Brain capillary endothelial cells (BCECs) form the blood-brain barrier (BBB), which is essential for maintaining homeostasis of the brain. Net cellular turnover, which results from the balance between cell death and proliferation, is important in maintaining BBB homeostasis. Here we report a novel mechanism that underlies ATP-induced cell proliferation in t-BBEC 117, a cell line derived from bovine brain endothelial cells. Application of 0.1–30 μM ATP to t-BBEC 117 concentration-dependently increased intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}) in two phases: an initial transient phase and a later and smaller sustained one. These two phases of [Ca^{2+}]_{i} rise were mainly due to Ca^{2+} release and sustained Ca^{2+} influx, respectively. The pretreatment with apamin, a selective blocker of small conductance Ca^{2+}-activated K^{+} channels (SK), significantly reduced both the [Ca^{2+}]_{i} increase and K^{+} current induced by ATP. Transcripts corresponding to P2Xs, SK2, and transient receptor potential channels were detected in t-BBEC 117. Knock down of SK2 protein, which was the predominant Ca^{2+}-activated K^{+} channel expressed in t-BBEC 117, by siRNA significantly reduced both the sustained phase of the [Ca^{2+}]_{i} rise and the K^{+} current induced by ATP. Cell proliferation was increased significantly by the presence of the stable ATP analogue ATP_{γ}S. This effect was blunted by UCL1684, a synthesized SK blocker. In conclusion, in brain endothelial cells ATP-induced [Ca^{2+}]_{i} rise activates SK2 current, and the subsequent membrane hyperpolarization enhances Ca^{2+} entry presumably through transient receptor potential channels. This positive feedback mechanism can account for the augmented cell proliferation by ATP.

Under physiological conditions, the blood-brain barrier (BBB) restricts movement of substances from the circulation, blocks the invasion of noxious matters to the brain, and maintains the homeostasis of the brain. Brain capillary endothelial cells (BCECs) form the BBB whose functional characteristics include the presence of intercellular tight junctions and a relating low transcellular vesicular transport induced by surrounding astrocytes (1). In the BBB cell homeostasis requires a delicate balance between formation of new cells by proliferation and their elimination by cell death.

Cell proliferation is stimulated by ATP in a variety of cells (2, 3). There is a line of evidence for a critical role of intracellular Ca^{2+} activity in the regulation of cell proliferation (4–6). ATP is known to increase intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}) via activation of metabotropic P2Y receptors (7), which in turn can trigger Ca^{2+} entry (8). A variety of K^{+} channels have also been implicated in the regulation of cell proliferation (9). In peripheral endothelial cells, ion channels such as Ca^{2+}-activated K^{+} channels (K_{Ca}) (10, 11), inward rectifier K^{+} channels (K_{ir.2.x}) (12, 13) and transient receptor potential (TRP) channels (14, 15) have been reported to be present, and some of their functions have been identified. In contrast, electrophysiological characteristics, including ion channel expression and their functions in BCECs, have not been studied extensively. Only the accumulation of information about transporters such as P-glycoprotein in BCECs has been recently well accelerated (16). Experimental difficulties in preparing freshly isolated BCECs and identifying BCECs among various types of cell are major reasons why the electrophysiology of BCECs using single cell preparation and patch clamp methods has progressed rather slowly, despite its importance (17).
In this study, we have used t-BBEC 117, an immortalized bovine BCEC line, which was established by transfection of SV40 large T antigen and then by isolating a single clone. In vitro culture of these cells has been accomplished, and the BBB phenotypes have been confirmed based on the following criteria: (i) spindle-shape morphology, (ii) rapid uptake of acetylated-LDL, (iii) formation of tight junction-like structures, (iv) high alkaline phosphatase activity, and (v) expression of multidrug resistance and glucose transporter-1 mRNA (18). We have analyzed the functional and molecular expression of ion channels and purinoceptors in t-BBEC 117. Our findings revealed a novel mechanism of positive feedback in the regulation of $[\text{Ca}^{2+}]$ by ATP. This involves the activation of two types of ion channels, Ca$^{2+}$-activated K$^+$ channels ($K_{\text{Ca}}$) and Ca$^{2+}$-permeable channels, which are, presumably, TRP channels. We therefore postulate that ATP, which is released into the cleft between BCEC and astrocytes, regulates and sustains cell proliferation of BCECs by the positive feedback mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—An immortalized endothelial cell line, t-BBEC 117, was established from primary cultured bovine brain endothelial cells, using the transfection of SV40 large T antigen expressing vector as described previously (18). t-BBEC 117 were maintained at 37 °C, 5% CO$_2$ in low glucose (1000 mg/liter) Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Sigma), 100 units/ml penicillin (Meiji Seika Kaisha, Ltd., Tokyo, Japan), and 0.2 mg/ml streptomycin (Meiji Seika Kaisha, Ltd.).

**RT-PCR**—Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription (RT) was carried out using random hexamers and SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen). The resultant cDNA was subjected to PCR-based amplification. Our oligonucleotide primers for the K$^+$ channels and $\beta$-actin, and for the purinoceptors, are shown in supplemental Tables S1 and S2, respectively. The reactions for K$^+$ channels and $\beta$-actin contained 1 μl of RT reaction product as template DNA and was carried out using 1 μM primers and 100 μM reverse primers. The reactions for K$^+$ channels and $\beta$-actin contained 1 μl of RT reaction product as template DNA and were carried out for 35 cycles, using a 95 °C, 45-s denaturing step; a 56 °C, 30-s annealing step; and a 72 °C, 60-s extension step. The reaction for purinoceptors contained 1 μl of RT reaction product as template DNA and was carried out for 35 cycles, using a 95 °C, 15-s denaturing step and a 60 °C, 60-s annealing and extension step. The PCR products were visualized by ethyldium bromide staining following separation on 2% agarose gels. Amplified products were sequenced using BigDye terminator v3 kit (Applied Biosystems) with an ABI PRIZM 3100 genetic analyzer (Applied Biosystems).

