membrane potential.

Charybdotoxin reduced the activity of the Ca^{2+}-activated K^+ channel and the oscillation of the membrane current, but apamin had no apparent effect. The application of tetraethylammonium (TEA) from outside and $BaCl_2$ from inside of the cell diminished the activity of the channel. The properties of this channel were different from those of both the large conductance (BK or MAXI K) and small conductance (SK) type Ca^{2+}-activated K^+ channels.

Key Words Ca^{2+}-activated K^+ channel · Ca^{2+} oscillation · inward rectification · charybdotoxin · apamin

Introduction

Many hormones, neurotransmitters and growth factors evoke oscillations in the intracellular Ca^{2+} concentration in a variety of cells (*see* Berridge & Galione, 1988; Jacob et al., 1988; Pandiella et al., 1989; Rink & Jacob, 1989). These fluctuations of the intracellular Ca^{2+} concentration produced spontaneous oscillations of the membrane potential in many electrically excitable and unexcitable cells (*see* Kuba, 1980; Adams et al., 1982; Petersen & Maruyama, 1984). These potential changes are presumably a consequence of the fact that increases in intracellular Ca^{2+} stimulate several Ca^{2+}-activated channels, including the large conductance (BK or MAXI K) and small conductance (SK) Ca^{2+}-activated K^+ channels (Blatz & Magleby, 1987). The membrane potential of the mammary gland cells cultured in the presence of epidermal growth factor (EGF) showed spontaneous hyperpolarizing oscillations (Enomoto et al., 1986*a*); EGF is known to stimulate proliferation of the murine mammary epithelial cells (Taketani & Oka, 1983; Oka & Yoshimura, 1986). The involvement of the Ca^{2+}-activated K^+ channel in the hyperpolarizing potential is indicated by pharmacological (Enomoto et al., 1986*b*) and patch-clamp studies (Furuya et al., 1989). The characteristics of this Ca^{2+}-activated K^+ channel differ from those of the SK and BK types (Furuya et al., 1989). To learn more about the characteristics of this unique Ca^{2+}-activated K^+ channel, we investigated single channels and the whole-cell currents of primary cultured normal and established cancerous mammary cells. The characteristics of the Ca^{2+}-activated K^+ channels in the two cell types were also compared.

Materials and Methods

Cell Culture

Normal mammary cells were cultured with a slight modification of the methods described previously (Furuya et al., 1989). Mammary glands of late pregnant or lactate ICR mice were dissected, minced with a surgical knife and then incubated in DME/F12 (Sigma) including 0.1% collagenase (Wako), 1% BSA (Seikagaku Kougyou) and 0.0001% DNase (Sigma) for 30–40 min in 37°C. Organoids including mammary epithelial gland cells were filtered with 150-μM nylon mesh and then centrifuged in a density gradient of 45% Percoll (Pharmacia) at 20,000 x g for 1 hr (Yang et al., 1980). The collected organoids were washed three times and cultured on collagen-coated dishes with DME/F12 supplemented with 1% low protein serum replacement (LPSR, Sigma) + 50 ng/

ml EGF (Collaborative Research) in 37°C with 5% CO_2 for 3–7 days. Collagen gel (0.5–1 ml/35-mm petri dish) was prepared by mixing 9 volumes of collagen solution (Koken) or rat tail extracted collagen solution (Yang et al., 1979) and 1 volume of 10 × DME/F12 and adding several drops of 1 N NaOH to neutralize.

The murine mammary tumor cell line, MMT060562, was obtained from American Type Culture Collection. The cells were cultured in collagen-coated dishes with DME/F12 or DME (4.5 g/liter glucose + 10 mM HEPES) supplemented with 5% FCS or 1% LPSR. To prevent contamination, 100 Units penicillin G (Sigma), 0.025 μg/ml amphotericin B (Sigma) and 100 μg/ml streptomycin (Meiji Seika) were added.

