Modulation of Ca$^{2+}$ oscillation and melatonin secretion by BK$_{Ca}$ channel activity in rat pinealocytes

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Mizutani H, Yamamura H, Muramatsu M, Hagihara Y, Suzuki Y, Imaizumi Y. Modulation of Ca$^{2+}$ oscillation and melatonin secretion by BK$_{Ca}$ channel activity in rat pinealocytes. Am J Physiol Cell Physiol 310: C740–C747, 2016.—The pineal glands regulate circadian rhythm through the synthesis and secretion of melatonin. The stimulation of nicotinic acetylcholine receptor due to parasympathetic nerve activity causes an increase in intracellular Ca$^{2+}$ concentration and eventually downregulates melatonin production. Our previous report shows that rat pinealocytes have spontaneous and nicotine-induced Ca$^{2+}$ oscillations that are evoked by membrane depolarization followed by Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels (VDCCs). These Ca$^{2+}$ oscillations are supposed to contribute to the inhibitory mechanism of melatonin secretion. Here we examined the involvement of large-conductance Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$) channel conductance on the regulation of Ca$^{2+}$ oscillation and melatonin production in rat pinealocytes. Spontaneous Ca$^{2+}$ oscillations were markedly enhanced by BK$_{Ca}$ channel blockers (1 μM paxilline or 100 nM iberiotoxin). Nicotine (100 μM)-induced Ca$^{2+}$ oscillations were also augmented by paxilline. In contrast, spontaneous Ca$^{2+}$ oscillations were abolished by BK$_{Ca}$ channel opener [3 μM 12,14-dichlorodehydroabietic acid (diCl-DHAA)]. Under whole cell voltage-clamp configurations, depolarization-elicted outward currents were significantly activated by diCl-DHAA and blocked by paxilline. Expression analyses revealed that the activity of BK$_{Ca}$ channels modulated melatonin secretion from whole pineal gland of the rat. Taken together, BK$_{Ca}$ channel activation attenuates these Ca$^{2+}$ oscillations due to depolarization-synchronized Ca$^{2+}$ influx through VDCCs and results in a recovery of reduced melatonin secretion during parasympathetic nerve activity. BK$_{Ca}$ channels may play a physiological role for melatonin production via a negative-feedback mechanism.

calcium oscillation; calcium-activated potassium channel; pineal gland; parasympathetic nerve

THE PINEAL GLANDS CAN PLAY A PIVOTAL ROLE in the generation of circadian rhythm through the synthesis and secretion of melatonin. Melatonin production begins with adrenergic $\beta_1$ receptor stimulation by norepinephrine (NE), which is released from nerve endings of sympathetic axons in the superior cervical ganglia (33). The stimulation increases cAMP production and stimulation by norepinephrine (NE), which is released from Ca$^{2+}$ subunits of BK$_{Ca}$ channel were highly expressed in rat pinealocytes. Expression analyses revealed that the activity of BK$_{Ca}$ channels modulated melatonin secretion in rat pinealocytes. Spontaneous Ca$^{2+}$ oscillations were markedly enhanced by BK$_{Ca}$ channel blockers (1 μM paxilline or 100 nM iberiotoxin). Nicotine (100 μM)-induced Ca$^{2+}$ oscillations were also augmented by paxilline. In contrast, spontaneous Ca$^{2+}$ oscillations were abolished by BK$_{Ca}$ channel opener [3 μM 12,14-dichlorodehydroabietic acid (diCl-DHAA)]. Under whole cell voltage-clamp configurations, depolarization-elicted outward currents were significantly activated by diCl-DHAA and blocked by paxilline. Expression analyses revealed that the activity of BK$_{Ca}$ channels modulated melatonin secretion from whole pineal gland of the rat. Taken together, BK$_{Ca}$ channel activation attenuates these Ca$^{2+}$ oscillations due to depolarization-synchronized Ca$^{2+}$ influx through VDCCs and results in a recovery of reduced melatonin secretion during parasympathetic nerve activity. BK$_{Ca}$ channels may play a physiological role for melatonin production via a negative-feedback mechanism.

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addition to this adrenergic pathway, parasympathetic neurons, which originate from the pterygopalatine ganglia, are involved in the regulation of melatonin production (28). The stimulation of nicotinic acetylcholine receptors (nAChRs) elicits membrane depolarization, and thus induces Ca$^{2+}$ influx mediated by voltage-dependent Ca$^{2+}$ channels (VDCCs). The increase in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) enhances the release of glutamic acid from pinealocytes (37). The released glutamic acid is likely to activate metabotropic glutamate receptor type 3 (mGluR3), which couples to G$_i$ protein and reduces adenylate cyclase activity (38). The decrease in cAMP synthesis reduces AANAT activity and melatonin synthesis. Therefore, the parasympathetic innervation is considered to regulate negatively the NE-dependent melatonin synthesis in pineal glands.