**Western Blot Analysis**—Cells cultured under the specified conditions were harvested into Ca$^{2+}$- and Mg$^{2+}$-free phosphate-buffered saline and centrifuged for 10 min at 800 × g at 4 °C. Cell pellets were suspended in 100 μl of Tris-buffered saline (TBS, 20 mM Tris-HCl buffer, pH 7.6, 137 mM NaCl) containing 100 μM phenylmethanesulfonyl fluoride, 10 μM pepstatin A, 10 μM leupeptin, 2 mM EDTA. The quantity of protein was measured using a BCA protein assay regent kit (Pierce). 30-μg aliquots from protein sample were separated by 12% SDS-PAGE and transferred to a Clear Blot Membrane-P (ATTO, Tokyo, Japan). The membrane filters were blocked for 1 h with 5% skim milk in TBS-T (20 mM Tris-HCl buffer, pH 7.6, 137 mM NaCl, 0.1% Tween 20), and then incubated overnight at 4 °C with the following antisera: SK1 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), SK2 (1:500, Alomone Laboratories, Jerusalem, Israel), or SK3 (1:200, Alomone Laboratories). After washing the membranes for 15 min with TBS-T, they were incubated for 1 h with anti-rabbit IgG (GE Healthcare Bio-Sciences Corp.,) or anti-goat IgG (Santa Cruz Biotechnology) coupled to horseradish peroxidase diluted 1:4000 in TBS-T. Bands were visualized using an ECL detection system (GE Healthcare Bio-Sciences Corp.).

**Measurement of $[\text{Ca}^{2+}]$**—t-BBEC 117 was incubated with 10 μM fura-2 acetoxymethyl ester (Fura-2 AM, Molecular Probes Inc., Eugene, OR) in normal HEPES solution for 30 min at room temperature. Fura-2 fluorescent signals was measured using the ARUGAS/HiSCA imaging system (Hamamatsu Photonics, Hamamatsu, Japan). The frequency of image acquisition was selected to be one image every ~1 s for $[\text{Ca}^{2+}]$, measurement. In some experiments, the ratios of fluorescence intensity were transformed into $[\text{Ca}^{2+}]$, using Equation 1,

$$[\text{Ca}^{2+}] = 224 \cdot B \cdot ((R - R_{\text{min}})/(R_{\text{max}} - R)),$$  

where $R$ is the fluorescence ratio 340 nm/380 nm, $R_{\text{min}}$ and $R_{\text{max}}$ are the fluorescence ratios determined by addition of 1 mM EGTA and 2 mM Ca$^{2+}$, respectively, after the permeabilization of cells with 10 μM ionomycin, and $B$ is the averaged fluorescence proportionality coefficients obtained at 380 nm under $R_{\text{min}}$ and $R_{\text{max}}$ conditions.

**Electrophysiological Recordings**—Whole cell membrane currents were applied to single t-BBEC 117 with patch pipettes using a CEZ-2400 (Nihon Koden, Tokyo, Japan) amplifier. The resistance of microelectrodes filled with pipette solution was ~3–5 MΩ. Membrane currents were stored and analyzed as described previously (10, 19). Briefly, membrane currents were monitored on a storage oscilloscope (VC-6041, Hitachi, Tokyo, Japan) and stored on videotape after being digitized by a PCM-recording system (modified to acquire a DC signal, PCM 501ES, SONY, Tokyo, Japan). The data on the tape were later loaded into a computer (IBM-AT compatible) through an analog-digital converter (DT2801A; Data Translation, Marlboro, MA). Data acquisition and analysis for whole cell current were carried out using AQ/Cell-soft, developed in the laboratory of Dr. Wayne Giles (University of Calgary). In some experiments, ramp waveforms were applied using a voltage clamp command using a multipulse generator (FS-1915, NF Electronics, Tokyo, Japan). All currents recorded were filtered at 3 kHz (four-pole Bessel filter, NF Electronics).

**Membrane Potential Measurements Using a Voltage-sensitive Fluorescent Dye**—The measurement of membrane potential changes by DiBAC$_4$(3), which is a bisbbarbituric acid oxonol dye with excitation maximal at ~490 nm, was performed as described previously (20). Before the fluorescence measurements, cells were incubated with 100 nM DiBAC$_4$(3) in HEPES-buffered solution for 30 min at room temperature. Experiments were carried out in the constant presence of DiBAC$_4$(3). Hyperpolarization results in the extrusion of the dye from cells and a
SK2 Function in Brain Endothelial Cell Proliferation

subsequent decrease in fluorescence intensity. The decrease in fluorescence intensity by 1% corresponded to ~0.5 mV hyperpolarization in the membrane potential range of 20 and 70 mV (20). Data collection and analyses were performed using ARGUS-HisCA. The sampling interval of DiBAC$_4$(3) fluorescence measurements was 5 s.

**Specific Knockdown of SK2 by RNA Interference**—The sequences of short interference RNA (siRNA) used in this study, selected according to the criteria suggested previously (21), were as follows: 5′-GGUACAUAGUUCAACAGG-ATT-3′ for sense strand, and 5′-GCAUCAGCUCUAGCAGGAAATT-3′ for antisense strand. These oligonucleotides were annealed and labeled by Cy5. The siRNA target site for SK2 was 5′-GGUACAUAGUUCAACAGGAAATT-3′ (nucleotides 1004–1022). The cells in a 35-mm dish were washed with 2 ml of the fresh culture medium 30 min prior to transfection. 25 μl of siRNA solution (1 μM) and 5 μl of Lipofectamine2000 (Invitrogen) were each mixed with 250 μl of Opti-MEM. The two mixtures were combined and incubated for 20 min at room temperature for complex formation. The entire mixture was added to the cells, resulting in a final concentration of 10 nM for the siRNA. The cells were incubated for 5–8 h in a CO2 chamber, washed once, and supplied with 2 ml of the fresh culture medium.