Patch- and Whole-Cell Clamp Experiments

The culture dish was mounted on the stage of an inverted phase-contrast microscope. The temperature of the bath was maintained at 35°C by a temperature control circuit which used a thermosenser and a heater. The whole-cell currents and the single-channel currents in the inside-out, outside-out and cell-attached modes were recorded using the patch-electrode method (Hamill et al., 1981).

Composition of the Ringer solution was (in mM): 152 NaCl; 5.4 KCl; 1.8 $CaCl_2$; 0.8 $MgSO_4$; 10 HEPES/NaOH (pH = 7.4) unless otherwise noted. The high-K^+ (HK) solution contained (in mM): 150 KCl; 10 NaCl; 1.8 $CaCl_2$; 0.8 $MgSO_4$; 10 HEPES/NaOH (pH = 7.4) and 2.4, 2.10, 1.92 or 1.86 EGTA to give free Ca^{2+} concentrations of 0.1, 0.2, 0.5 and 1 μM, respectively. In the whole-cell clamp and cell-attached modes, 2–5% FCS was sometimes added to the Ringer solution, or culture medium supplemented with 2–5% FCS was used. To measure the potassium-dependent shift of the null potential, half of the KCl in HK solution was replaced with Tris chloride. The effects of $BaCl_2$ were monitored in the inside-out mode and $BaCl_2$ was added to the HK solution in the bath. To examine the effects of tetraethylammonium (TEA, Nakarai), charybdotoxin (gift of Dr. Nakayama) and apamin (Sigma) on the Ca^{2+}-activated K^+ channel, the outside-out and whole-cell clamp modes were used, and the substances were added to the bath.

Patch-Clamp Circuits and Data Analysis

A patch-clamp amplifier was built after the circuit described by Auerbach and Sachs (1985). A Warner PC-501 patch/whole-cell clamp amplifier (Hamden, CT) was also used. The currents recorded were positive for outward membrane current and negative for inward. The current signals were passed through 3 kHz Butterworth and 1 kHz Bessel (24 dB/oct) high cut filters. The voltage and the current signals from patch-clamp amplifiers were stored with a PCM video tape recorder (Bezanilla, 1985).

In this report, membrane potential (*Em*) denotes the *trans* membrane potential measured inside the cell. In the inside-out mode, note that $Em = -PV$, where PV is the pipette potential. In the outside-out and whole-cell clamp modes, $Em = PV$. In the cell-attached mode, $Em = -PV + RP$, where RP is a resting membrane potential. Since the RP varied from cell to cell, the current-voltage (*I-V*) relationships varied with RP (Figs. 1*B* and 5*A*). However, when HK-filled pipettes were used, the RP could be estimated from the null potential of each record; because the null "membrane" potential of K^+ current measured with HK pipette was close to 0 mV, the observed null "pipette" potential should be nearly equal to the RP in cell-attached mode. The null potential (NP) of each record was obtained by extrapolation of *I-V* relations in Fig. 5*A* by eye. This is to say, $RP = -NP$, so the *trans* membrane potential is assumed to be $-PV - NP$ (Fig. 5*B*).

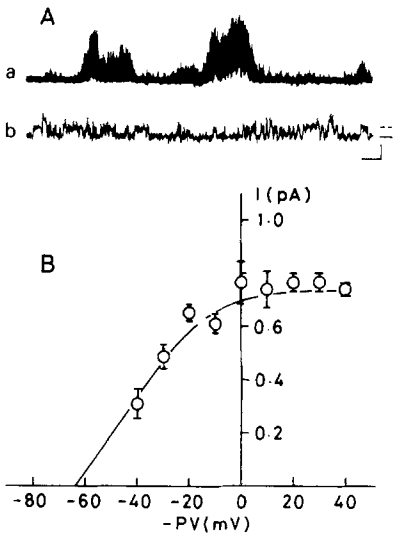

Fig. 1. Spontaneous activity of Ca^{2+}-activated K^+ channel in the mammary tumor cell (MMT060562), recorded with cell-attached mode. Electrode was filled with Ca^{2+}-free Ringer with 1 mM EGTA and bath was perfused with Ringer solution. (*A*) *a*: Trace of the single-channel activity. *b*: Expansion of the time scale of *a*. Pipette potential was 0 mV. Continuous and broken lines on the right side show the closed and open states of the channel. Calibrations are 2 pA, 5 sec for *a*, 1 pA, 20 msec for *b*. (*B*) Current-voltage (*I-V*) relationships. −PV, reverse sign of the pipette potential. Each data point was the average of 4–10 records. Vertical bar shows SE

