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Characterization of a Functionally Expressed Stretch-activated BKca Channel Cloned from Chick Ventricular Myocytes

Q.Y. Tang[1,2], Z. Qi[2], K. Naruse[1,2], M. Sokabe[1,2]
[1]Department of Physiology, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan
[2]ICORP Cell Mechanosensing, JST. Nagoya 466-8550, Japan

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Abstract. We have characterized electrophysiological and pharmacological properties of a stretch-activated BKca channel (SAKcaC) that was cloned from cultured chick ventricular myocytes (CCVM) and expressed in chinese hamster ovary cells (CHO) using the patch-clamp technique. Our results indicate that the cloned SAKcaC keeps most of the key properties of the native SAKcaC in CCVM, such as conductance, ion selectivity, pressure-, voltage- and Ca^{2+}-dependencies. However, there was a slight difference between these channels in the effects of channel blockers, charybdotoxin (CTX) and gadolinium (Gd^{3+}). The native SAKcaC was blocked in an all-or-none fashion characterized as the slow blockade, whereas the conductance of the cloned SAKcaC was gradually decreased with the blockers' concentration, without noticeable blocking noise. As the involvement of some auxiliary components was suspected in this difference, we cloned a BK β-subunit from CCVM and coexpressed it with the cloned SAKcaC in CHO cells to examine its effects on the SAKcaC. Although the pharmacological properties of the cloned SAKcaC turned out to be very similar to the native one by the coexpression, it also significantly altered the key characteristics of SAKcaC, such as voltage- and Ca^{2+}-dependencies. Therefore we concluded that the native SAKca in CCVM does not interact with the corresponding endogenous β-subunit. The difference in pharmacological properties between the expressed SAKcaC in CHO and the native one in CCVM suggests that the native SAKca in CCVM is modulated by unknown auxiliary components.

Correspondence to: M. Sokabe; email: msokabe@med.nagoya-u.ac.jp

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Introduction

Voltage-dependent large-conductance Ca^{2+}-activated K^{+} channels (BKcaCs) are a unique class of ion channels that couple intracellular chemical signaling to electric signals (McManus, 1991; Robitaille & Charlton, 1992; Latorre, 1994; Vergara et al., 1998; Brenner et al., 2000). BK channels are widely expressed in both excitable and non-excitable cells and known to be involved in many physiological processes, such as vascular tone regulation (Brayden, 1996; Rusch, Lin & Pleyte, 1996; Brenner et al., 2000; Pluger et al., 2000), smooth muscle tone regulation (Nelson & Quayle, 1995; Brenner et al., 2000), neuronal firing (Robitaille & Charlton, 1992; Robitaille et al., 1993; Golding et al., 1999; Poolos & Johnston, 1999), and endocrine cell secretion (Marty, 1989; Lingle et al., 1996). Furthermore, it has been shown that BK channels exhibit differential properties in different cell types (Reinhart et al., 1991; Chung et al., 1991). The reasons for the diversities of BK channels may, in part, be due to the molecular diversities of their pore-forming α-subunits, which contain all of the gating and ion permeation functions (Atkinson, Robertson & Ganetzky, 1991; Adelman et al., 1992; Butler et al., 1993). In contrast to the multiple gene families of other voltage-dependent channels, BK channels are encoded by a single *Slo1* gene (Atkinson et al., 1991; Adelman et al., 1992; Butler et al., 1993) and form a functional channel through the tetrameric assembly of four α-subunits, which contain three functionally important domains: ion-permeation pore, voltage sensor (Atkinson et al., 1991; Butler et al., 1993; Jan & Jan, 1997), and "calcium-bowl"

(Schreiber & Salkoff, 1997). Several lines of evidence have shown that the diverse properties of BK channels depend on different isoform types derived from this gene (Adelman et al., 1992; Lagrutta et al., 1994; Tseng-Crank et al., 1994; McCobb et al., 1995; Clark, Hall & Shipston, 1999). In addition, auxiliary β-subunits may interact with the α-subunit, probably in 1:1 stoichiometry (Knaus et al., 1994), and modify the biophysical and pharmacological properties of the α-subunit. Recently, several β-subunit family members have been identified (Wallner, Meera & Toro, 1999; Xia et al., 1999, 2000; Brenner et al., 2000; Meera, Wallner & Toro, 2000; Uebele et al., 2000; Weiger et al., 2000), and it is increasingly clear that this family of auxiliary subunits plays a major role in defining the tissue-specific phenotypic properties of BK channels.

It has been reported that five distinct types of SA channels are expressed in cultured chick ventricular myocytes (CCVM) (Ruknudin, Sachs & Bustamante, 1993; Kawakubo et al., 1999). Kawakubo et al. found that the predominant channel among them was the channel with the largest conductance and characterized it as a stretch-activated BKca channel (referred as SAKcaC here). Although the role of SAKcaC in heart has not been clarified yet, this new member of K channels in heart may have a distinct role in shaping heart action potentials (AP) due to its unique gating properties, including Ca^{2+}-, voltage- and stretch-dependencies. Apart from its physiological functions, the structure-function relationship of this channel is particularly interesting as a new member of SA channels. Therefore, we cloned a gene encoding SAKcaC from CCVM (Naruse, Tang, Sokabe, submitted). The primary aim of this study is to test if the cloned gene is really responsible for the SAKcaC functioning in CCVM through the comparison of electrophysiological and pharmacological properties between the cloned and functionally expressed SAKcaC in CHO cells and the native SAKcaC in CCVM.

Materials and Methods

Gene Cloning

Chick SAKcaC gene was cloned from the cDNA library made from chick embryonic hearts (GenBank accession number AB072618). This was subcloned into mammalian expression vectors (pTarget®, Promega, Madison, WI) for the transfection studies described below. A chick BK β-subunit was PCR-cloned from a 10-day chick-heart cDNA library using primer sets based on the sequence previously reported in chick cochlea (AF420468 Balt, S.L & Hudspeth, A.J., direct submission) and was subcloned in pENTR (Invitrogen, Carlsband, CA). The pENTR-BK-β was recombinated into pDEST42 vector according to the manufacturer's instruction.

Cell Culture

Chick ventricular myocytes were cultured as described previously (Kawakubo et al., 1999). Briefly, ventricles were dissected from 10–12 day old White Leghorn embryos under sterile conditions. Cell suspensions were prepared by exposing the ventricles to normal saline for 10 min at 37°C and were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL) supplemented with heat-inactivated horse serum (10% vol/vol) and chick embryo extract (2% vol/vol). Cells from 4 to 10 days of culture were used for experiments. Chinese hamster ovary (CHO-K_1) cells were routinely grown in Ham's F-12 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum and incubated at 37°C in a 5% CO_2 humidified environment. Cells were passaged every 2–3 days using 0.02% trypsin in Hanks' buffered salt solution (HBSS) containing 0.1% EDTA.

Heterologous Expression

CHO cells were transiently transfected with the SAKcaC cDNA in a mammalian expression vector (pTarget®, Promega) using the Lipofectamine Plus(TM) reagent according to the manufacturer's protocol (GIBCO). Briefly, the day before transfection, CHO cells were seeded onto glass coverslips in a 35 mm culture dish at a density to allow cells to reach 50–70% confluence after 24 hrs. To monitor successfully transfected cells, pEGFP (Clontech Laboratories, Palo Alto, CA) was coexpressed with the SAKcaC cDNA at the ratio of 5:1 (weight/weight). In the co-transfection experiments with the β-subunit, an excess amount of β-subunit over the SAKcaC α-subunit (10:1 mole/mole) was used to maximize their interaction. The expected stoichiometry between them was 1:1 (Wang et al., 2002). The transfected cells grown on the cover slip for 1–2 days were used for patch-clamp experiments without any further treatment. We could not detect any endogenous BK channel activity in untransfected CHO cells.

Single-Channel Recordings and Analysis

All experiments were performed in the excised inside-out configuration of the patch-clamp technique. Unfortunately, outside-out patches were too unstable to get reliable data and thus were not employed here. Patch electrodes were fabricated from disposable micro-pipettes (Drummond Scientific, PA.) in two stages on a vertical electrode puller (PP-83, Narishige Scientific Instrument Lab, Tokyo) to a tip diameter of ~0.5–1.0 μm. The patch pipettes were fire-polished on a microforge (MG-83, Narishige Scientific Instrument Lab, Tokyo) to give an average resistance of 5–15 MΩ in recording solution. Negative pressure was applied in the patch pipette using a pneumatic transducer tester (DPM-IB, BIO-TEK Instruments INC.VT.). All the recordings were made at room temperature (22–25°C). Currents were amplified using an EPC9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany), sampled at 2–5 kHz and filtered at 1.5–2.9 kHz via a 4-pole low-pass Bessel filter. The program package PULSE + PULSEFIT and TAC 4.0 (HEKA Elektronik) were used for data acquisition and analysis. Single-channel currents were measured as a difference between the current levels at the closed and open states which were determined from current amplitude histograms. In most cases the probability of the channel being open (P_o) was simply calculated from the total time spent in the open state divided by the total time of the recording for the patches containing a single channel. Continuous recordings of 2,000–6,000 ms were used to estimate P_o values. When multiple channels were present in the patch, the probability of the channel being open (P_o) was calculated from the amplitude histogram as $P_o\ (\%) = (1 - P_C^{1/N}) \times 100$ (Sokabe, Sachs & Jing, 1991), where P_C is the fraction of area under the closed state, and N is the number of channels, which was estimated from the maximum observed current level at relatively higher voltage and/or bath Ca^{2+} concentration. Data are presented as the mean ± standard error (SE), unless otherwise noted.