**MTT Assay**—Cell viability was monitored by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) assay as described (22). MTT was dissolved in phosphate-buffered saline at 5 mg/ml and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. Cells (4 × 10$^3$ cells/well) were seeded onto 96-well plates and incubated with test substances for a selected time at 37 °C in 5% CO$_2$. At the time points indicated, stock MTT solution (10 μl per 100-μl medium) was added to all wells of an assay plate, and the plates were incubated at 37 °C for 4 h. After 4 h, 20% w/v SDS (50% N,N-dimethyl formamide and demineralized water) was added to all wells, and the plates were incubated at 37 °C for 6–8 h. The plates were then analyzed following absorption on a Multiscan JX (Ver1.1 system; Thermo Labsystems), using a test wavelength of 595 nm and a reference wavelength of 650 nm. Unless specified in the text, no mixing was performed, and no medium was removed prior to the addition of any ingredient.

**[3H]Thymidine Incorporation**—Cells (5 × 10$^3$ cells/well) were seeded onto 96-well plates and incubated with test substances for a selected period at 37 °C in 5% CO$_2$. Cells were labeled with 20 nCi/well of [3H]thymidine (24 h, 37 °C) and then trypsinized for isolation after the washout of 3H. Cells were harvested on glass fiber filters by using a multiple cell harvester (Labo mash). Filters were air-dried and placed in scintillation vials containing 2 ml of ACSII (GE Healthcare Bioscience) scintillation fluid. The cell-associated [3H]radioactivity was determined on a model 5801 liquid scintillation counter (Beckman).

**Solutions**—For electrophysiology bath solutions, we used normal HEPES solution, of the following composition, in mM: NaCl 137, KCl 5.9, CaCl$_2$ 2.2, MgCl$_2$ 1.2, glucose 14, HEPES 10, NaOH to pH 7.4. For Ca$^{2+}$ imaging, we used normal HEPES solution and Ca$^{2+}$-free HEPES solution, of the following composition, in mM: NaCl 137, KCl 5.9, MgCl$_2$ 1.2, glucose 14, HEPES 10, EGTA 5, NaOH to pH 7.4. For DiBAC$_4$(3) fluorescence imaging, we used 140 mM K$^+$ solution, of the following composition, in mM: NaCl 2.9, KCl 140, CaCl$_2$ 2.2, MgCl$_2$ 1.2, glucose 14, HEPES 10, NaOH to pH 7.4. All whole cell patch clamp pipettes contained, in mM: KCl 140, MgCl$_2$ 4, ATP-Na$_2$ 5, EGTA 5, CaCl$_2$ 4.3, HEPES 10, KOH to pH7.2 (pCa 6.0) and KCl 22, potassium aspartate 118, MgCl$_2$ 4, ATP- Na$_2$ 5, EGTA 0.05, HEPES 10, KOH to pH 7.2.

**Statistics**—Pooled data were expressed as mean ± S.E. Statistical significance was examined using the paired or unpaired Student’s t test for two groups, respectively, and Scheffe’s multiple comparisons for three groups. In all figures, * and #, and ** and #*, indicate statistical significance at p values of 0.05 and 0.01, respectively.

**Drugs and Chemical Agents**—The following compounds were used in this study: apamin, ATP, UDP, ATPγS, thapsigargin, adenosine 3′-phosphate 5′-phosphosulfate (AP35PS), UCL1684, U0126, Go6983, and PD98059 (Sigma); barium chloride (Ba$^{2+}$) and lanthanum chloride (La$^{3+}$) (Wako, Osaka, Japan); DCEBIO (Tocris); UTP (Yasama shouyu, Chiba, Tokyo); ADP (Oriental Yeast Co., Ltd., Tokyo, Japan); SK&F 96365 (Funakoshi, Tokyo, Japan); and L-NAME (Dojindo, Osaka, Japan). The solvents (distilled water and Me$_2$SO) had no involvement of P2YR in this response (7). The contribution of P2Y$R_s$ were used in this study: apamin, adenosine 3′-phosphate 5′-phosphosulfate (AP35PS), UCL1684, U0126, Go6983, and PD98059 (Sigma); barium chloride (Ba$^{2+}$) and lanthanum chloride (La$^{3+}$) (Wako, Osaka, Japan); DCEBIO (Tocris); UTP (Yasama shouyu, Chiba, Tokyo); ADP (Oriental Yeast Co., Ltd., Tokyo, Japan); SK&F 96365 (Funakoshi, Tokyo, Japan); and L-NAME (Dojindo, Osaka, Japan). The solvents (distilled water and Me$_2$SO) had no effect on [Ca$^{2+}$]$_i$, membrane current, and membrane potential, when the corresponding amount was applied by superfusion.

**RESULTS**

Identification of Purinoceptor Subtypes in t-BBEC 117—Application of 10 μM ATP to t-BBEC 117 consistently resulted in a marked rise of [Ca$^{2+}$]$_i$ (Fig. 1A, panel a). ATP-induced [Ca$^{2+}$]$_i$ rise (805.0 ± 102.2 nm) in the control was almost abolished by pretreatment with 5 μM thapsigargin (TG) (33.8 ± 7.9 nm), suggesting that it is due to Ca$^{2+}$ release from intracellular storage sites (Fig. 1A, panels a and b). To determine subtype of purinoceptor expressed in t-BBEC 117, RT-PCR analysis was performed using primers for 16 subtypes of P2X$R_s$ and P2Y$R_s$. Transcripts of P2X1, P2X4, P2X6, P2X7, P2Y1, P2Y2, and P2Y12 were detected (Fig. 1B). Based on these results, the purinoceptor subtypes functionally expressed in t-BBEC 117 are considered to be P2Y1, P2Y2, and/or P2Y12, because P2Y$R_s$s are known to couple with G$_{q/11}$ proteins and inositol 1,4,5-trisphosphate signal transduction pathways to induce Ca$^{2+}$ release from endoplasmic reticulum. β-actin primers were used to confirm that the products generated were representative of RNA (542 bp) and not contaminated with genomic DNA (intron containing 767-bp band), because these primers were designed to span an intron as well as three exons (data not shown).