Recently, we have found both spontaneous and nAChR-mediated Ca$^{2+}$ oscillations in tissue slice preparations from pineal glands and also in single pinealocytes from the rat (27). These Ca$^{2+}$ oscillations are mediated by Ca$^{2+}$ influx through VDCCs and involved in the inhibitory mechanism of melatonin secretion, which is known to be regulated by the parasympathetic activity in mammalian pineal glands. In addition to VDCCs (1, 9, 22, 40), molecular and functional expressions of voltage-dependent K$^+$ channels (1, 4, 7, 8), nonselective cation channels (12, 29), store-operated Ca$^{2+}$ channels (21), and cyclic nucleotide-gated channels (32) have been suggested in mammalian pineal glands. Furthermore, membrane currents presumably due to the large-conductance Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$; also known as K$_{Ca}$1.1) channel activation have been recorded in mammalian pinealocytes (7, 8, 21, 22). However, the molecular basis of pineal BK$_{Ca}$ channel and its physiological significances remain to be fully elucidated.

The present study was undertaken to elucidate the regulation of Ca$^{2+}$ oscillation and melatonin secretion by the activity of BK$_{Ca}$ channels during parasympathetic activation in mammalian pineal glands, using Ca$^{2+}$ imaging techniques, electrophysiological recordings, expression analyses, and melatonin assay. Here we report that spontaneous and nicotine-evoked Ca$^{2+}$ oscillations in rat pinealocytes are modulated by the BK$_{Ca}$ channel conductance. Moreover, melatonin secretion in rat pineal glands is also affected by the BK$_{Ca}$ channel activity.

MATERIALS AND METHODS

Ethical approval. All experiments were approved by the Ethics Committee of Nagoya City University, Japan, and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Japanese Pharmacological Society.

Cell culture. The pineal glands were removed from male Wistar/ST rats (6–9 wk) and then incubated in PBS containing 0.1% collagenase.

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(Wako Pure Chemical Industries, Osaka, Japan) and 0.02% trypsin (Type I: Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C (27). The dispersed pinealocytes were cultured on coverslips coated with 5 μg/ml poly-l-lysine (Sigma-Aldrich) in DMEM supplemented with 10% heat-inactivated FBS (Invitrogen/Gibco, Carlsbad, CA), 20 U/ml penicillin, and 20 μg/ml streptomycin (Wako Pure Chemical Industries). Experiments were performed at 24–96 h after cell culture.

**Ca**$^{2+}$ imaging. [Ca$^{2+}$]$_{i}$ measurements were performed using an Argus/HISCAM imaging system (Hamamatsu Photonics, Hamamatsu, Japan). Single pinealocytes were loaded with 10 μM fura-2 acetoxymethyl ester (Invitrogen/Molecular Probes, Eugene, OR) for 40 min at room temperature (23 ± 2°C). The HEPES-buffered solution had an ionic composition of 137 mM NaCl, 5.9 mM KCl, 2.2 mM CaCl$_{2}$, 1.2 mM MgCl$_{2}$, 14 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with 10 N NaOH. Ca$^{2+}$ fluorescence ratios in the absence of and with saturation of Ca$^{2+}$ were calculated using the formula: 

$$
\frac{[\text{Ca}^{2+}_{i}]}{[\text{Ca}^{2+}_{i} + \text{Ca}^{2+}_0]} = \frac{K_d \text{R(min)} + R_{0}}{R_{0} + \text{R(max)}}
$$

where $K_d$ is the dissociation constant of fura-2 (224 nM), $R$ is the fluorescence ratio ($F_{135} - F_{380}$), and $R_{0}$ and $R_{\text{max}}$ are the fluorescence ratios in the absence of and with saturation of Ca$^{2+}$, respectively.