Solutions for Electrophysiological Measurements

All the recordings were made with excised inside-out patches. The pipette solution facing the extracellular surface of the patch contained (in mM): 145 K-gluconate, 1 EGTA, 10 HEPES, 5 glucose, pH 7.4. One mM EGTA was used to chelate free Ca^{2+} to eliminate the possibility of SAKcaCs activation by the increased Ca^{2+} at the cytoplasmic surface via putative Ca^{2+}-permeable stretch-activated channels and/or stretch-induced Ca^{2+} leakage. The bath solution (facing the cytoplasmic surface of the patch) contained the same solution except for various Ca^{2+} concentrations (referred to as $[Ca^{2+}]_i$ hereafter). When Ca^{2+} concentrations lower than 1 μM were needed, proper concentrations of $CaCl_2$ with 1 mM EGTA were used on the basis of calculations using the program EQCAL (Biosoft, MO) with the stability constants from references (Chaberek & Martell, 1959; Owen, J.D. 1976). The osmolality of the solutions was routinely measured with an osmometer (μ-Osmette, Precision Systems, Natick, MA), and adjusted with sucrose to 310 ± 5 mosmol kg^{-1}. All salts and reagents were purchased from Sigma (Sigma, St. Louis MO).

Results

Conductance and Ion Selectivities

Representative current traces of single channels from the native SAKcaC (*left*) and the cloned SAKcaC (*right*) in symmetric 145 mM K^+ solutions at different holding membrane potentials and corresponding *I-V* curves are shown in Fig. 1*A* and *B*, respectively. The slope conductances (−80 to +80 mV) were 273.74 ± 2.61 pS ($n = 16$) for the native SAKcaC and 271.82 ± 3.1 pS ($n = 22$) for the cloned SAKcaC, showing no significant difference between them ($P = 0.01$). The ion selectivity of the cloned SAKcaC was determined from current-to-voltage (*I-V*) curves under various ionic conditions (Fig. 1*C*). All the *I-V* curves were well fitted with the Goldman-Hodgkin-Katz current equation and the permeability ratios of various ions with respect to K^+ were calculated using the following equations with estimated reversal potentials as

$$P_{Na}/P_K = ([\alpha_K]_o/[\alpha_{Na}]_i)\ \exp(-V_{rev}F/RT) \quad (1)$$

$$P_{Cl}/P_K = \frac{[\alpha_K]_i - [\alpha_K]_o\ \exp(-V_{rev}F/RT)}{[\alpha_{Cl}]_i\ \exp(-V_{rev}F/RT) - [\alpha_{Cl}]_o} \quad (2)$$

$$P_{Ca}/P_K = ([\alpha_K]_i/4[\alpha_{Ca}]_o)\ \exp(2V_{rev}F/RT) \quad (3)$$

where V_{rev} is the reversal potential, $[\alpha_x]_i$ and $[\alpha_y]_o$ are the activity of ion x in the bath and ion y in the pipette, respectively. The activity of ion A was defined as $\alpha_A = \gamma_A$ [A], where [A] is the molar concentration of ion A, γ_A is the activity coefficient of ion A, which could be calculated with a program on the web (http://www.chem.vt.edu/chem-ed/simulations/activity-coefficients.xls). F is Faraday's constant, R, gas constant and T, absolute temperature (°K). The obtained permeability ratios were as follows: $P_{Na}/P_K = 0.028$ ($V_{rev} = +87.5$ mV, $n = 6$; Eq. 1); $P_{Cl}/P_K = 0.014$ ($V_{rev} = +15.4$ mV, $n = 6$; Eq. 2) and $P_{Ca}/P_K = 0.017$ ($V_{rev} = -51.8$ mV, $n = 5$; Eq. 3). These values are not significantly different from those for the native SAKcaC in cultured CCVM ($P_{Na}/P_K = 0.030$; $P_{Cl}/P_K = 0.026$ and $P_{Ca}/P_K = 0.025$. Kawakubo et al., 1999).

Pressure Dependency

Figure 2*A* shows typical current traces of the native (*left*) and cloned (*right*) SAKcaCs at different negative pressures with 0.25 μM $[Ca^{2+}]_i$. Negative pressure in the pipette was applied in a stepwise fashion with 10 mmHg increments. The channel open probability (P_o) plotted against negative pressure is shown in Fig. 2*B*. The P_o of both the native (■, $n = 7$) and cloned (○, $n = 6$) SAKcaCs increased in a dose-dependent manner, which could be fitted with the Boltzmann equation of the form, $P_o = 1/[1 + \exp(P_{1/2} - P)\ K]$, where P is the pressure in the pipette, $P_{1/2}$ is the pressure required to induce half-maximal channel activation, and K is the inverse of curve steepness. The best fit to the data was obtained with values of e-fold increase in P_o: $K^{-1} = 15.48 \pm 0.30$ mmHg for the native and $K^{-1} = 13.22 \pm 0.21$ mmHg for the cloned SAKcaC, showing no significant difference between the native and cloned SAKcaCs ($P = 0.05$). The response of the channel activity frequently lagged behind the pressure change by a few seconds, which might come from the slowness of our pressure application system and/or the slow area change of the patch to pressure change, as observed in our earlier work with a high-speed pressure-clamp apparatus (Sokabe, et al. 1991). This may raise the possibility that the observed channel activation by negative pressure just arises from the increased number of channels in the stretched patch area. However, this may not be the case for the following two reasons. First, the open and closed times are actually changed in response to pressure, as clearly shown in Fig. 2*A*. Second, at a high activation level of the channel (e.g., with 1 mM $[Ca^{2+}]_i$, at −50 mV, Fig. 2*C*), where we can reliably estimate the total number of the channels in the patch, negative pressure never changed the maximum current level, say, the total number of the channels in the patch.

Voltage- and Ca^{2+}-Dependencies

Voltage Dependency

Figure 3 shows the open probability (P_o) as a function of membrane potential at various $[Ca^{2+}]_i$ for the native (*A*; $n = 15$) and cloned (*B*; $n = 18$) SAKcaCs, where increasing $[Ca^{2+}]_i$ from 0.1 to 0.8 μM causes a

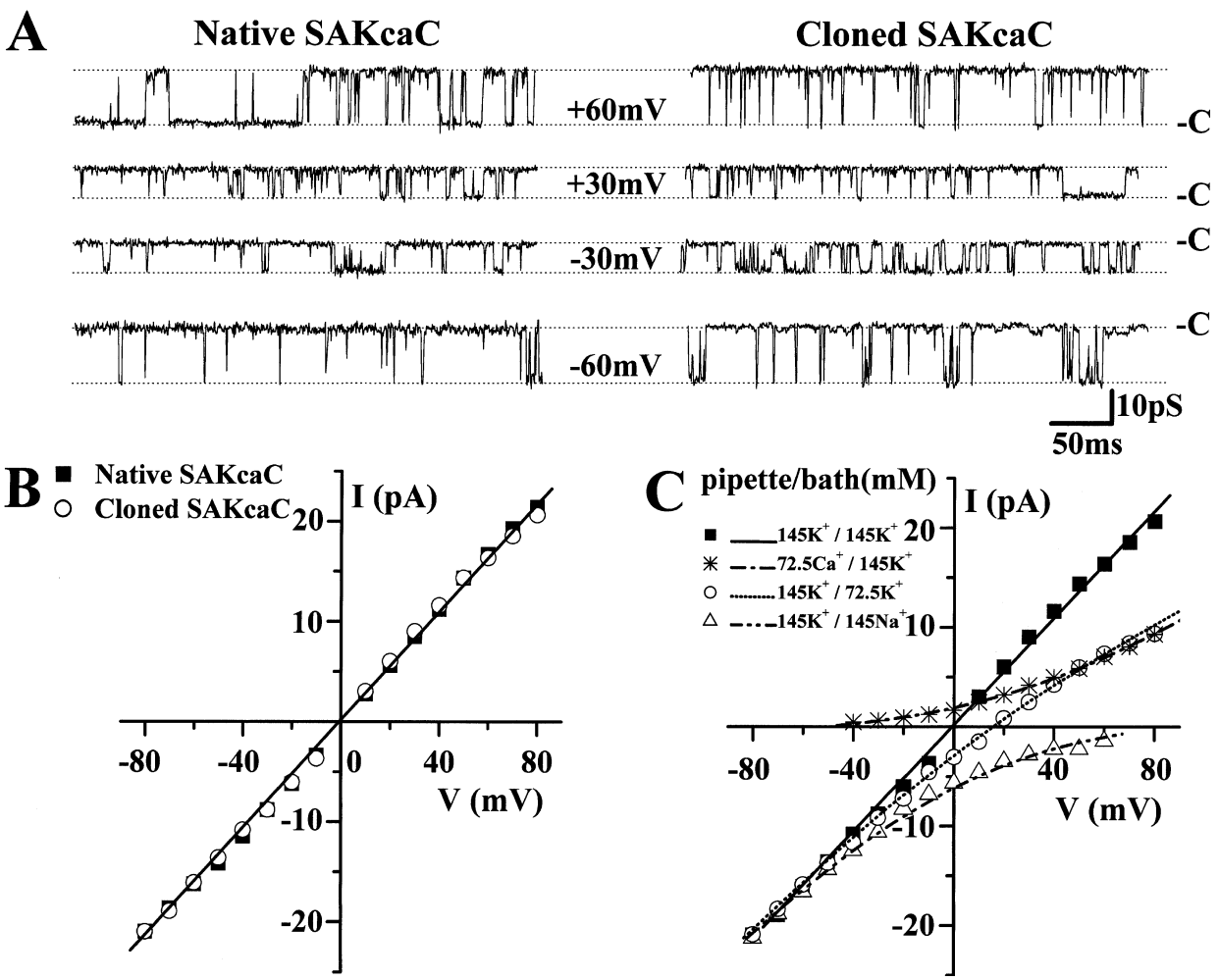

Fig. 1. Single-channel conductance and ion selectivity. (*A*) Single-channel current traces obtained from the native (*left*) and cloned (*right*) SAKcaCs in symmetric 145 mM K^+ solution with 1 mM $[Ca^{2+}]_i$ at −60, −30, +30 and +60 mV. Channel closed levels are indicated as "*C*." (*B*) Current-voltage relationships for the native (■) and cloned (◯) SAKcaCs. Single-channel conductances were 273.74 ± 2.61 ps (n = 16) and 271.82 ± 3.1 pS (n = 22) for the native and cloned SAKcaCs, respectively. (*C*) *I-V* relationships of the cloned SAKcaC with symmetrical 145 mM K^+ (■), 145 mM K^+ in the pipette and 145 mM Na^+ in the bath (□), 145 mM K^+ in the pipette and 72.5 mM K^+ in the bath (◯), and 72.5 mM Ca^{2+} in the pipette and 145 mM K^+ in the bath (□). Data points in individual conditions represent averages from 22, 6, 6, and 5 patches, respectively. Curves are drawn according to the Goldman-Hodgkin-Katz current equation.