To examine further the subtypes of purinoceptor in t-BBEC 117, the potency of P2R agonists in eliciting these [Ca$^{2+}$]$_i$ responses were compared. ATP, ADP, UDP, UDP, and ATPγS at concentrations from 0.1 to 100 μM were applied consecutively. The dose-response curves are shown in Fig. 1C. The 50% effective concentrations (EC$_{50}$) were (in μM): ATP: 3.0 ± 0.7, ADP: 0.82 ± 0.03, UDP: 0.93 ± 0.05, UDP: 36.5 ± 1.67, and ATPγS: 62.6 ± 0.4. The order of the potency was: ADP = UTP > ATP > ATPγS > UDP. This pattern of results suggests the involvement of P2YR in this response (7). The contribution of P2Y1 for this Ca$^{2+}$ response was examined using ADP and
A3P5PS, because these compounds are specific agonist and antagonist for P2Y, respectively (23). The ADP-induced Ca\(^{2+}\) response was markedly suppressed by 10 \(\mu M\) A3P5PS (Fig. 1D). Taken together, these results strongly suggested that P2Y is mainly responsible for the [Ca\(^{2+}\)] response to ATP in t-BBEC 117.

Involvement of SK Channel Activation in Ca\(^{2+}\) Entry following ATP-evoked Ca\(^{2+}\) Release—It has been reported that a [Ca\(^{2+}\)] rise via metabotropic P2Y receptors can trigger additional Ca\(^{2+}\) entry in airway epithelium (8). We examined this possibility in t-BBEC 117. The ATP-induced [Ca\(^{2+}\)] response occurred in two distinct phases, an initial transient phase and a larger sustained phase (Fig. 2A, panel a). When Ca\(^{2+}\) was omitted from the extracellular solution, only the transient [Ca\(^{2+}\)] rise remained (Fig. 2A, panel b). Summarized data (Fig. 2A, panel c) are presented as the percentage of integrated [Ca\(^{2+}\)] rise in the second application of ATP versus that in the first one. Note that the second Ca\(^{2+}\) rise was significantly reduced from 58.5 ± 1.6% \((n = 106)\) to 28.9 ± 1.1% \((n = 86, p < 0.01)\), in Ca\(^{2+}\)-free solution. Next, the susceptibility of the ATP-induced [Ca\(^{2+}\)] rise to blockers of cation channels was examined (Fig. 2B, panel a). The addition of 10 \(\mu M\) lanthanum (La\(^{3+}\)) or 10 \(\mu M\) gadolinium (Gd\(^{3+}\)) markedly reduced the integrated [Ca\(^{2+}\)] signal, but 10 \(\mu M\) SK&F96365 did not change this. These results suggest that [Ca\(^{2+}\)] rise triggered by stimulation of P2Y in t-BBEC 117 consists of two components, the Ca\(^{2+}\) release mainly in the initial transient phase and the Ca\(^{2+}\) entry mainly in the sustained phase.

The Ca\(^{2+}\) entry through cation channels, presumably TRP channels, is often not voltage-dependent. Instead it is driven by membrane potential gradient (24). Accordingly a membrane hyperpolarization by activation of K\(^+\) channel can enhance Ca\(^{2+}\) entry through non-voltage-dependent cation channels (24). To examine the possibility that the Ca\(^{2+}\) rise due to the initial release activates Ca\(^{2+}\)-activated K\(^+\) \((K_{Ca})\) channels, these effects of specific blockers of K\(_{Ca}\) on the [Ca\(^{2+}\)] were examined. Iberiotoxin and TRAM-34, selective blockers of large and intermediate conductance K\(_{Ca}\) (BK and IK) channels, respectively,
were not effective (not shown). In contrast apamin, a blocker of small conductance KCa (SK) channels significantly reduced the $\left[\text{Ca}^{2+}\right]_i$ rise (Fig. 2C). Apamin significantly reduced the relative value of $\left[\text{Ca}^{2+}\right]_i$ from 58.5 ± 1.6% to 31.0 ± 1.2%. These important new findings suggest the possibility that the activation of SK2 can enhance $\text{Ca}^{2+}$ entry and form part of a positive feedback mechanism for the regulation of $\left[\text{Ca}^{2+}\right]_i$ in t-BBEC 117.

Activation of SK2 Current by Stimulation of P2YRs—To directly demonstrate that the $\left[\text{Ca}^{2+}\right]_i$ rise can activate SK channels, ATP-induced membrane currents in t-BBEC 117 were analyzed under whole cell voltage clamp. Ramp voltage clamp waveform from −120 to +60 mV (0.72 V/s) were applied once every 10 s from a holding potential of −40 mV. Fig. 3A (panel a) shows the time course of changes in membrane current ampli-
SK2 Function in Brain Endothelial Cell Proliferation

FIGURE 3. Activation of Ca\textsuperscript{2+}-activated K\textsuperscript{+} current or membrane hyperpolarization by ATP. A, panel a, effects of 10 \mu M ATP on membrane current were examined under whole cell voltage clamp in the absence and presence of 10 nm apamin. Ramp voltage clamp waveform from −120 to +60 mV was applied once every 10 s (holding potential, −40 mV). The inward current amplitude measured at −120 mV was plotted against time. b, the relationship between current density and voltage for the ATP-activated current component was obtained by subtraction of currents in the absence (i) and presence (ii) of ATP. The I-V relationships in the absence and presence of apamin were measured at the time shown in A, panel a by two pairs of black and gray dots. c, the relationship between current density and voltage of apamin-sensitive component was obtained by subtraction using the data shown in b at membrane potential from −120 mV to +60 mV, in 20-mV steps. Data were obtained from six cells. B, measurement of changes in DiBAC\textsubscript{4}(3) fluorescence in response to apamin. a, fluorescence intensity of DiBAC\textsubscript{4}(3) was measured from clusters of tBBEC 117 in the continuous presence of 100 nm DiBAC\textsubscript{4}(3) after loading for 30 min. The fluorescence intensity measured at 1 min before the application of 10 \mu M ATP was taken as 1.0. b, summarized values of DiBAC\textsubscript{4}(3) fluorescence intensity in the control, in the presence of 10 \mu M ATP, and in the simultaneous presence of 10 \mu M ATP and 100 nm apamin are illustrated from the experiments shown in a. Data were obtained from six dishes. **, p < 0.01. C, panel a, measurement of changes in DiBAC\textsubscript{4}(3) fluorescence in response to 10 \mu M DCEBIO, and addition of 10 nm apamin. b, summarized data obtained from eight dishes. *, p < 0.05.