A personal computer with 12-bit A/D and D/A converters (NEC PC-9801 VX21) was used for control of the pipette potential, and acquisition and analysis of analog data in both the on-line and off-line modes (Vivaudau, Singer & Walsh, 1986; Enomoto, *unpublished*). The sampling frequency was between 2 and 5 kHz.

Results

In normal mammary epithelial cells, several spontaneously active channels were observed in the apical membrane of the mammary epithelial cell, but the Ca^{2+}-activated K^+ channel was recorded most frequently (Furuya et al., 1989). Transient activation of the Ca^{2+}-activated K^+ channel for periods of 5 to 20 sec was sometimes observed (Furuya et al., 1989). The similarity of the time courses of the spontaneous activities of the single-channel currents and of the hyperpolarizing potential (Enomoto et al., 1986*a*,*b*, 1987) in EGF-treated mammary cells led us to conclude that the activation of this Ca^{2+}-activated K^+ channel probably produced the hyperpolarizing potential.

In mammary tumor cells, similar spontaneous oscillations of the channel activity were sometimes observed by cell-attached mode patch clamp (Fig.

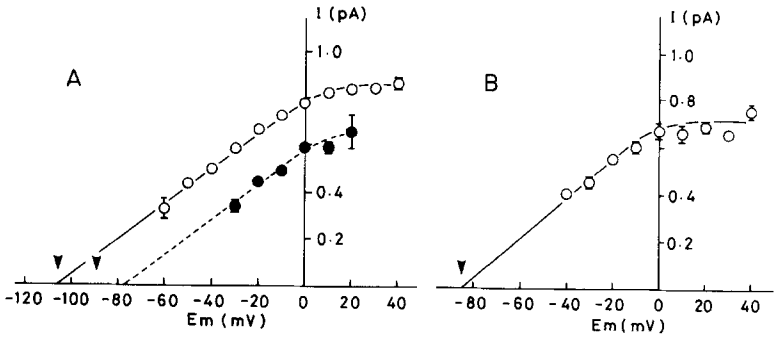

Fig. 2. Current-voltage (*I-V*) relationships of the Ca^{2+}-activated K^+ channel of the normal (*A*) and cancerous (*B*) mammary epithelial cells recorded with inside-out mode. In *A*, electrode was filled with (in mM): 150 NaCl; 2.8 KCl; 1 $MgCl_2$; 6 $CaCl_2$; 10 HEPES/NaOH (pH = 7.4), and in *B*, electrode was filled with Ringer solution (5.4 KCl; *see* Materials and Methods). Bath was perfused with HK solution containing 150 mM K^+ (open circles in *A* and *B*), or 75 mM K^+ (filled circles in *A*). The concentrations of Ca^{2+} were 0.1 to 10 μM. Arrowheads show the reversal potential expected from Nernst equation for K^+. Slope conductances between -10 to -40 mV of membrane potential (E_m) were 8 and 9 pS for *A* and *B*. Averages of 4–10 records. Vertical bar shows SE, and symbols without bar mean that SE was smaller than the size of symbol

1*A*). The electrode was filled with the Ca^{2+}-free Ringer solution, and the outside was perfused with the normal Ringer solution. Depletion of Ca^{2+} in the patch pipette was helpful to make a tight seal and had no apparent effect on the activity and slope conductance of the single channel. In the illustration, the periods of activation lasted about 35 sec and a maximum of eight channels were open. The relationship between the amplitude of the single-channel current and the reverse sign of the pipette potential ($-$PV) in Fig. 1*B* had a slope conductance was 12 pS for the potentials more negative than -20 mV. However, the slope conductance decreased and finally reached zero as the potential became more positive. The null potential of this channel obtained by linear extrapolation of the relationship at more negative membrane potentials was -63 mV. Since an average resting membrane potential of this cell recorded with whole-cell clamp was -48 mV, the real null potential of this channel was estimated to be -111 mV. It was suggested that this was a K^+-selective channel.