systematic leftward shift of the P_o-V curves. Voltage-dependent behavior of the channel open probability (P_o) was modeled with the Boltzmann function of the form,

$$P_o = 1/\{1 + \exp\,[-ZF/RT\,(V - V_{1/2})]\} \qquad (4)$$

where $V_{1/2}$ is the membrane potential for the half-maximal channel activation. When the P_o-V relationship is well fitted with this Boltzmann equation, a plot of ln (P_o) as a function of voltage becomes linear at low P_o values (Dopico, Anathaman & Treistman, 1998). The reciprocal of the slope of this plot gives the potential needed to produce an e-fold change in P_o, which is routinely used as a measure of the voltage dependency of channel gating (Reinhart, Chung & Levitan, 1989; Toro, Ramos-Franco & Stefani, 1990; Dopico, Lemos & Treistman, 1996; Dopico et al., 1998). Calculated membrane potentials required for the e-fold change of P_o for the native SAKcaC was 21.48 ± 2.71 mV and 22.07 ± 2.42 mV for the cloned SAKcaC. The gating charge (Z) calculated using the relation, 1/slope = RT/ZF (Toro et al., 1990), was 1.21 ± 0.153 for the native SAKcaC and 1.176 ± 0.13 for the cloned one. Thus, no significant difference was observed in the voltage dependency between the native and cloned SAKcaCs (P = 0.01).

Calcium Dependency

A key feature of BK channels is that the channel activity is regulated by $[Ca^{2+}]_i$ and membrane potential (McManus, 1991). Thus, the goal here is to

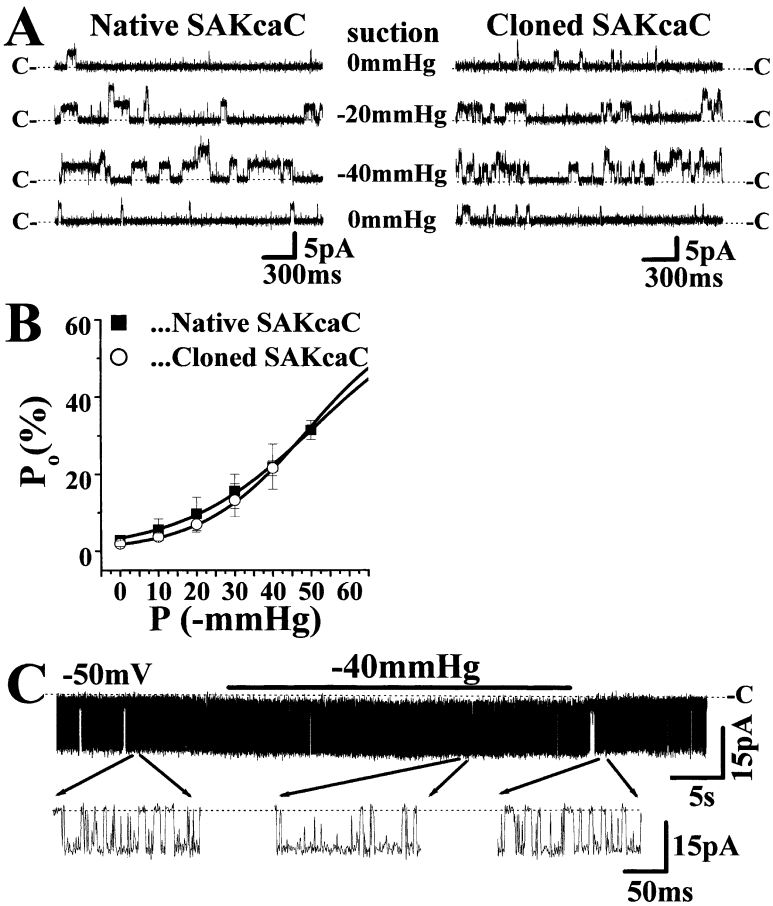

Fig. 2. Pressure dependency of the native and cloned SAKcaCs. (*A*) Single-channel activities of the native (*left*), and cloned (*right*) SAKcaCs at different pressures, as indicated. Pipette contained 145 mM K^+, 0 μM Ca^{2+} (1 mM EGTA), bath contained 145 mM K^+ and 0.25 μM Ca^{2+}. Membrane potential was held at +10 mV. (*B*) Pressure dependency of P_o for the native (■, $n = 7$) and cloned (○, $n = 6$) SA KcaCs. Lines were drawn according to the Boltzmann equation: $P_o = 1/[1 + \exp(P_{1/2} - P)\ K]$, where P is the pressure applied in the pipette, $P_{1/2}$ is the pressure required to induce half-maximal activation. (*C*) A continuous current trace of the cloned SAKcaCs in a maximally activated state with 1 mM $[Ca^{2+}]_i$ at −50 mV to demonstrate that application of even a relatively high pressure (−40 mmHg) does not change the number of the channels in the patch. Bar indicates the period of pressure application.

examine the calcium- and voltage-dependent gating properties using a simple two-state model in an effort to extract the parameters that can be used to compare the two channel types. The model assumes that voltage-dependent binding of calcium as a first order process, leading to the channel gating scheme,

$$\mathrm{C} \underset{K^{-1}}{\overset{K^1[\mathrm{Ca}^{2+}]_i \exp(2\delta FV/RT)}{\rightleftharpoons}} \mathrm{O} \qquad \text{(A)}$$

where K^1 and K^{-1} are the forward and reverse rates for the binding of calcium at 0 mV, and δ is the electrical distance traveled by calcium in the membrane electric field. Using scheme *A*, the activation of the channel can be defined as a function of calcium concentration as

$$P_o = 1/(1 + K_d(0)\ \exp(-2\delta FV/RT)/[\mathrm{Ca}^{2+}]_i) \qquad (5)$$

The dissociation constant for calcium at zero voltage, K_d (0), is the ratio of K^{-1} (0) to K^1 (0). Equation (5) may be rearranged to represent the half-activation voltage ($V_{1/2}$) as a logarithmic function of calcium concentration (Cui, Cox & Aldrich, 1997) as

$$V_{1/2} = -(2.302RT/2\delta F)\log[\mathrm{Ca}^{2+}]_i + (2.303RT/2\delta F)\log K_d(0) \qquad (6)$$

Figure 3*C* shows the $V_{1/2}$ plotted as a function of calcium concentration for the native and cloned SAKcaCs. It is apparent that the shift of $V_{1/2}$ is not parallel over the range of the physiological $[Ca^{2+}]_i$ tested. The coefficients of the straight line fitted in the inset were determined in the range of 0.1 to 0.8 μM $[Ca^{2+}]_i$, and were compared with the parameters in Eq. 6 to determine the values of K_d (0) and δ for each type of channel. The estimated K_d (0) values were 0.529 μM and 0.607 μM for the native and cloned SAKcaCs, respectively. The electrical distances (δ) were 0.185 and 0.186 for the native and cloned SAKcaCs, respectively. From above results, we concluded that there were no significant difference in the voltage and calcium sensors between the native SAKcaC in CCVM and the cloned one in CHO.

To estimate the apparent number of bound Ca^{2+} to the SAKcaC, P_o as a function of calcium concentration was fitted to the Hill equation (Fig. 3*D*),

$$P_o = \frac{[\mathrm{Ca}^{2+}]_i^{\eta H}}{(K_d^{\eta H} + [\mathrm{Ca}^{2+}]_i^{\eta H})} \qquad (7)$$

where P_o is the channel open probability ηH the Hill coefficient, and K_d the dissociation constant. The relationship between $[Ca^{2+}]_i$ and P_o at the membrane potential of +30 mV is shown in Fig. 3*D*. The calcium