tude at −120 mV. Application of 10 \mu M ATP transiently increased the inward current at −120 mV, and pretreatment with 10 nm apamin strikingly reduced the amplitude of this ATP-induced current. Fig. 3A (panel b) shows current-voltage (I-V) relationships of ATP-activated current component. These were obtained by subtracting the I-V relationships before the application of ATP from those after ATP application, in the absence (gray) and presence of apamin (black), respectively. The reversal potentials of ATP-activated current in the absence and presence of apamin were −28.2 ± 4.3 and −3.6 ± 2.8 mV, respectively (n = 6, p < 0.01). It is notable that the addition of apamin shifted the apparent reversal potential to the positive direction by −25 mV. The relationship between current density and voltage of the current component, which was ATP-activated and apamin-sensitive, is shown in Fig. 3A (panel c). The reversal potential was −61.5 ± 9.2 mV (n = 6), indicating the component is K\textsuperscript{+} current.

The activation of SK channels by an ATP-induced [Ca\textsuperscript{2+}], increase would be expected to result in membrane hyperpolarization. In the next series of experiments, membrane potential changes induced by ATP were measured from mass of t-BBEC 117 using voltage-sensitive fluorescent dye, DiBAC\textsubscript{4}(3). In Fig. 3B (panel a) changes in fluorescence intensity ratio to that at time 0 in t-BBEC 117 are shown. Application of 10 \mu M ATP induced a decrease in the fluorescence ratio (F/F\textsubscript{0}), which corresponds to membrane hyperpolarization, and the addition of 100 nm apamin abolished the ATP-induced hyperpolarization. Fig. 3C shows the effect of DCEBIO, an opener of SK channels. Addition of 10 \mu M DCEBIO elicited membrane hyperpolarization, and this hyperpolarization was also blocked by 10 nm apamin. In combination, results clearly indicate that SK channels are functionally expressed in t-BBEC 117, and are activated by ATP-induced [Ca\textsuperscript{2+}], rise to elicit membrane hyperpolarization.

Molecular Identification of SK2 Channel and Effects of SK2 Channel Knockdown by siRNA in t-BBEC 117—To identify the molecular basis of K\textsubscript{Ca} channels in t-BBEC 117, mRNA expression of five types of K\textsubscript{Ca} channel \alpha-subunits; BK, SK1, SK2, SK3, and IK (SK4), were examined by RT-PCR. Expression of BK and IK were barely detectable (Fig. 4A). In contrast, the expression of SK1, SK2, and SK3 could be detected unambiguously. The sequence analysis of PCR products for SK1 and SK2 revealed that the sequences of
bovine SK1 and SK2 are highly homologous to those of human and mouse, and variants with a 3-amino acid insertion were found both in SK1 and SK2 (data not shown). Those sequences were submitted to the DNA Data Bank of Japan (DDBJ), EMBL, and GenBank as the accession number AB176709 for bovine SK1 and AB114474 for bovine SK2.

We also analyzed expressions of SK1, SK2, and SK3 proteins in t-BBEC 117 by Western blot. The protein lysates obtained from HEK293 cells, which were stably expressing hSK1, hSK2, and hSK3, were used as the positive control. As shown in Fig. 4B, SK1 was faintly detected, SK2 was considerably observed, but SK3 was not detected. Molecular masses of SK1 and SK2 in t-BBEC 117 were almost the same as those of positive controls traces of [Ca\textsuperscript{2+}]\textsuperscript{i}, response to ATP in the cells transfected with siRNA-SK2 or -random. The sustained phase of [Ca\textsuperscript{2+}]\textsuperscript{i}, was markedly reduced by SK2-specific knockdown. The concentration of [Ca\textsuperscript{2+}]\textsuperscript{i}, at the time to withdrawal of ATP (arrowhead in Fig. 4D, panel a) is summarized in Fig. 4C (panel b): 144.1 ± 11.2 nM in siRNA-SK2 and 261.2 ± 25.2 nM in siRNA-random. These results indicate that SK2 channels can contribute to sustained phase of Ca\textsuperscript{2+} entry induced by ATP stimulation and that SK2 channels play a key role in the positive feedback mechanism in Ca\textsuperscript{2+} regulation.

**Contribution of SK2 Channels to the Enhancement of Cell Proliferation and \textsuperscript{3}H]Thymidine Incorporation by ATP**—In HaCaT keratinocytes, it has been reported that cell prolifera-
SK2 Function in Brain Endothelial Cell Proliferation