**Electrophysiological recording.** Electrophysiological studies were performed using a whole cell voltage-clamp technique with a CEZ-2400 amplifier (Nihon Kohden, Tokyo, Japan), an analog-digital converter (Digitida 1440A), and pCLAMP software (version 10; Molecular Devices/Axon Instruments, Foster City, CA) (15, 39). The HEPES-buffered solution was used as an extracellular recording solution. The pipette solution for whole cell currents had the following ionic composition: 137 mM KCl, 2.8 mM MgCl$_{2}$, 14 mM glucose, 10 mM HEPES, 4.2 mM CaCl$_{2}$, and 5 mM MgCl$_{2}$. The pH was adjusted to 7.2 with 1 N KOH. When BKCa currents were recorded using the pipette solution (pCa 6.0), 50 μM CdCl$_{2}$ was added to the extracellular solution. The pipette solution (pCa 6.0) contained 140 mM KCl, 2.8 mM MgCl$_{2}$, 2 mM ATP$_{Na2}$, 10 mM HEPES, 4.2 mM CaCl$_{2}$, and 5 mM MgCl$_{2}$. The pH was adjusted to 7.2 with 1 N KOH.

**Quantitative real-time PCR.** The total RNA extraction from homogenates of rat pineal glands, the RT method, and quantitative real-time PCR analysis were performed as reported previously (27). Specific primers for rat BKCa genes were designed as follows: a (GenBank Accession number NM_019273), (+) AGA GAC ATC CTG CCA GA, (−) GCA ATA AAT CGC AAG CCA AA; b (NM_019273), (+) AGA AGA CAC TCG GGA TCA AA, (−) GAA ATT GGC TCT GAC CTT CTC; b (NM_178661), (+) AAG AGC GTC ATC CTG ACC AAA, (−) GTT TCA CCA TAG CAA CGA TTG C; c (NM_001104560), (+) CCT AGC AAT TCG GGA CAG AAA G, (−) ACC TCT GCC TCC GTG ACA TGT G; d (NM_023960), (+) ATG GGT TCC CAG CCA TTC A, (−) GAA GCA GTG CAG GAG AAT; e (NM_031144), (+) AGG CCA ACC GTG AAA AGA TG, (−) ACC AGA GCC ATA CAG GGA CA.

**Immunocytochemistry.** Immunocytochemical staining was performed as reported previously (27). In brief, pinealocytes were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. These pinealocytes were treated with primary antibody [1:100 dilution; anti-BK(α) (APC-107), anti-BKβ1 (APC-036), anti-BKβ2 (APC-034), and anti-BKβ4 (APC-061), Alomone Lab, Jerusalem, Israel; and anti-BKβ3 (H00027094-A01), Abnova, Taipei, Taiwan] for 12 h at 4°C, and then covered with Alexa Fluor 488-labeled secondary antibody solution (1:1,000 dilution; Invitrogen/Molecular Probes) for 1 h at room temperature. Confocal images were obtained using a laser scanning confocal microscope (A1R; Nikon, Tokyo, Japan).

**Melatonin secretion assay.** Freshly dissected pineal glands were incubated for 1 h at 37°C and then exposed to 1 μM NE or vehicle control (2 h). Test compounds were added at the beginning of incubation prior to NE addition. The amount of melatonin secreted from the whole pineal gland was quantitatively determined using a melatonin ELISA kit (IBL International, Hamburg, Germany) and then normalized by that of NE-induced melatonin release (100%).

**Drugs.** Pharmacological reagents were obtained from Sigma-Aldrich except for 4-aminoypyridine, CdCl$_{2}$ (Wako Pure Chemical Industries), 12,14-dichlorohydroxyacetic acid (diCl-DHAA; Helix Biotech, New Westminster, Canada), EGTA, HEPES (Dojin, Kumamoto, Japan), iberiotoxin (IbTx; Peptide Institute, Osaka, Japan), nicotine, and tetraethylammonium (TEA) chloride (Tokyo Chemical Industry, Tokyo, Japan). All hydrophobic compounds were dissolved in DMSO at a concentration of 1 or 3 mM as a stock solution.

**Statistics.** All pooled data are shown as means ± SE with the numbers of cells (n) or glands (N) examined. Statistical significance between two groups was determined by Student’s t-test. Statistical significance among groups was determined by Scheffé’s test after one-way ANOVA or Wilcoxon’s rank sum test (U-test) after the nonparametric Kruskal-Wallis test. Significant differences are expressed as P < 0.05 or P < 0.01.

**RESULTS**

**Enhanced Ca$^{2+}$ oscillations by BKCa channel blockers in rat pinealocytes.** When resting [Ca$^{2+}$]$_{i}$ in normal bath solution was measured in primary cultured rat pinealocytes, a subset of cells (15–20%) showed spontaneous and oscillatory [Ca$^{2+}$]$_{i}$ changes (Figs. 1 and 2), as reported previously (27). The amplitude (100–500 nM) and frequency (0.3–1.0 min$^{-1}$) of spontaneous Ca$^{2+}$ oscillations varied somewhat among cells. Spontaneous Ca$^{2+}$ events usually continued for >30 min during [Ca$^{2+}$]$_{i}$ measurements.