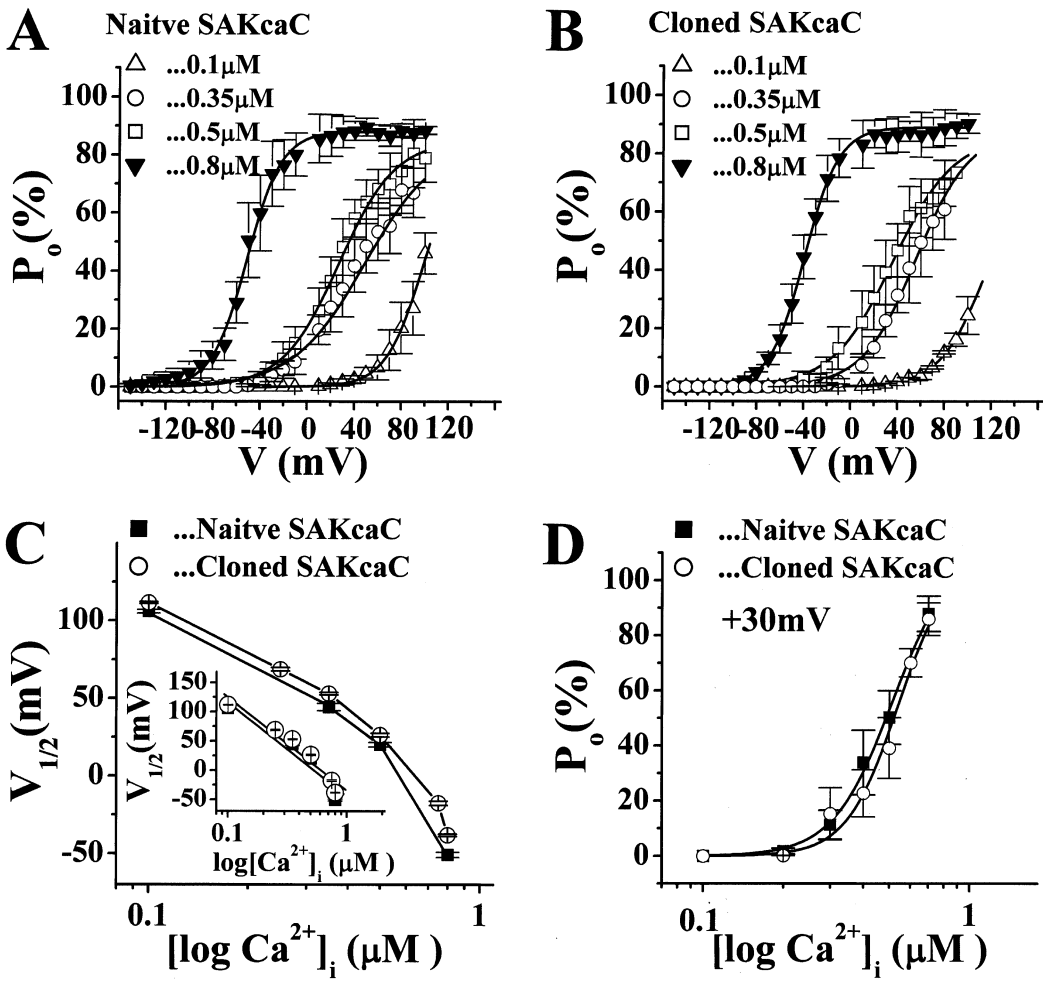

Fig. 3. Calcium and voltage dependencies. (*A*), (*B*) Channel open probability (P_o) *vs.* membrane potential for the native ($n = 15$) and cloned ($n = 18$) SAKcaCs at different $[Ca^{2+}]_i$, as indicated. Lines were drawn according to the best fits with the Boltzmann equation: $P_o = 1/\{1/ + \exp[-ZF/RT(V-V_{1/2})]\}$ (Eq. 4). (*C*) $V_{1/2}$ as a function of $[Ca^{2+}]_i$ for the native (■) and cloned (○) SAKcaCs ($V_{1/2}$ was taken from *A* and *B*). Standard errors are comparable to the size of the symbols used. Lines in the inset were drawn based on the least-square fit and can be expressed as a linear function as $V_{1/2} = A \log[Ca^{2+}] +$ *B*, from which zero-voltage dissociation constants $K_d(0)$ for Ca^{2+} could be estimated. The obtained $K_d(0)$ values were 0.529 μM and 0.60 μM for the native and cloned SAKcaCs, respectively. (*D*). P_o (as measured in *A* and *B*) for the native (■) and cloned (○) SAKcaCs at +30 mV is plotted as a function of $[Ca^{2+}]_i$. The data points were fitted with the Hill equation (Eq. 7) to obtain the calcium concentration necessary to open half of the channels (K_d) and the Hill coefficient (ηH). K_d was 0.488 μM for the native SAKcaC and 0.589 μM for the cloned one; ηH was 2.786 for the native and 2.8617 for the cloned SAKcaCs.

concentration (K_d) for half-maximal channel activation in the native SAKcaC was 0.490 ± 0.030 μM and 0.640 ± 0.013 μM for the cloned SAKcaC. At all tested membrane potentials (−40 to +80 mV), K_d ranged from 0.741 to 0.216 μM for the native SAKcaC and 0.807 to 0.238 μM for the cloned one, showing no significant difference between them ($P = 0.01$). The Hill coefficient (ηH) provides a minimum estimate of the number of bound Ca^{2+} at the maximal channel-activation level and highly depends on the degree of cooperativity among binding sites (McManus, 1991; Cui et al., 1997). At +30 mV, the Hill coefficient for the native SAKcaC was 2.786 and 2.861 for the cloned one. The Hill coefficients calculated at different voltages between −40 to +80 mV ranged from 1.58 to 3.97 for the native SAKcaC and 1.541 to 3.72 for the cloned one, showing no significant difference between the native and cloned SAKcaCs ($P = 0.01$).

The Pharmacological Properties Regarding TEA, CTX and Gd^{3+}

Effects of TEA

First we examined the effect of extracellularly applied TEA (TEA_o) on the native and cloned SAKcaCs, using the backfill method (Auerbach, 1991): the pipette tip was first filled with 145 mM K^+ and then backfilled with the same solution containing 1 mM TEA, followed by an immediate approach to the cell. The bath contained 145 mM K^+ and 1 mM $CaCl_2$. As shown in Fig. 4*A*, TEA_o gradually decreased the

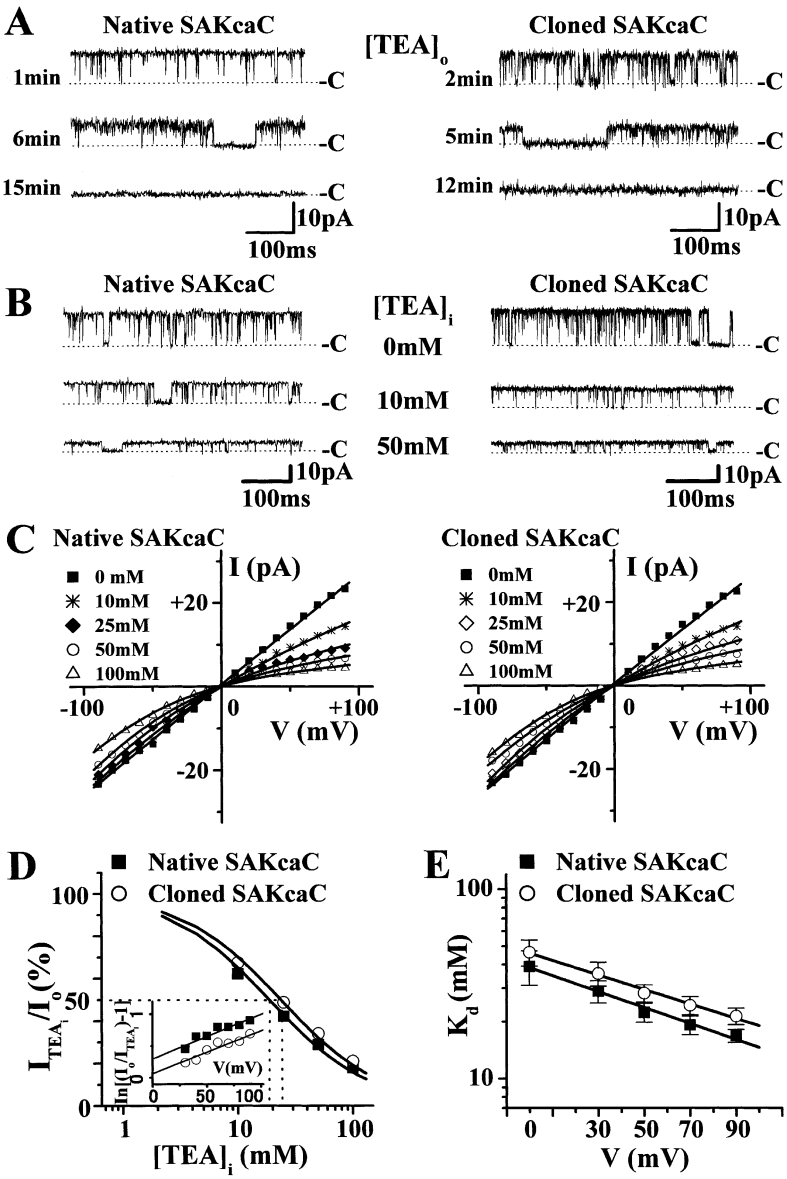

Fig. 4. Channel blockade by TEA. (*A*) The effect of TEA at the extracellular surface: TEA_o reduced the current amplitude with time both in the native (*left*) and cloned (*right*) SAKcaCs essentially in the same manner. Traces were obtained at indicated times after the starting of diffusion of backfilled solution in the pipette. The pipette tip was first filled with 145 mM K^+ and then backfilled with the same solution containing 1 mM TEA; the bath contained 145 mM K^+ and 1 mM Ca^{2+}. Membrane potential was held at +30 mV. (*B*) Single-channel current traces of the native (*left*) and cloned (*right*) SAKcaCs at various concentrations of TEA in the bath (TEA_i). Dose-dependent blockade can clearly be seen in both channel types. Membrane potential was held at +70 mV. (*C*) Current-to-voltage relationships at various $[TEA]_i$ for the native (*left*, $n = 7$) and cloned (*right*, $n = 7$) SAKcaCs, demonstrating voltage-dependent TEA blockade. (*D*) Dose-dependent inhibition of single-channel currents by TEA_i at +70 mV. Data were taken from *C*, which could be well fitted with the Langmuir function (Eq. 8) with K_d (70 mV) values of 19.22 ± 2.15 for the native SAKcaC (■), and 24.41 ± 2.68 mM for the cloned SAKcaC (○). The inset is a linearized plot of ln $[(I_o/I_{TEAi}) - 1]$ against membrane potential at 50 mM TEA_i. *Y*−intercepts of the lines give estimated $K_d(0)$ values of 35.98 mM and 46.64 mM for the native and cloned SAKcaCs, respectively. The electrical distance (δ) of the TEA_i binding site could be estimated from the slope of the lines as 0.205 for the native SAKcaC and 0.203 for the cloned one. (*E*) Mean $K_d(V)$ (± SEM) values plotted against membrane potential for the native (■) and cloned (○) SAKcaCs. Lines were drawn based on the best fit to the function, $K_d(V) = K_d(0)\exp(-Z\delta FV/RT)$.