**FIGURE 5. Effects of inhibitor on cell proliferation and $[^3H]$thymidine incorporation promoted by ATP-$S$.** A, panel a, the proliferation of t-BBEC 117 was increased by ATP-$S$ in a time-dependent manner. Cell growth was measured with the MTT method. These values were normalized to data obtained at the start of cell culture (1.0). Data were obtained from four sample wells. *, $p < 0.05$ versus control. B, proliferation of t-BBEC 117 was increased by ATP-$S$ in a dose-dependent manner. Cells were cultivated for 48 h in the absence (control) or presence of 10, 30, and 100 $\mu$M ATP-$S$. Data were obtained from four sample wells. *, $p < 0.05$ versus control. C, effects of 10 $\mu$M La$^{3+}$, 100 $\mu$M UCL1684, and both of these agents on ATP-$S$-evoked cell proliferation measured after the treatment with ATP-$S$ for 48 h. Data were obtained from four separate wells. *, $p < 0.05$ versus control; # and ##, $p < 0.05, 0.01$ versus ATP-$S$. B, panel a, the $[^3H]$thymidine incorporation was enhanced by 100 $\mu$M ATP-$S$ in a time-dependent manner in t-BBEC 117. Data were obtained from four separate wells. *, $p < 0.05$ versus control; b, effects of La$^{3+}$, UCL1684, and both together as a mixture on ATP-$S$-induced enhancement of $[^3H]$thymidine incorporation measured after 48-h treatment. C, effects of 300 $\mu$M L-NAME and 1 $\mu$M Go6983 on ATP-$S$-induced enhancement of $[^3H]$thymidine incorporation measured after 48-h treatment. D, effects of 100 $\mu$M U0126 and 1 $\mu$M PD98059 on ATP-$S$-induced $[^3H]$thymidine incorporation measured after 48-h treatment. Data were obtained from five separate wells. ***, $p < 0.01$ versus control; # and ##, $p < 0.05$ and 0.01 versus ATP-$S$.

...tion is enhanced by the activation of metabotropic P2Y receptors and the subsequent increase in [Ca$^{2+}$], (2). In the next series of experiments, we examined whether the sustained [Ca$^{2+}$], rise in response to ATP enhances cell proliferation of t-BBEC 117. The MTT assay, based on the principle that the tetrazolium ring is cleaved in active mitochondria, was used. ATP-$S$, a non-hydrolyzed ATP analogue, UCL1684, a non-peptide SK channel blocker (26), and La$^{3+}$, a TRP channel blocker, were used to stimulate P2YRs, to block SK2, and to inhibit Ca$^{2+}$ entry, respectively. In Fig. 5A (panel a), cells were cultivated for 0, 24, 48, or 72 h in the absence or presence of 100 $\mu$M ATP-$S$, and the cell growth was expressed as relative value based on time 0 data (see “Experimental Procedures”). The cell proliferation for 24 and 48 h was significantly increased by the presence of ATP-$S$. The cell proliferation for 48, 48, and 72 h was enhanced by incubation with ATP-$S$ to 220, 190, and 170% of the control, respectively. Fig. 5A (panel b) shows that ATP-$S$ in the concentration range (10–100 $\mu$M) was applied just after the start of cell culture for 48 h. These results show that ATP-$S$ promoted cell proliferation in a dose-dependent manner (control, 1.499 ± 0.052; 10 $\mu$M ATP-$S$, 1.523 ± 0.206; 30 $\mu$M ATP-$S$, 1.586 ± 0.176; and 100 $\mu$M ATP-$S$, 1.937 ± 0.164). This enhancement of cell proliferation by 100 $\mu$M ATP-$S$ was significant. Addition of 10 $\mu$M La$^{3+}$ reduced this 100 $\mu$M ATP-$S$-induced enhancement (Fig. 5A, panel c). Addition of 100 $\mu$M UCL1684 and a mixture with 10 $\mu$M La$^{3+}$ abolished the ATP-$S$-induced enhancement (control, 1.499 ± 0.052; ATP-$S$, 1.937 ± 0.164; ATP-$S$ plus La$^{3+}$, 1.592 ± 0.069; ATP-$S$ plus UCL1684, 1.575 ± 0.073; mixture, 1.545 ± 0.079). Only UCL1684 or La$^{3+}$ without ATP-$S$ did not change the cell proliferation (data not shown).

To confirm the mitogenic effects of ATP, we tested the ability of ATP-$S$ to stimulate $[^3H]$thymidine uptake in t-BBEC 117. Application of 100 $\mu$M ATP-$S$ stimulated $[^3H]$thymidine incorporation in a time-dependent manner (Fig. 5B, panel a). The $[^3H]$thymidine incorporation for 48 and 72 h was significantly increased by the presence of ATP-$S$. Moreover, to survey the signal pathway for ATP-induced enhancement of DNA synthe-
**SK2 Function in Brain Endothelial Cell Proliferation**

sis, we used several inhibitors; 300 μM l-NAME as an eNOS inhibitor, 1 μM Go6983 as a protein kinase C inhibitor, 100 nM U0126 and 1 μM PD98059 as MAPK kinase 1/2 (MEK) inhibitors, in addition to channel blockers, 100 nM UCL1684, 10 μM La3+. As shown in Fig. 5B (panels b–d), all of them except Go6983 significantly reduced [3H]thymidine incorporation (data not shown). These results suggest that ATP-γS-stimulated [3H]thymidine incorporation was mediated through the activation of SK2, TRP channels, eNOS, MEK1/2, and MAPK in t-BBEC 117.

**DISCUSSION**

BCECs form an essential component of the BBB. They exhibit distinct characteristics that differ from peripheral endothelial cells. These include the presence of intercellular tight junctions with very low transcellular vesicular transport activity by surrounding astrocytes (17). Recently, evidence has been reported showing that several endogenous substances, including ATP, function as signal transmitters in the intercellular information transmission pathway between neurons, glial cells, and presumably BCECs (27, 28). Signals are transmitted between BCECs and astrocytes by substances such as intracellular inositol 1,4,5-trisphosphate (through gap junctions) and by extracellular ATP, which has been released into the intercellular clefts (29, 30). In t-BBEC 117, the activation of purinoreceptors resulted in a [Ca2+]i rise, due to the release from endoplasmic reticulum and a later Ca2+ influx. This second response was elicited as the consequence of the entry from store-operated Ca2+ channel (SOC), as has been reported previously from studies on human bronchial epithelial cells (8). Based on our RT-PCR analysis, the strong suppression of ATP-induced [Ca2+]i rise by pretreatment with TG, and the pharmacological characterization using agonists and antagonist, the predominant purinoreceptors in t-BBEC 117 are P2Y1 and/or P2Y2. Although P2XR transcripts were also detected by RT-PCR, their contribution to ATP-induced [Ca2+]i rise is rather unlikely based on these results.