The current-voltage (*I-V*) relationship of this K^+ channel was compared with that of the normal mammary cell in the inside-out mode (Fig. 2*A* and *B*). In both cases, when the membrane potentials were negative, the slopes of the *I-V* relationship were linear and were about 8–9 pS. In addition, as the membrane potential became more positive, the slope conductances decreased and finally reached zero. This nonlinearity was reduced when intracellular Na^+ was removed (Furuya et al., 1989). The values of the null potentials obtained by extrapolation were similar to those expected by the Nernst equation for K^+ (arrowheads in Fig. 2*A* and *B*) indicating that these were K^+-selective channels. This evidence was supported by the fact that the null potential shifted when the K^+ concentration in the bath was

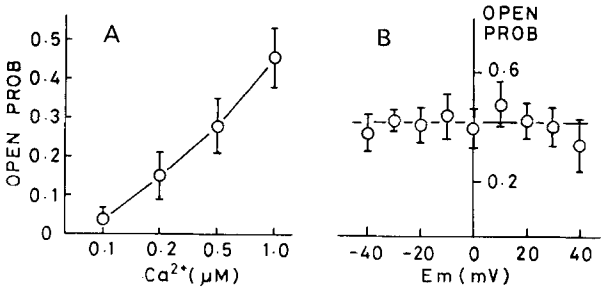

Fig. 3. Changes in the open probability of the Ca^{2+}-activated K^+ channel in the mammary tumor cell with the intracellular Ca^{2+} concentration (*A*) and the membrane potential (*B*). Single channels were recorded in the inside-out mode. Patch electrode was filled with Ringer solution and the bath was perfused with HK solution. In *B*, concentration of free Ca^{2+} in HK solution was 1 μM. Values are shown with average $\pm$ SE ($n \geqq 4$)

decreased (Fig. 2*A*, filled circles) but was unaltered with changes in the Na^+ concentration (Furuya et al., 1989).

In the normal mammary cell, the open probability of this Ca^{2+}-activated K^+ channel increased with intracellular Ca^{2+} between 0.1 to 1 μM, but it did not depend on the membrane potential (Furuya et al., 1989). In the mammary tumor cell, the channel was also stimulated by similar Ca^{2+} levels (Fig. 3*A*), and again no dependence on membrane potential was observed (Fig. 3*B*). The suction of the patch pipette in the inside-out mode did not stimulate the channel activity, and so this channel was not stretch-activated (*data not shown*).

With HK solution in the pipette, this channel in both normal and cancerous mammary cells showed large inward currents in the cell-attached mode, and these currents were sometimes oscillating (Fig. 4*A*). With whole-cell clamp, spontaneous oscillations of the outward membrane currents were also observed