mean single-channel current of the native ($n = 4$) and cloned ($n = 7$) SAKcaCs. There was no significant difference in the TEA_o blockade between the native and cloned SAKcaCs, though we could not make a rigorous test because of the uncertainty of the final concentration of the drug at the membrane surface due to the technical limitation of the backfill method. In contrast, we could make more precise examination of the effects of TEA applied from the intracellular surface (TEA_i) with inside-out patches. Figure 4*B* represents current traces at +70 mV from the native and cloned SAKcaCs in the presence of 0, 10, and 50 mM of TEA_i (at 1 mM $[Ca^{2+}]_i$). The amplitude of the single-channel currents decreased in a dose-dependent manner without discrete blocking events, referred to the fast blocking ("flickery blocking" due to a relatively fast kinetics). Figure 4*C* is a plot of single-channel current amplitude against membrane potential for the native and cloned SAKcaCs. In both channel types, TEA_i reduced the amplitude in a voltage-dependent way: greater blockade at more positive membrane potentials. Figure 4*D* shows the relative current (I_{TEAi}/I_o) versus $[TEA]_i$ for the native (■) and cloned (○) SAKcaCs. The solid lines represent the best fits to the Langmuir function,

$$I_{TEAi}/I_o = \{(1 + [TEA]_i/K_d(V))\}^{-1} \quad (8)$$

where K_d (V) is the dissociation constant at a given voltage V, $[TEA]_i$ is the concentration of TEA in the bath solution. Taking the reciprocal of each side of Eq. 8 and substituting $K_d(V)$ for zero voltage dissociation constant $K_d(0)$, leads to the Woodhull equation (1973) as

$$I_o/I_{TEAi} = \frac{1 + [TEA]_i\ [\exp(Z\delta FV/RT)}{K_d(0)} \quad (9)$$

where I_o and I_{TEAi} represent the single-channel conductance in the absence and presence of TEA_i, Z is blocker valence, and δ is the fraction of voltage drop at the blocking site measured from the intracellular side of the membrane (Coronado & Miller, 1982; Blatz & Magleby, 1984). When Eq. 9 is linearized by taking the natural log of each side, the slope of ln [I_o/I_{TEAi}−1] versus membrane potential equals $Z\delta F/RT$. Thus, δ = slope (RT/FZ), and K_d (0) = $[TEA]_i$/exp (y−intercept). As shown in Fig. 4*D*, I_{TEAi}/I_o plotted against $[TEA]_i$ is well fitted with Eq. 8, suggesting that TEA_i interacts with both the native and cloned SAKcaCs in a one-to-one fashion. These fits yielded K_d values of 19.22 ± 2.16 mM for the native SAKcaC and 24.41 ± 2.69 mM for the cloned one at a membrane potential of +70 mV. K_d values were also calculated with the data obtained at +30, +50 and +90 mV using the same method, and at 0 mV, using the Woodhull (1973) equation (Eq. 9). The inset in Fig. 4*D* shows the plot of ln [I_o/I_{TEAi} −1] against membrane potential. Analysis of the y-axis intercepts yielded K_d (0) values of 35.98 and 46.84 mM at 50 mM $[TEA]_i$ for the native and the cloned SAKcaCs, respectively. At 50 mM $[TEA]_i$, the electrical distance $Z\delta$ was calculated as 0.179 for the native SAKcaC and 0.173 for the cloned one. K_d values are plotted against membrane potential for the native (■) and cloned (◯) SAKcaCs in Fig. 4*E*. Note that the K_d values for the cloned SAKcaC were larger than those for the native one at every membrane potential examined ($P < 0.05$). In addition, for both channel types, K_d decreased as the potential became more positive, indicating TEA_i undergoes a typical voltage-dependent channel-blocking. The mean values of electrical distance (δ) for the TEA_i binding site at various $[TEA]_i$ with the known TEA valence ($Z = 1$) were 0.21 ± 0.04 and 0.20 ± 0.05 for the native and cloned SAKcaCs, respectively, indicating no significant difference ($P = 0.001$).

Effects of CTX and Gd^{3+}

We investigated the effect of CTX, a specific blocker for BKcaCs (Anderson et al., 1988). The pipette was backfilled with the same solution as above, except for 20 nM CTX as a substitute for TEA_o. Figure 5*A* shows the effect of 20 nM CTX on the single-channel currents at 1 mM $[Ca^{2+}]_i$ for the native and cloned SAKcaCs. The blockade of the native SAKcaC occurred without significant reduction in current amplitude, showing a blocking process with relatively slow kinetics ($n = 5$) referred to the slow blockade (with an "all-or-none" type of gaps in the open-channel currents). This result was consistent with our previously published data (Kawakubo et al., 1999). However, CTX reduced gradually the current amplitude of the cloned SAKcaC as a function of time, in other words, with increasing CTX concentration, due to its diffusion towards the patch ($n = 10$). We suspected that this gradual conductance inhibition arose from the fast blockade of the channel and that we could not resolve the fast blocking events just because of the limited bandwidth of our recording setup (3.33 kHz sampling, 1.6 KHz cutoff frequency in this case). Therefore we increased the sampling rate as high as 100 kHz; however, we could not resolve blocking events. Therefore it may arguably indicate a "nonspecific effect" in which the CTX is bound nonspecifically around the pore and occludes the passage of ions (*see* Discussion and Fig. 8*A*). Next, we investigated the effect of gadolinium (Gd^{3+}) on the native and the cloned SAKcaCs with the same backfill procedure as above (Fig. 5*B*). Gd^{3+} is a potent blocker of SA channels in a variety of tissues, acting principally at the extracellular surface of the channels (Yang & Sachs, 1989). Figure 5*B* shows the effect of Gd^{3+} on the native and cloned SAKcaCs. All the conditions were the same as that in Fig. 5*A* except that the pipette was backfilled with the solution containing 20 μM Gd^{3+}. As in the above case with CTX, the open probability of the native SAKcaC decreased without significant reduction in current amplitude by Gd^{3+} ($n = 3$), whereas this drug gradually reduced the current amplitude of the cloned SAKcaC ($n = 10$), essentially in the same manner as in CTX blockade.

Effects of β-Subunit Cloned from CCVM

Above results indicated that the cloned SAKcaC in CHO has almost the same features, including ion permeation and gating, as those of the native one in CCVM, except for some pharmacological properties against TEA_i, CTX and Gd^{3+}. Since it has been reported that the β-subunit (KCNMB4) modified the binding constant of CTX to a BK channel (*hslo*) expressed in *Xenopus laevis* oocytes (Meera et al., 2000), one possibility causing this difference may be a contribution of a β-subunit. To test this hypothesis, we cloned a β-subunit from chick heart and co-expressed it with the cloned SAKcaC and examined its effect on the pharmacological and electrophysiological properties of the cloned SAKcaC (α-subunit).

Effects on Channel Blockade by TEA, CTX and Gd^{3+}

As shown in Fig. 6*A* the *I-V* relationship and the single-channel conductance (268.9 ± 1.8 pS, $n = 6$) of the cloned SAKcaC co-expressed with the β-subunit (termed α/β channel) were almost the same as those of the cloned SAKcaC (α-subunit alone). First we examined the channel blockade by TEA. Figure 6*B* shows the effect of extracellularly applied TEA (TEA_o), where TEA_o decreases the single-channel current with time, indicating essentially the same result of TEA_o blockade of the cloned SAKcaC (α-subunit alone). Next we tested the TEA_i blockade of

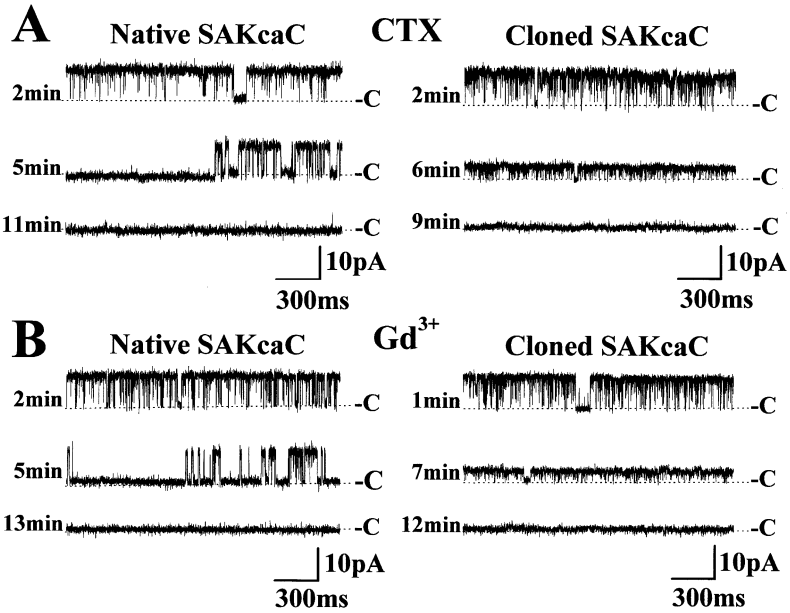

Fig. 5. Effect of CTX and Gd^{3+} on single-channel currents. (*A*) Effect of 20 nM CTX on the single-channel currents from the native (*left*) and cloned (*right*) SAKcaCs showing an all-or-none-type "slow blockade" in the native SAKcaC, while graded inhibition of the current amplitude in the cloned SAKcaC. (*B*) Effect of 20 μM Gd^{3+} on the single-channel currents from the native (*left*) and cloned (*right*) SAKcaCs showing an all-or-none-type "slow blockade" in the native SAKcaC, while graded inhibition of the current amplitude in the cloned SAKcaC, just as occurred with CTX. Both drugs were back-filled in the pipette, thus diffused toward the patch surface with time.

the α/β channel, which we can quantitatively analyze only with inside-out patches, as shown above. Figure 6*C* shows the plots of relative current (I_{TEA}/I_o) *vs.* $[TEA]_i$ at +70 mV. The well-fitted data to the Langmuir function suggests that TEA_i interacts with the α/β channel in a one-to-one fashion as in the native and cloned SAKcaCs. From this fitting we can get a K_d value of 19.79 ± 1.29 mM, almost the same K_d of the native SAKcaC at +70 mV in CCVM (K_d = 19.22 ± 2.16 mM). K_d values at different membrane potentials (+30, +50 and +90 mV) are shown in Fig. 6*D*, where the solid and broken lines represent the regression fit of K_d values of TEA_i for the α/β, cloned, and native SAKcaCs, respectively. Note that the β-subunit shifts the K_d value of the cloned SAKcaC (α-subunit alone) close to the native one over the range of membrane potential applied. The electrical distance (δ) of the TEA_i binding site was estimated to be 0.23 ± 0.04 (average value of the data at 10, 25, 50 and 100 mM $[TEA]_i$), suggesting no significant difference compared to the native (δ = 0.21 ± 0.04) and the cloned α-alone channel (δ = 0.20 ± 0.05) (P = 0.001). Next, we examined the effect of co-expression of β-subunit on the channel blockade by CTX and Gd^{3+}. Surprisingly, as shown in Fig. 6*E* and *F*, CTX and Gd^{3+} blocked the α/β channel in an all-or-none fashion, as in the native SAKcaC shown in Fig. 5 (*left column*). Although this is just a qualitative observation, combining the above result on TEA_i the β-subunit seems to recover the original pharmacological property of the native SAKcaC in CCVM.