It has been shown that ATP promotes proliferation in several types of cells (2, 3). This occurs via P2YR activation and both Ca2+ release and influx. The increase in [Ca2+]i, has been suggested to be the central signal pathway for the promotion of cell proliferation. However, the specific mechanism underlying ATP-promoted cell proliferation has not been determined. On the other hand, the evidence for a significant role of K+ channels in cell proliferation has been obtained with various types of cells, particularly in non-excitable cells. In these preparations, a membrane hyperpolarization due to K+ channel activation enhances Ca2+ influx through voltage-independent Ca2+-permeable channels (4). As an example, the activation of SK4 channels is essential for the differentiation of T-lymphocytes in immune response (31) and the proliferation of vascular smooth muscle cells under pathophysiological conditions (32, 33).

Our results clearly show that SK2 is predominantly expressed, compared with other selected K+ channels (SK1–3, IK, and BK channels) in t-BBEC 117. Although the expression of BK channels and its function in regulation of the permeability of blood-brain tumor barrier has been reported (34), expression and functions of K+ channels in BCECs has not been reported previously. The knock down of SK2 channels by siRNA markedly reduced apamin-sensitive current and decreased the sustained phase of [Ca2+]i rise induced by ATP. In contrast, this maneuver did not affect the transient phase of [Ca2+]i rise. One of the most important results in this study is that the application of 100 nM UCL1684, a stable and specific blocker of SK1–3, almost completely removed the ATP-γS-induced enhancement of proliferation (Fig. 5A) and [3H]thymidine incorporation (Fig. 5B) in t-BBEC 117. To explain the causal relationship between these two observations, we prepared the novel hypothesis that the activation of SK2 channels (due to Ca2+ release by inositol 1,4,5-trisphosphate formation via G-protein-coupled receptor stimulation) plays an obligatory role for the secondary Ca2+ influx through voltage-independent Ca2+-permeable channels. These form a novel positive feedback mechanism for ATP-induced Ca2+ influx in non-excitable cells, in which voltage-dependent Ca2+ channels are not expressed (15, 24, 37, 38). In t-BBEC 117, we detected neither voltage-dependent Ca2+ channel currents nor [Ca2+]i rise blocked by nicardipine (data not shown). Based on the RT-PCR analysis (supplemental Fig. S1), t-BBEC 117 expressed transcripts of TRPC1, TRPC3, and TRPC5. Any of these could be possible molecular candidates for SOC channels (36, 39, 40). Although TRPC5 homotetramers are activated by Gd3+ in 10 μM (41), ATP-induced [Ca2+]i rise in t-BBEC 117 was blocked by 10 μM Gd3+. In addition, we could not detect the TRPC5 protein in t-BBEC 117 (data not shown). Moreover, the lack of effects to SK&F96365 is similar to SOC in cerebral arteriole smooth mus-
ea cells, where TRPC1 is suggested to be the responsible molecule (42). In contrast, it has been shown that TRPC3 expressed in HEK293 cells is strongly blocked by 10 μM SK&F96365 (43). The possibility that TRPM7, which was also detected as a transcript in t-BBEC 117 by RT-PCR, contributes to the Ca2+ entry cannot be ruled out completely, although so far no literature suggests that TRPM7 works as the SOC (40). We suggest that the TRP channel subtype, which is responsible for Ca2+ entry during application of ATP in t-BBEC 117, is possibly the TRPC1 and/or the complex of TRPC1 and TRPC3. However, the molecular basis for the Ca2+ entry channel remains to be determined.

It has been reported that the mitogenic effect of UTP is mediated through a P2Y2 receptor that involves the activation of Ras/Raf/MEK/MAPK pathway associated with cell proliferation in cultured C6 glioma cells (44). UTP-induced MAPK activation of SK2 channels hyperpolarized the endothelial cells and hence increased the driving force for Ca2+ entry through TRPC in t-BBEC 117. The sustained [Ca2+]i is also mediated by eNOS, MEK1/2, and MAPK, whereas protein kinase C activation may not be involved in the pathway.

In conclusion, SK2 channels have an obligatory role in a novel positive feedback mechanism, which is responsible for the sustained [Ca2+]i, rise in response to P2Y1/2 activation. The activation of SK2 channels hyperpolarized the endothelial cells and hence increased the driving force for Ca2+ entry through TRPC in t-BBEC 117. The sustained [Ca2+]i is responsible for the promotion of cell proliferation by ATP. This ATP-induced feedback mechanism via SK2 activation may contribute to turnover of BCECs to maintenance of BBB function under physiological conditions.

Acknowledgments—We thank Manami Yamamoto for technical assistance and Dr. Wayne R. Giles (University of California, San Diego, CA) for providing data acquisition and analysis programs and for reading the manuscript.