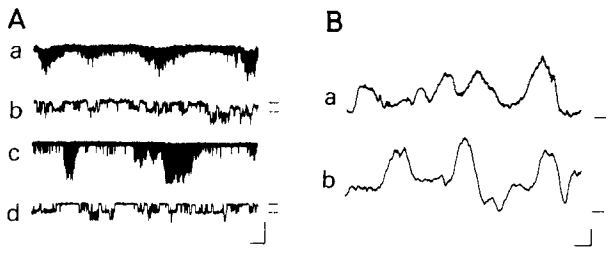

Fig. 4. Spontaneous oscillations of the single-channel (*A*) and whole-cell (*B*) currents in normal and cancerous mammary cells measured with HK solution in the pipette. (*A*) Spontaneous activities of Ca^{2+}-activated K^+ channels in normal (*a*,*b*) and cancerous (*c*,*d*) mammary cells, recorded with cell-attached mode. Electrode was filled with HK (0.1 μM Ca^{2+}) solution, and bath was perfused with Ringer solution. Pipette potential was 0 mV. *b* and *d*: Expansion of the time scale of traces *a* and *c*. Continuous and broken lines on the right side show the closed and open states of the channel. Calibrations are 10 pA for *a–d*, 5 sec for *a* and *c*, 20 msec for *b* and *d*. (*B*) Spontaneous outward currents in the normal (*a*) and the cancerous (*b*) mammary cells recorded with whole-cell clamp. *a*: Normal mammary cell was cultivated for five days in the presence of EGF. Bath was perfused with Ringer solution. *b*: Mammary tumor cells. Bath was perfused with DME/F12 + 2% FCS. Electrode was filled with HK (0.1 μM free Ca^{2+}) solution. Holding potentials were −24 and −42 mV for *a* and *b*. Lines on right side show possible baseline. Calibrations were 50 pA and 5 sec

in normal and cancerous mammary cells (Fig. 4*B*). There was no clear difference in the active pattern of the single- and whole-cell currents between normal and cancerous mammary cells. In the EGF-treated normal and the cancerous mammary cells, the probability of observing these spontaneous oscillations was 10–50% of the recorded cells and it varied from preparation to preparation.

The *I-V* relationships of the single channels in cancerous mammary cells measured with the HK solution filled pipette in the cell-attached mode were nonlinear (Fig. 5*A*). Each curve in Fig. 5*A* was varied by the difference of the resting potential of each cell. Using the null potential of each curve (*see* Materials and Methods), the normalized *I-V* relationship of each channel was obtained (Fig. 5*B*). The *I-V* relation was nonlinear, as is the case for the normal mammary cell (Furuya et al., 1989). In both cases, the slope conductances for the inward K^+ current were 30–40 pS at the assumed *trans* membrane potential (−PV − NP) between −60 to −20 mV, and increased to 70 pS at more negative than −80 mV (Fig. 5*B*). This value was 3–9 times larger than the slope conductance of the same channel for outward K^+ currents (Figs. 1 and 2). Generally there was no clear reversal in this mode, even when the membrane potential shifted 30–40 mV more positive than the expected null potential.

To learn more about the characteristics of the Ca^{2+}-activated K^+ channels of mammary cells, the effects of TEA and charybdotoxin, which block the BK-type Ca^{2+}-activated K^+ channel (Adams et al., 1982; Miller et al., 1985), and apamin, which blocks the SK-type channel (Romey et al., 1984; Blatz & Magleby, 1986), were tested with the outside-out mode on the normal (Fig. 6*A–C*) and cancerous (Fig. 6*D–F*) mammary cells. TEA (>5 mM; Fig. 6*A* and *D*) and charybdotoxin (>0.1 μM; Fig. 6*B* and *E*) decreased the channel activities, but 20–50 μM apamin had no apparent effect (Fig. 6*C* and *F*). Whole-cell clamp of the cancerous mammary cells also showed that charybdotoxin (>0.4 μM) blocked the oscillating outward current (Fig. 7*A*) but 25 μM apamin was without effect (Fig. 7*B*). The application of $BaCl_2$ (100 μM) to the inside of the membrane decreased the open probability of the Ca^{2+}-activated K^+ channel of both the normal mammary (Furuya et al., 1989) and the mammary tumor cells (*data not shown*).