The involvement of BKCa channel activity in the regulation of spontaneous Ca$^{2+}$ oscillations was examined in rat pinealocytes. The application of 1 μM paxilline, a selective BKCa channel blocker (13, 18), significantly increased the amplitude of spontaneous Ca$^{2+}$ oscillations from 167 ± 35 to 464 ± 67 nM (n = 15, P < 0.01; Fig. 1, A and B). Application of another BKCa channel blocker, 100 nM IbTx (13, 18), showed the same effect (211 ± 35 to 576 ± 52 nM, n = 5, P < 0.01; Fig. 1, D and E) and frequency of spontaneous Ca$^{2+}$ oscillations was slightly but not significantly increased in the presence of paxilline (0.36 ± 0.07 to 0.47 ± 0.06 min$^{-1}$, n = 15, P > 0.05; Fig. 1, A and C) or IbTx (0.43 ± 0.04 to 0.53 ± 0.08 min$^{-1}$, n = 5, P > 0.05; Fig. 1, D and F). These findings indicated that the mechanism underlying the generation of spontaneous Ca$^{2+}$ oscillations was modulated by the activity of BKCa channels in rat pinealocytes.

**Attenuated Ca$^{2+}$ oscillations by BKCa channel opener in rat pinealocytes.** In addition to BKCa channel blockers, effects of a BKCa channel opener, diCl-DHAA (31), on spontaneous Ca$^{2+}$ oscillations were also examined in rat pinealocytes. The addition of 3 μM diCl-DHAA markedly reduced the amplitude (461 ± 76 to 159 ± 63 nM, n = 5, P < 0.05; Fig. 2, A and B) and frequency (0.70 ± 0.06 to 0.36 ± 0.05 min$^{-1}$, n = 5, P < 0.05; Fig. 2, A and C) of spontaneous Ca$^{2+}$ oscillations. The attenuation by diCl-DHAA was partly removed by its withdrawal. The inhibitory effects by 0.1 μM diCl-DHAA on the amplitude of spontaneous Ca$^{2+}$ oscillations (from 182 ± 26 to 80 ± 12 nM, n = 6, P < 0.05) were reversed by the addition of 1 μM paxilline (to 511 ± 96 nM, n = 6, P < 0.01 vs. diCl-DHAA alone; Fig. 2, D–F). These results suggested that the activation of BKCa channel conductance decreased VDCC-mediated Ca$^{2+}$ influx, presumably due to membrane hyperpolarization in rat pinealocytes.
Depolarization-evoked outward currents were significantly reduced in rat pinealocytes. Effects of BKCa channel blockers on spontaneous Ca2+ oscillations were examined in rat pinealocytes. A: representative recording of changes in Ca2+ oscillations before and after the application of 1 µM paxilline (Pax). B and C: amplitude (B) and frequency (C) of spontaneous Ca2+ oscillations in the presence of paxilline (n = 15). D: spontaneous Ca2+ oscillations in the absence and presence of 100 nM iberiotoxin (IbTx). E: amplitude (E) and frequency (F) of spontaneous Ca2+ oscillations after the application of IbTx (n = 5). **P < 0.01, statistical significance vs. control (Cont). [Ca2+]i, intracellular Ca2+ concentration.

**Effect of BKCa channel blocker on nicotine-induced Ca2+ oscillations in rat pinealocytes.** In some pinealocytes (20–25%), Ca2+ oscillations were observed following a short application of nicotine, which induced a transient Ca2+ rise, as reported previously (27). Therefore, effect of BKCa channel blockade on cytosolic Ca2+ mobilization during stimulation of nAChRs was examined in rat pinealocytes. The application of 100 µM nicotine caused a transient increase in [Ca2+]i in pinealocytes (by 2,575 ± 48 nM, n = 7; Fig. 3A). After withdrawal of nicotine, Ca2+ oscillations were observed in some pinealocytes. The amplitude of nicotine-induced Ca2+ oscillations was markedly enhanced by 1 µM paxilline (152 ± 43 to 742 ± 220 nM, n = 7, P < 0.05; Fig. 3, A and B). There was no significant change in frequency in the presence of paxilline (0.36 ± 0.12 to 0.46 ± 0.17 min−1, n = 7, P > 0.05; Fig. 3, A and C). These observations suggest that the pharmacological properties of nicotine-induced Ca2+ oscillations are similar to those of spontaneous Ca2+ oscillations.