Effects on Ca^{2+}- and Voltage-dependencies

Figure 7*A* shows a plot of channel open probability (P_o) against membrane potential of the α/β channel at various $[Ca^{2+}]_i$. To depict the difference among different channel types, P_o *vs.* V relationships at 0.8 μM $[Ca^{2+}]_i$ for the native, cloned (α) and α/β channels are shown in Fig. 7*B*. In contrast to the similarity between the native and α channels, the much higher voltage sensitivity of the α/β channel is apparent. The average voltage (K^{-1}) for e-fold change of P_o at all tested $[Ca^{2+}]_i$ was estimated as 14.67 ± 1.12 mV, and the average gating charge (Z) 1.77 ± 1.154, indicating an increased voltage dependency of the α/β channel compared to the native SAKcaC (K^{-1} = 21.48 ± 2.71 mV; Z = 1.21 ± 0.15), and the α channel (K^{-1} = 22.07 ± 1.18 and Z = 1.18 ± 0.13). Next, we analyzed the alteration of the Ca^{2+} sensitivity of the α channel by the β subunit. Figure 7*C* shows the plot of the half-maximal voltage ($V_{1/2}$) versus ln $[Ca^{2+}]_i$ for the α/β channel with the dotted lines representing the feature for the native and cloned SAKcaCs as shown in the inset of Fig. 3*C*. Note that the β-subunit significantly decreases the half-maximal voltages over the range of $[Ca^{2+}]_i$ tested. The value of zero voltage dissociation constant for Ca^{2+}, K_d (0), was estimated as 0.298 μM, an apparently lower K_d (0) value than those of the native SAKcaC (K_d (0) = 0.529 μM) and the α channel (K_d (0) = 0.607 μM). The same trend can be more clearly seen in the P_o versus $[Ca^{2+}]_i$ plot for the three types of channels in Fig. 7*D*, where P_o steeply increases from 0.232% to 93.09% with the increase in $[Ca^{2+}]_i$ from 0.1 μM to 0.8 μM. The estimated K_d for Ca^{2+} at +30 mV was 0.223 μM and Hill coefficient (ηH) was 3.852. The K_d value ranged from 0.144–0.575 μM over the tested membrane potentials (−40 ∼ +80 mV), indicating the significantly increased Ca^{2+}-sensitivity of the α/β channel compared to the native SAKcaC (0.216∼0.741 μM) and the α channel (0.238∼0.807 μM) (P < 0.05). Hill coefficient for the α/β

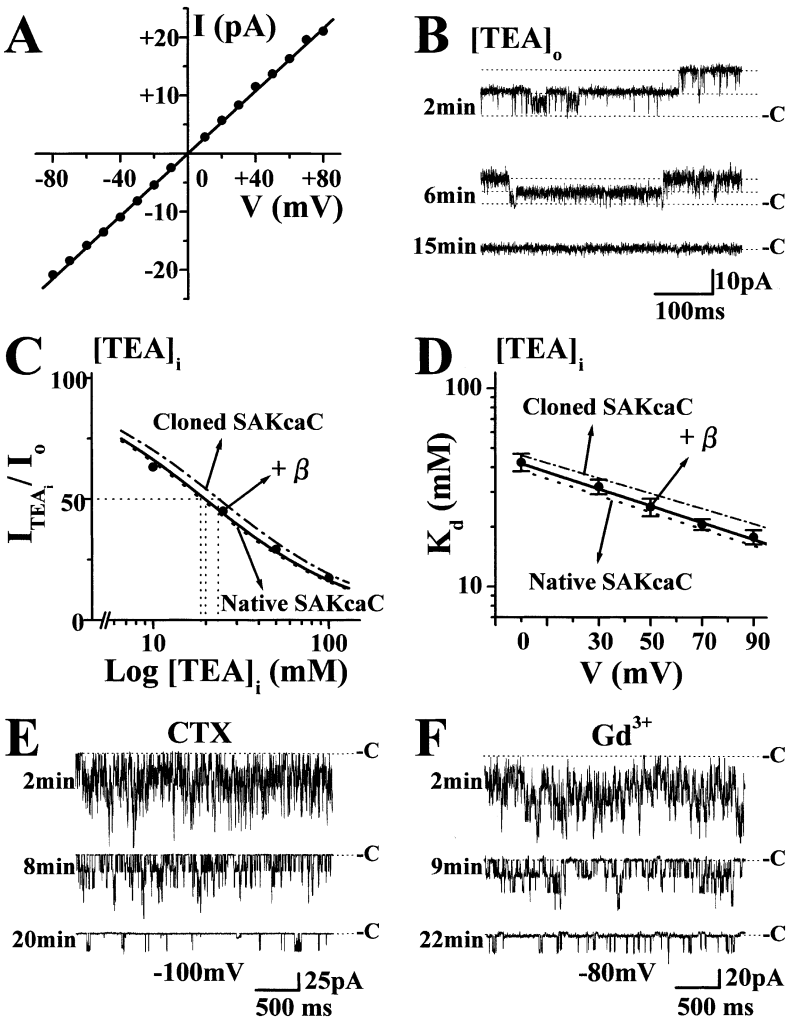

Fig. 6. The effects of β-subunit on the action of channel blockers. (*A*) Current-voltage relationship of the α/β channel, from which single-channel conductance was calculated as 268.9 ± 1.8 pS ($n = 6$). (*B*) The effect of TEA at the extracelluar surface: 1 mM TEA reduced the current amplitude of the α/β channel with time, just as observed in the native and cloned SAKcaCs. Open-channel noise due to the fast channel blocking by TEA can be seen in the middle trace. (*C*). Dose-inhibition of single-channel current from the α/β channel by TEA_i at +70 mV, which could be well fitted with the Langmuir function (Eq. 8) with a K_d(70 mV) value of 19.79 mM, almost the same value (19.22 mM) as from the native SAKcaC in CCVM. For comparison, results from the native and cloned SAKcaCs in Fig. 4*D* are shown as dotted curves. (*D*) $K_d(V)$ values for TEA_i plotted against membrane potential for the α/β channel (*solid line*), the native and the cloned SAKcaCs (*broken lines*).The data for the latter two channels were taken from Fig. 4*E*. (*E*) Blockade of the α/β channel by extracellularly applied 1 μM CTX ($n = 4$), which occurred without significant reduction in current amplitude, like as in the native SAKcaC in CCVM. (*F*) Blockade of the α/β channel by extracellularly applied 200 μM Gd^{3+} ($n = 3$), which proceeded without significant reduction in current amplitude, as in the native SAKcaC in CCVM.

channel ($\eta H = 2.89–5.73$) under the same voltage range as above was also much larger than the native SAKcaC ($\eta H = 1.58–3.89$) and the α channel ($\eta H = 1.54–3.72$) ($P < 0.01$). Collectively, in contrast to the interpretation made in pharmacology, it is strongly suggested that the β-subunit employed here does not interact with the native SAKca in CCVM and that the β-subunit is not responsible for the difference in the pharmacological property between the cloned SAKcaC (α channel) in CHO and the native SAKcaC in CCVM.

Discussion

In this study we have characterized a SAKcaC that was cloned from CCVM and expressed in CHO and compared its properties with those of the native one in CCVM to learn whether the cloned *SAKcaC* gene is responsible for coding the native SAKcaC. The cloned SAKcaC (α-subunit alone) in CHO showed almost the same electrophysiological features as the native one except for a difference in the blocking mode by CTX and Gd^{3+}.

Similarity of Electrophysiological Properties Between Native and Cloned SAKcaCs

The cloned SAKcaC expressed in CHO showed essentially the same *I-V* relationship, single-channel conductance, ion selectivity and pressure dependency as those of the native one in CCVM. Moreover, rigorous examinations revealed that its voltage- and Ca^{2+}-dependencies were nearly the same as those of the native one, too (Table 1). One of the notable features common to both channels is their dramatically steep Ca^{2+} sensitivity (Table 1). We think this may arise from the 59-amino acid STREX (stress axis regulated exon, Chang et al., 1998) sequence between S8–S9 domains identified in our cloned SAKcaC (Naruse, Tang, Sokabe, unpublished observation). Previous reports showed that the STREX sequence contributes to the dramatically high Ca^{2+} sensitivity of BK channels containing this sequence. For example, gBK (*hSlo* from human glioma cell) shows a Ca^{2+} sensitivity with a half-activation calcium concentration (K_d) of 0.137 μM (±0.223) at +80 mV (Liu et al., 2002), an even higher Ca^{2+} sensitivity than ours (K_d is 0.238 μM for