Discussion

Our results show that the cancerous mammary cells (MMT060562) have a Ca^{2+}-activated K^+ channel which is very similar to that in normal mammary cells. It is interesting that the cancerous cells exhibited spontaneous outward currents or intermittent activation of the Ca^{2+}-activated K^+ channels with periods of about 20–30 seconds, which is very similar to the observations in the EGF-treated normal mammary cells in primary culture. Recently, we observed oscillations of the intracellular Ca^{2+} concentrations in normal and cancerous mammary cells. In addition, there is the linear relationship between the concentration of intracellular Ca^{2+} and the activity of Ca^{2+}-activated K^+ channel (Furuya & Enomoto, 1990). These facts suggest that the oscillations of intracellular Ca^{2+} and the presence of Ca^{2+}-activated K^+ channels lead to the slowly oscillating membrane potentials in normal and cancerous mammary cells. It is possible that these oscillations may play a role in the cell proliferation, because both the cancerous cells and the EGF-treated normal cells proliferated rapidly.

The Ca^{2+}-activated K^+ channels have been found in various cells and must have been classified into two types (BK and SK) by conductance, voltage dependency and sensitivity to some channel blockers (Blatz & Magleby, 1987; Castle, Haylett & Jenkinson, 1989). The single-channel conductances of BK and SK are 100–250 and 6–14 pS. The SK has no voltage dependence and is blocked by apamin. On the other hand, the BK is usually voltage dependent and is blocked by TEA and charybdotoxin.

Other Ca^{2+}-activated K^+ channels have been

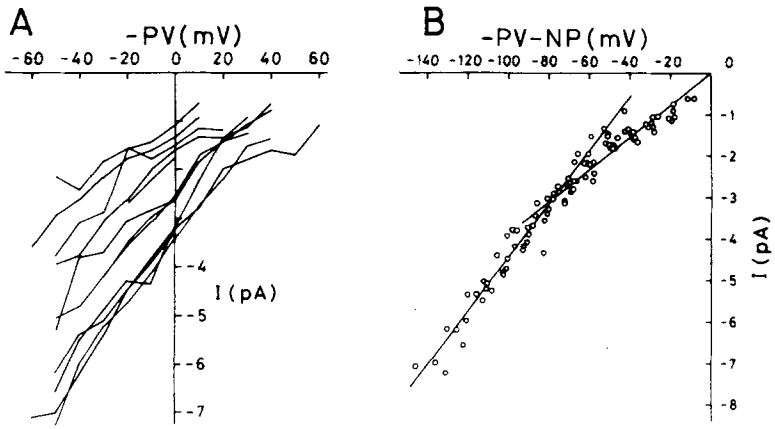

Fig. 5. *I-V* relationships of the single Ca^{2+}-activated K^+ channel of cancerous mammary cells recorded with cell-attached mode. Electrode was filled with HK (0.1 μM free Ca^{2+}) solution, and bath was perfused with Ringer solution. (*A*) The amplitude of single-channel current in each record *versus* −PV, where PV is a pipette potential. Null potentials (NP) of each record were obtained by linear extrapolation between 0 to −3 pA of each curve by eye. (*B*) The corrected *I-V* relations of single-channel currents were plotted as a function of the assumed *trans* membrane potentials (−PV − NP) (*see* Materials and Methods)

described in rat skeletal muscle (4 pS; Blatz & Magleby, 1986), red blood cells (40 pS; Hamill, 1983), HeLa Cells (10–50 pS; Sauve et al., 1986) snail neurons (20–35 pS; Lux, Neher & Marty, 1981; Hermann & Erxleben, 1987). These have been denoted other K (OK) (Blatz & Magleby, 1987) or intermediate K (IK) channels (Castle et al., 1989) but this group probably includes several different types. Snail neurons have a voltage-dependent IK-type Ca^{2+}-activated K^+ channel (Lux et al., 1981; Hermann & Erxleben, 1987) but the channel in HeLa cells is voltage-independent (Sauve et al., 1986).

In mammary cells, the single-channel conductances of Ca^{2+}-activated K^+ channels for outward current recorded with cell-attached and inside-out mode were between 8–12 pS (Figs. 1*B* and 2*A* and *B*; Furuya et al., 1989) and this value was close to that of the SK type. The lack of voltage dependence of the open probability (Fig. 3*B*) is also a property of the SK type (Lang & Ritchie, 1987). However, the absence of effect of apamin (Figs. 6*C* and *F* and 7*B*) precludes the Ca^{2+}-activated K^+ channel in the mammary gland being the SK type.