REFERENCES

1. Abbott, N. J. (2002) J. Anat. 200, 629–638
2. Lee, W. K., Choi, S. W., Lee, H. R., Lee, E. J., Lee, K. H., and Kim, H. O. (2001) J. Dermatol Sci. 25, 97–105
3. Franke, H., and Illes, P. (2006) Pharmacol. Ther. 109, 297–324
4. Parekh, A. B., and Penner, R. (1997) Physiol. Rev. 77, 901–930
5. Berridge, M. J., Bootman, M. D., and Lipp, P. (1998) Nature 395, 645–648
6. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) Nat. Rev. Mol. Cell. Biol. 1, 11–21
7. King, B. F., Townsend-Nicholson, A., and Burnstock, G. (1998) Trends Pharmacol Sci. 19, 506–514
8. Bahra, P., Mesher, J., Li, S., Poll, C. T., and Danahay, H. (2004) Br. J. Pharmacol. 143, 91–98
9. Lang, F., Foller, M., Lang, K. S., Lang, P. A., Ritter, M., Gulbins, E., Vereni-

SK2 Function in Brain Endothelial Cell Proliferation

cipulated

10. Muraki, K., Imaiizumi, Y., Ohya, S., Sato, K., Takii, T., Onozaki, K., and Watanabe, M. (1997) Biochem. Biophys. Res. Commun. 236, 340–343
11. Sollini, M., Frieden, M., and Beny, J. L. (2002) Br. J. Pharmacol. 136, 1201–1209
12. Forsyth, S. E., Hoger, A., and Hoger, J. H. (1997) FEBS Lett. 409, 277–282
13. Yang, D., MacCallum, D. K., Ernst, S. A., and Hughes, B. A. (2003) Invest. Ophthalmol. Vis. Sci. 44, 3511–3519
14. Muraki, K., and Imaiizumi, Y. (2001) J. Physiol. 537, 431–441
15. Antoniotti, S., Lovisolo, D., Fiorio Pla, A., and Munaron, L. (2002) FEBS Lett. 510, 189–195
16. Hosoya, K., Ohtsuki, S., and Terasaki, T. (2002) Int. J. Neurosci. 124, 25–39
17. Abbott, N. J., Ronnback, L., and Hansson E. (2006) Nat. Neurosci. 7, 41–53
18. Sobue, K., Yamamoto, N., Yoned, K., Hodgson, M. E., Yamashiro, K., Tsuruoka, N., Tsuda, T., Katsuya, H., Miura, Y., Asai, K., and Kato, T. (1999) Neurosci. Res. 35, 155–164
19. Imaiizumi, Y., Muraki, K., and Watanabe, M. (1989) J. Physiol. 411, 131–159
20. Yamada, A., Gaja, N., Ohya, S., Muraki, K., Narita, H., Ohwada, T., and Imaiizumi, Y. (2001) Jpn. J. Pharmacol. 86, 342–350
21. Elbashir, R. M. S., Harbort, H. J., Webe, R. K., and Tusch, L. T. (2002) Methods 26, 199–213
22. Hansen, M. B., Nielsen, S. E., and Berg, K. (1989) J. Immunol. Methods 119, 203–210
23. Takasaki, J., Kamiyama, M., Saito, T., Matsumoto, M., Matsumoto, S., Ohishi, T., Soga, T., Matsuishi, H., and Furuchi, K. (2001) Mol. Pharmacol. 60, 432–439
24. Nilius, B., and Droogmans, G. (2001) Physiol. Rev. 81, 1415–1459
25. Sailer, C. A., Hu, H., Kaufmann, W. A., Trieb, M., Schwarzer, C., Storm J. F., and Knaus, H. G. (2002) J. Neurosci. 22, 9698–9707
26. Malik-Hall, M., Gannell, C. R., Galanakis, D., and Jenkinson, D. H. (2000) Br. J. Pharmacol. 129, 1431–1438
27. Haydon, P. G. (2001) Nat. Rev. Neurosci. 2, 185–193
28. Hansson, E., and Ronnback, L. (2003) FEBS Lett. 577, 341–349
29. Paemeleire, K., and Leybaert, L. (2000) J. Neurotrauma 17, 345–358
30. Berra, R., Paemeleire, K., D’Herde, K., Sanderson, M. J., and Leybaert, L. (2001) Eur. J. Neurosci. 13, 79–91
31. Ghanshani, S., Wulff, H., Miller, M. J., Rohm, H., Neben, A., Gutman, G. A., Cahalan, M. D., and Chandy, K. G. (2000) J. Biol. Chem. 275, 37137–37149
32. Cheong, A., Bingham, A. J., Li, J., Kumar, B., Sukumar, P., Munsch, C., Buckley, N. J., Neylon, C. B., Porter, K. E., Beech, D. J., and Wood, I. C. (2005) Mol. Cell. Biol. 20, 45–52
33. Srivastava, S., Li, Z., Lin, L., Liu, G., Ko, K., Coetzee, W. A., and Skolnik, E. Y. (2005) Mol. Cell. Biol. 25, 3630–3638
34. Ningaraj, N. S., Rao, M., Hashizume, K., Asotra, K., and Black, K. L. (2002) J. Pharmacol. Exp. Ther. 301, 838–851
35. Dubyak, G. R., and -moatassim, C. (1993) Am. J. Physiol. 265, C577–C606
36. Beech, D. J., Muraki, K., and Fleming, R. (2004) J. Physiol. 559, 685–706
37. Kamouchi, M., Philipp, S., Flockerzi, V., Wissenbach, U., Mamin, A., Raeye-

mackers, L., Eggermont, J., Droogmans, G., and Nilius, B. (1999) J. Physiol. 518, 345–358
38. Beech, D. J. (2005) Pflugers Arch. 451, 53–60
39. Nilius, B., Viana, F., and Droogmans, G. (1997) Annu. Rev. Physiol. 59, 145–170
40. Parekh, A. B., and Putney, J. W., Jr. (2005) Physiol. Rev. 85, 757–810
41. Xu, S. Z., Zeng, F., Boulay, G., Grimm, C., Harteneck, C., and Beech, D. J. (2005) Br. J. Pharmacol. 145, 405–414
42. Fleming, R., Xu S. Z., and Beech, D. J. (2003) Br. J. Pharmacol. 139, 955–965
43. Zhuo, X., Jiang, M., and Birnbaumer, L. (1998) J. Biol. Chem. 273, 133–142
44. Tu, M. T., Luo, S. F., Wang, C. C., Chien, C. S., Chiou, C. T., Lin, C. C., and Yang, C. M. (2000) Br. J. Pharmacol. 129, 1481–1489
45. Rojas, S., Rojas, R., Lamperti, L., Casanello, P., and Sobrevia L. (2003) Exp. Physiol. 88, 209–219