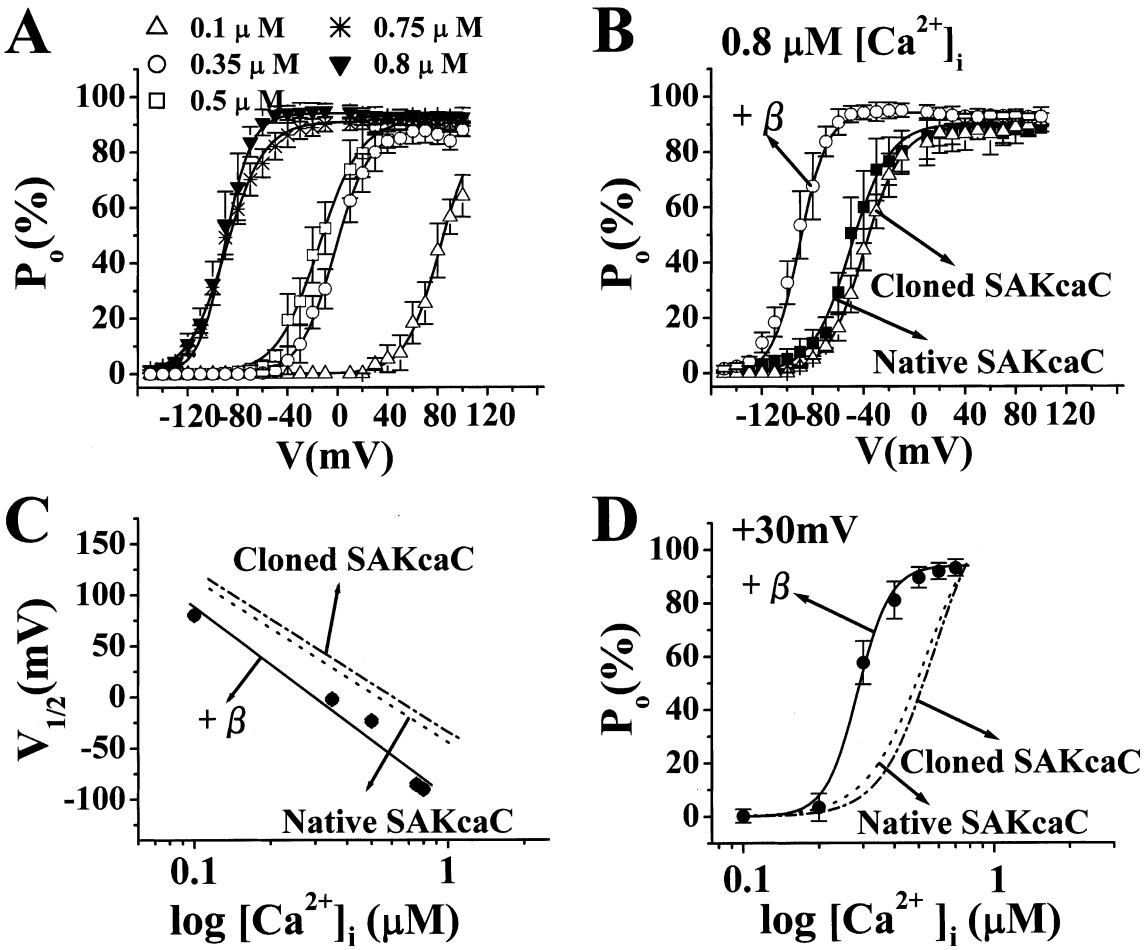

Fig. 7. The effects of β-subunit on voltage- and Ca^{2+}-dependencies. Channel open probability (P_o) plotted against membrane potential for the α/β channel ($n = 15$) at different $[Ca^{2+}]_i$. Lines are the best fits of the Boltzmann equation: $P_o = 1/\{1 + \exp[-ZF/RT(V-V_{1/2})]\}$ (Eq. 4). Comparison of P_o-V relationships among the native ($n = 5$), cloned (α-subunit alone, $n = 5$) and α/β ($n = 6$) SAKcaCs at 0.8 μM $[Ca^{2+}]_i$. Note that the α/β channel shows higher Ca^{2+}- and voltage-sensitivities than others. (*C*) $V_{1/2}$ as a function of $[Ca^{2+}]_i$ for the α/β channel ($V_{1/2}$ were from *A*). For comparison, results from the native and cloned SAKcaCs in Fig. 3*C* are shown as dotted lines. (*D*) P_o (as measured in *A*) for the α/β channel at +30 mV is plotted as a function of $[Ca^{2+}]_i$. Results from the native and cloned SAKcaCs in Fig. 3*D* are shown as dotted curves to make the higher Ca^{2+} sensitivity of the α/β channel stand out.

the cloned SAKcaC at +80 mV). Another example can be seen in the mouse AtT20 BK channel (*mSlo*) with the STREX-1 exon: the half-maximal activation voltage ($V_{1/2}$) at 0.1 μM $[Ca^{2+}]_i$ was 30.7 ± 1.6 mV (Shipston et al., 1999), a higher Ca^{2+} sensitivity than ours at this Ca^{2+}-concentration (*ca.* 100 mV). We prepared a "STREX deletion mutant" to know the role of STREX in our observed high Ca^{2+} sensitivity and found that the deletion mutant showed a dramatically decreased Ca^{2+} sensitivity (e.g. $V_{1/2} = 98.97 \pm 1.20$ ($n = 4$) compared with $V_{1/2} = 67.5 \pm 1.17$ mV ($n = 6$) for control (unpublished observation). Another intriguing feature common to the cloned and native SAKcaCs is their stretch sensitivity. Again the STREX sequence is critical for the stretch sensitivity of the cloned SAKcaC because the STREX deletion mutant is devoid of stretch sensitivity (Naruse, Tang, Sokabe, unpublished observation). However, other mammalian *slo* variants containing a STREX sequence, like *mslo* or *rslo*, did not show any stretch sensitivity when expressed in CHO cells (*ibid.*). Although the sequences are essentially identical among various STREX variants, subtle variations in the STREX sequence, even a single amino acid at certain positions, seem to be responsible for the stretch sensitivity of the channel (*ibid.*). In summary, essentially the same electrophysiological features among the cloned and native SAKcaCs strongly suggest that the cloned SAKca (α-subunit) is responsible for the native SAKcaC in CCVM.

Differences in Pharmacological Properties Between Native and Cloned SAKcaCs

In contrast to the similarity of electrophysiological properties between the cloned and native SAKcaCs, there were noticeable differences in their pharmacology against TEA_i, CTX, and Gd^{3+} (Table 1). Because we could not get stable outside-out patches

Table 1. Comparison of electrophysiological and pharmacological parameters between native SAKcaC, cloned SAKcaC(α subunit) and α/β channel

Parameter	Native SAKcaC	Cloned SAKcaC	α/β channel
Conductance (pS)	273.7 ± 2.2 (16)	271.8 ± 3.1 (22)	268.4 ± 1.8 (6)
Pressure-dependency			
K^{-1} (e-fold change in P_o) (mmHg)	15.48 ± 0.30 (7)	13.22 ± 0.21 (6)	13.35 ± 0.32 (6)
Gating			
V-Dependency			
$V_{1/2}$ (mV)	−50.9 ± 1.7 (5)	−38.3 ± 0.75 (5)	−93.3 ± 0.78* (6)
K^{-1} (e-fold change in P_o) (mV)	21.48 ± 2.71 (5)	22.07 ± 2.42 (5)	14.67 ± 1.12* (6)
Gating charge *Z*	1.21 ± 0.15 (5)	1.18 ± 0.13 (5)	1.77 ± 1.15* (6)
Ca^{2+}-Dependency			
K_d (*V*) (μM)	0.49 ± 0.03 (15)	0.64 ± 0.01 (18)	0.22 ± 0.01* (15)
K_d (0) (μM)	0.529	0.607	0.298*
Hill coefficient (η_H)	1.58–3.97	1.54–3.72	2.89–5.73*
Pharmacology			
Blockade by TEA_o	Fast	Fast	Fast
Blockade by TEA_i	Fast	Fast	Fast
Dissociation constant K_d (mM):	19.22 ± 2.16 (7)	24.41 ± 2.69* (7)	19.79 ± 1.29 (6)
Electrical distance Zδ	0.21 ± 0.04 (7)	0.20 ± 0.05 (7)	0.23 ± 0.04 (6)
Blockade by CTX	Slow	Graded	Slow
Blockade by Gd^{3+}	Slow	Graded	Slow

All parameters were obtained from excised inside-out patches with 0 $[Ca^{2+}]$ (1 mM EGTA) in the pipette. Pressure dependency was examined at +10 mV with 0.25 μM $[Ca^{2+}]_i$ except for α/β channel (0.15 μM $[Ca^{2+}]_i$). Voltage dependency is characterized by half-activation voltage $V_{1/2}$ at 0.8 μM $[Ca^{2+}]_i$, e-fold change in P_o (K^{-1}), and the gating charge (*Z*). Ca^{2+} dependency is characterized by K_d (*V*) representing $[Ca^{2+}]_i$ required to achieve $P_{1/2}$ at +30 mV, zero voltage K_d (0), and Hill coefficient η_H. The dissociation constant K_d for TEA_i blockade is given at +70 mV. Each value is the mean ± SEM with the number of experiments in parentheses; *indicates statistically significant difference ($P < 0.05$).

with our preparations, all the measurements were done with inside-out patches in combination with the back-fill method. Therefore, quantitative analysis could be made only with a TEA effect from the intracellular surface of the patch (TEA_i). TEA_i blocked the cloned and native SAKcaCs in a "fast blocking" manner but with a slightly stronger effect in the native SAKcaC (K_d was 19.22 mM for the native SAKcaC and 24.41 mM for the cloned one). However, even in cursory observations with inside-out patches, we could see an apparent difference in the blocking actions of CTX and Gd^{3+} between the cloned and native SAKcaCs. The drugs inhibited the conductance of the native SAKcaC in an "all-or-none"-type slow-blocking fashion, whereas they inhibited the conductance of the cloned one in a time-dependent (i.e., concentration-dependent) graded fashion. We initially thought that the graded conductance inhibition arises from a kind of "fast blocking", and that individual blocking events (blocking noise during channel opening) could not be resolved due only to the low sampling rate and cut-off frequency of our experimental setup. Thereupon we tried to record putative blocking noise during CTX and Gd^{3+} blockade by increasing the sampling rate as high as 10 μs, but failed. At this point we gave up to employ the "fast blocking mechanism". Instead, we rather took a moderate interpretation in which CTX and Gd^{3+} are bound nonspecifically around the channel entrance and occlude the passage of ions by electrostatic repulsion (Fig. 8*A*). This may not be an unrealistic idea since in extracellulary facing regions of the channel there are plenty of negatively charged amino acids (e.g., Asp^2, Glu^{11} and Asp^{15} on the N-terminal, Glu^{140}, Asp^{148} on the linker between S1 and S2, or Asp^{202}, Glu^{205}, Glu^{217}, Asp^{233} near the pore) that may bind polyvalent cations like Gd^{3+} and CTX (for positively charged residues of CTX, *see* Garcia-Valdes et al., 2001) (Fig. 8*A*). The continuous reduction in the amplitude of the conductance with concentration of both CTX and Gd^{3+} implies a large number of binding sites per channel, none of which is capable of shutting the channel. This model may also explain why the TEA undergoes a true channel blocking in a "fast blocking" manner. While CTX and Gd^{3+} are polyvalent cations, as TEA is a monovalent cation, it may weakly interact with the above putative negatively charged residues (termed nonspecific binding sites here), leading to a relatively easy access to the "TEA blocking site" in the pore.