Charybdotoxin is a potent blocker of BK-type Ca^{2+}-activated K^+ channels (3 nM; Miller et al., 1985). In addition, charybdotoxin blocks IK, Ca^{2+}-insensitive and volume-activated K^+ channels but at higher concentrations; SK channels are not affected (Castle et al., 1989). In normal and cancerous mammary cells, charybdotoxin blocked the activity of Ca^{2+}-activated K^+ channel (Fig. 6*B* and *E*) and the oscillating outward current (Fig. 7*A*) at submicromolar concentrations. It seems that the Ca^{2+}-activated K^+ channel in mammary gland cells was somewhat less sensitive to charybdotoxin, but a more pure toxin preparation will be needed to conclude this definitely.

TEA blocks the voltage-gated, receptor-controlled K^+ and BK-type Ca^{2+}-activated K^+ channels (Castle et al., 1989). In mammary gland cells TEA mimics the spontaneous hyperpolarizing potential monitored with intracellular recording (Enomoto et al., 1986*b*), and blocked the single Ca^{2+}-activated K^+ channels from the inside (Furuya et al., 1989) and the outside (Fig. 6*A* and *D*), but a concentration of 5 mM or more was needed. This is somewhat higher than the concentration effective on the BK-type channel from the outside (0.1–1 mM; Blatz & Magleby, 1987).

The *I-V* relations of the single channels in both normal and cancerous mammary cells showed nonlinearity: the conductances were large for inward currents (Fig. 5*B*) and small for outward currents (Figs. 1*B* and 2); the conductances were large at more hyperpolarizing membrane potentials and small at more depolarizing membrane potentials (Fig. 5*B*). From these results, it was concluded that the Ca^{2+}-activated K^+ channels in normal and MMT060562 cancerous mammary cells differed from the BK and SK types, and they were classified as the IK type by the criteria of Castle et al. (1989).

The properties of the Ca^{2+}-activated K^+ channels of the mammary cell are very close to those of HeLa cells. Both show a nonlinear *I-V* relationship, inward rectification and the voltage independence of the open probability (Sauve et al., 1986). In addition, mechanical stimuli induce a hyperpolarizing potential in both HeLa cell (Roy & Sauve, 1983), normal mammary gland cell (Enomoto et al., 1987) and mammary tumor cell (K. Enomoto & K. Furuya, *unpublished*). It is plausible that other epithelial cells and electrically nonexcitable cells including cancer cells also have similar type Ca^{2+}-activated K^+ channels.

In whole-cell clamp, the probability of observing the oscillation of the outward membrane current in EGF-treated mammary cell was somewhat smaller than the value observed previously with intracellular recording (Enomoto et al., 1986*a*). This may be due partly to the buffering of the intracellular Ca^{2+} con-

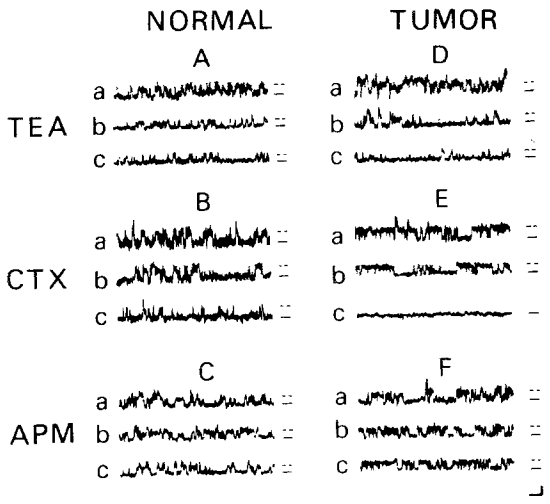

Fig. 6. Effects of tetraethylammonium (*TEA*) (*A* and *D*), charybdotoxin (*CTX*) (*B* and *E*) and apamin (*APM*) (*C* and *F*) on the Ca^{2+}-activated K^+ channels in the normal (*A–C*) and cancerous (*D–F*) mammary cells. Single-channel currents were recorded with the outside-out mode. Patch electrode was filled with HK (0.1 μM free Ca^{2+}) solution, and bath was perfused with Ringer solution. Drugs were added in the bath. Traces were recorded 1–5 min after addition of drugs. (*A*) Effects of TEA on the normal mammary cell. *a*, Before; *b*, 5 mM; *c*, 10 mM TEA. Membrane potential (E_m) was 20 mV. (*B*) Effects of charybdotoxin on the normal mammary cell. *a*, Before; *b*, 0.12 μM; *c*, 0.24 μM charybdotoxin. E_m = 0 mV. (*C*) Effects of apamin on the normal mammary cell. *a*, Before; *b*, 20 μM; *c*, 40 μM apamin. E_m = 20 mV. (*D*) Effects of TEA on the mammary tumor cell. *a*, Before; *b*, 5 mM; *c*, 10 mM TEA. E_m = 0 mV. (*E*) Effects of charybdotoxin on the mammary tumor cell. *a*, Before; *b*, 0.18 μM; *c*, 0.36 μM charybdotoxin. E_m = 0 mV. (*F*) Effects of apamin on the mammary tumor cell. *a*, Before; *b*, 25 μM; *c*, 50 μM apamin. E_m = 20 mV. Number of trials for each drug was 7 to 10, and similar results were obtained from >80% of preparations. Continuous and broken lines on the right side show the closed and open states of the channel. Calibrations are 1 pA and 20 msec

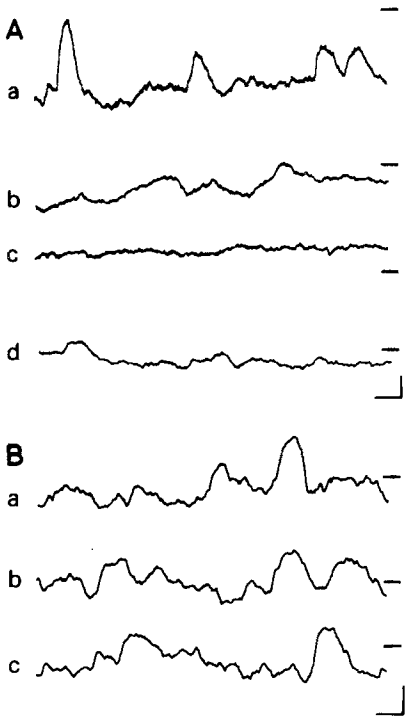

Fig. 7. Effects of charybdotoxin (*A*) and apamin (*B*) on the spontaneous outward current of the mammary tumor cell recorded with whole-cell clamp. Electrode was filled with HK (0.1 μM free Ca^{2+} was 0.1 μM Ca) solution. (*A*) *a* Before; *b*, 2 min after addition of 0.4 μM; *c*, 3 min after addition of 0.9 μM charybdotoxin; *d*, 3 min after washout. Five percent FCS was present in the Ringer solution. Holding potential was −49 mV. Same level of the trace is shown by the line on the right side. Similar results were obtained from three other preparations. Calibrations were 50 pA and 5 sec. (B) *a*, Before; *b*, 3 min after addition of 25 μM apamin; *c*, 2 min after washout. Bath was perfused with Ringer solution

centration by the HK solution in the patch electrode, and partly to the absence of the extracellular FCS. In fact, oscillations of the intracellular Ca^{2+} are inhibited by intracellular dialysis with 5.5 mM EGTA (Evans & Marty, 1986). Also, unknown factors possibly present in FCS and LPSR are important for oscillation, since the oscillations sometimes stopped after depletion of FCS and LPSR from the Ringer (Enomoto, *unpublished*). The factors affecting the oscillation and the difference of the single-channel activity with hormones and EGF are now under investigation.

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