Effects of β-Subunit on the Blocking Action of TEA_i, CTX and Gd^{3+}

Although we do not know the mechanism that generates pharmacological differences observed between the cloned and native SAKcaCs, it is natural to assume that an auxiliary protein(s) like the β-subunit in CCVM would contribute to the differences. To test

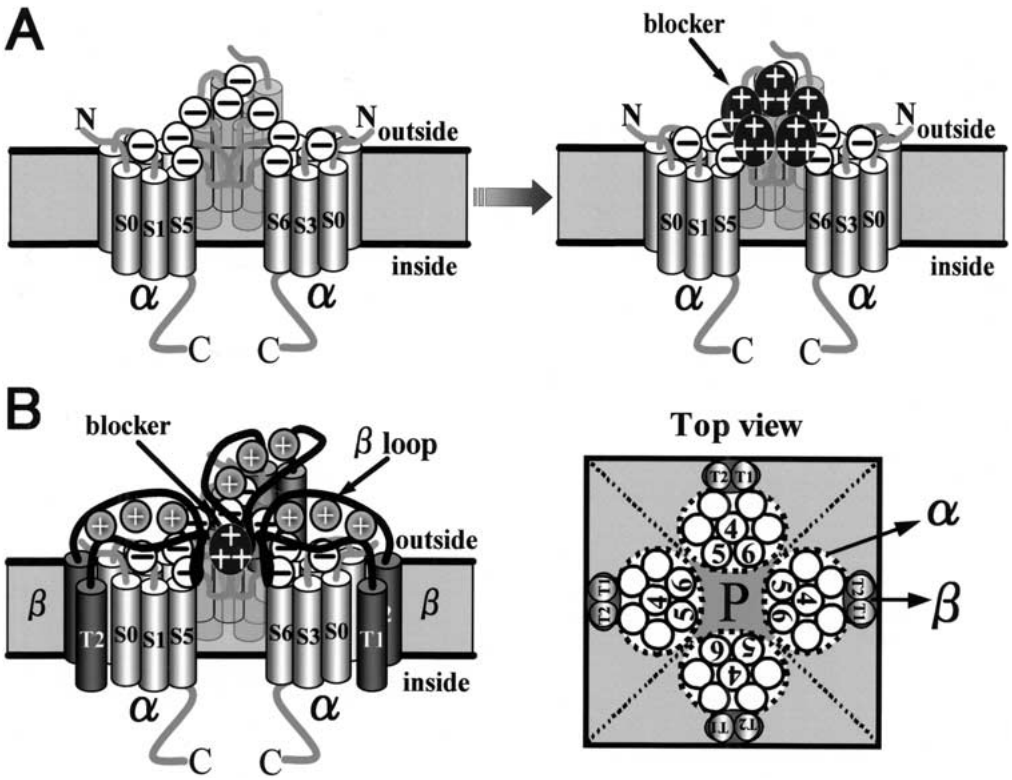

Fig. 8. A schematic model of the tetrameric α-subunits and α/β subunit complexes with one α-subunit removed to explain the β-subunit effect on the channel blockade by CTX and Gd^{3+}. (*A*). Left panel highlights negative charges (−) derived from extracellular loops of α-subunits, forming nonspecific binding sites for cations. Polyvalent cations like CTX and Gd^{3+} are assumed to be trapped by these binding sites and prevented from reaching the blocking sites of the channel (*right*). The bound cations will interfere with K permeation probably by electrostatic repulsion. (*B*). *Left*: A schematic model of the α/β channel, where positive charges (+) from extracellular β-loops are assumed to neutralize the above mentioned negative charges from α-subunits, letting polycationic blockers reach the intra-channel blocking sites. *Right*: top view of the α/β channel. (Diagrams were drawn based on Monks et al, 1999, Silberberg & Magleby, 1999 and Meera etal., 2000).

this assumption we coexpressed the cloned β-subunit from CCVM with the cloned SAKcaC α-subunit under the condition to form an α/β channel with 1:1 stoichiometry as described in Methods. As expected, the α/β channel exhibited a dissociation constant (K_d = 19.79 mM) for TEA_i very close to that of the native one (K_d = 19.22 mM) compared with the α subunit alone (K_d = 24.41 mM) without changing the electrical distance of the TEA_i binding site (*see* Table 1). More surprising effects of the β-subunit were seen in the blocking action of CTX and Gd^{3+}. The β-subunit changed the graded conductance inhibition by these drugs in the cloned SAKcaC (α-subunit alone) to an all-or-none-type slow blockade (Fig. 6), as observed in the native SAKcaC in CCVM. Based on the above interpretation of the graded conductance inhibition in the cloned SAKcaCα-subunit (Fig. 8*A*), where nonspecific binding of the blockers around the extracellular entrance of the channel is assumed to occlude the ion pathway, association of the β-subunit with the α-subunit may somehow prevent the blockers from the nonspecific binding to the channel, thus improving the accessibility of the drugs to the blocking site(s) of the channel. Mechanistically, the β-subunit-induced changes in "binding" of blockers may be explained by 1) an allosteric mechanism, or 2) an electrostatic contribution of the part of the β-subunit near the channel entrance. Since the ion permeation property of the cloned SAKcaC was not altered by the cloned β-subunit, structural changes in the ion pathway of the α-subunit might not be induced by the β-subunit. The fact that the cloned β-subunit did not alter the electrical distance of the binding site for TEA_i supports this idea. Therefore we prefer the second mechanism that a part of the β-subunit loop contributes to modify blocker-channel interaction as schematized in Fig. 8*B*, where long extracellular loops of the cloned β-subunit occlude the negatively charged "nonspecific binding site" for CTX and Gd^{3+} by "electrostatic neutralization" with the positively charged β-loop that contains 16 positively charged residues (Lys 45, Lys 55, Lys 58, Lys 62, Lys 64, Lys 111, Lys 123, Lys 126, Lys 128, Lys 137, Lys 138; His 80, His 85; Arg 108, Arg 159, Arg 164). In this way, the β-subunit can prevent the blockers from being trapped at the putative nonspe-

cific binding sites, improving the travel of polycationic blockers to the blocking sites of the channel. An alternative interpretation of the β-subunit effect on the CTX blockade may be conceivable based on a direct interaction of CTX with the β-subunit. Several reports describe the modification of CTX binding to BK channels by β-subunit. Hanner et al (1998) indicated that not only certain amino-acid residues (Leu90, Tyr91, Thr93, and Glu 94) in the extracellular loop of bovine BKβ but also conserved cysteine residues Lys 69 (cross-linking to CTX) are critical for the enhanced binding of CTX to the α/β channel. Amino-acid sequence analysis showed that the hβ1 subunit (NM04137) shares both of the "high-affinity-binding" and "cross-linking" cysteine residues. However, our cloned β-subunit, although it shares this "high-affinity binding" site to CTX at almost equivalent positions in the loop (Leu99, Tyr100, Thr102, and Glu 103), it lacks "cross-linking" cysteine residues. This may be the reason why nanomolar CTX could completely block the channel formed of the α/hβ1 channels (Xia et al., 1999; Meera et al., 2000; Garcia-Valdes et al., 2001), but not in our cloned α/β channel that needs few tens of nM CTX (Fig. 6). This speculation is consistent with the previous reports on hβ3 and hβ4 (both lack the conserved "cross-linking" cysteine residues) that hβ3 subunit confers a reduced sensitivity to CTX and that hβ4 subunit becomes resistant to CTX (Xia et al., 1999; Meera et al., 2000; Weiger et al., 2000; Garcia-Valdes et al., 2001).

Effect of β-Subunit on Channel Gating

Co-expression of the cloned β-subunit did not change the K^+ permeability of the cloned SAKcaC, indicating that the β-subunit does not affect the narrowest constriction (ion-selective filter) of the SAKcaC pore. However, the β-subunit significantly altered the gating property of the cloned SAKcaC (Table 1): gating charge was changed from 1.176 to 1.77, half-activation voltage ($V_{1/2}$) was shifted leftward by about 50 mV, and zero-voltage dissociation constant for Ca^{2+} was changed from 0.602 μM to 0.306 μM. Such effects of the β-subunit resemble those of β1-subunit on the properties of BK channels expressed heterogeneously in *Xenopus* oocytes (McManus et al., 1995; Cox & Aldrich. 2000). However, as the gating property altered by our β-subunit is far from the original gating property of the native SAKcaC, it is unlikely that the cloned β-subunit interacts with the native SAKcaC in CCVM under physiological condition. In addition, as the protein product of the cloned SAKcaC gene (α-subunit alone) expressed in CHO exhibited most of the fundamental electrophysiological properties of the native SAKcaC in CCVM, we prefer to draw the conclusion that the gene is responsible for the SAKcaC in CCVM. However, at the same time there is an undeniable observation of a pharmacological difference (e.g., CTX, Gd^{3+} blocking) between the cloned and native SAKcaCs. How can we reconcile this discrepancy? Is there any possibility that an unidentified auxiliary protein can modify the pharmacological property of SAKcaCs without changing its gating property? There is a report that currents from a fly BK channel (D*slo*) is not affected by the coexpression with a BK β-subunit (Toro et al., 1998). Therefore it is still possible that CCVM carries an unidentified β-subunit-like auxiliary protein, which meets the above criteria. A correct answer awaits further study.

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