**BKCa channel currents in rat pinealocytes.** Next, the function of BKCa channels in rat pinealocytes was examined by the whole cell patch-clamp technique. When single pinealocytes were depolarized from a holding potential of −40 to +40 mV in 10-mV steps for 150 ms every 15 s, using normal pipette solution containing 0.05 mM EGTA, outward currents were elicited at test potentials positive to −10 mV (Fig. 4, A–D). Depolarization-evoked outward currents were significantly reduced by a nonspecific BKCa channel blocker, 1 mM TEA (11) (101 ± 7 to 42 ± 7 pA/pF at +40 mV, n = 5, P < 0.01; Fig. 4, A and B). The outward currents were also decreased by 1 µM paxilline (86 ± 13 to 47 ± 12 pA/pF at +40 mV, n = 4, P < 0.01; Fig. 4, C and D). The paxilline-insensitive component was reduced by further addition of nonselective blockers of voltage-dependent K+ channels, 10 mM TEA, or 3 mM 4-aminopyrididine (18) (to ~20% of control, n = 3–5). Thus, paxilline-sensitive BKCa current was apparently the major component of outward currents upon depolarization in rat pinealocytes.

To analyze BKCa channel currents in detail, the pCa in the pipette solution was set at 6.0 with Ca2+/EGTA, andVDCCs were blocked by 50 µM Cd2+ in the external solution. Single pinealocytes were depolarized from a holding potential of −40 to +40 mV in 10-mV steps for 150 ms every 15 s (Fig. 4, E and F). Depolarization-evoked outward currents were significantly enhanced by 3 µM diCl-DHAA (100 ± 13 to 161 ± 10 pA/pF at +40 mV, n = 4, P < 0.05). Further addition of 1 µM paxilline reduced the outward currents to ~85% of the control (to 84 ± 4 pA/pF at +40 mV, n = 4, P < 0.05 vs. diCl-DHAA alone). These results strongly suggested that rat pinealocytes functionally expressed the BKCa channels that modulate spontaneous and nicotine-induced Ca2+ oscillations.

**Molecular basis of BKCa channels in rat pinealocytes.** BKCa channels are constituted from tetrameric sets of pore-forming α subunits and auxiliary β subunits in a reciprocal manner (3, 11, 35). Therefore, the molecular components of BKCa channels in rat pinealocytes were identified by quantitative real-time PCR and immunocytochemical analyses. Quantitative real-time PCR analysis showed the substantial expression of the α transcript (0.050 ± 0.005 ratio to β-actin, N = 5) in rat pineal...
glands (Fig. 5A). Among accessory subunits, mRNA expression of the β3 subunit (0.021 ± 0.002, N = 4) was clearly detected, but the β1, β2, and β4 subunits were not (<0.002, N = 3–4) in rat pineal glands. The expression of BKCa channel proteins in rat pinealocytes was confirmed by an immunocytochemical approach using subunit-specific polyclonal antibodies. Immunoreactivities to the α and β3 proteins were clearly observed at the plasma membrane of all pinealocytes examined (5 and 8 cells, respectively), but those of other subunits were not (β1, 1 of 10 cells; β2, all negative of 5 cells; β4, all negative of 6 cells; Fig. 5B). These findings strongly suggested that the BKCa channels in rat pinealocytes potentially consisted of the α and β3 subunits.

Modulation of melatonin secretion by BKCa channel activity. Melatonin secretion from rat pineal glands by neurotransmitters and its modulation by the activity of BKCa channels were quantitatively analyzed using a melatonin ELISA kit. Melatonin secretion from whole pineal glands was observed by treatment with 1 μM 12,14-dichlorodehydroabi-etic acid (diCl-DHAA) blocked spontaneous Ca2+ oscillations after application of diCl-DHAA (n = 5). D: inhibitory effect of 0.1 μM diCl-DHAA on spontaneous Ca2+ oscillations was removed by addition of 1 μM paxilline. E and F: amplitude (E) and frequency (F) of spontaneous Ca2+ oscillations after coapplication of diCl-DHAA and paxilline (n = 6). *P < 0.05 or **P < 0.01, statistical significance vs. control or diCl-DHAA, respectively.

Fig. 2. Attenuation of spontaneous Ca2+ oscillations by BKCa channel opener in rat pinealocytes. Effects of BKCa channel opener on spontaneous Ca2+ oscillations were examined in rat pinealocytes. A: application of 3 μM diCl-DHAA (diCl-DHAA) blocked spontaneous Ca2+ oscillations. B and C: amplitude (B) and frequency (C) of spontaneous Ca2+ oscillations after application of diCl-DHAA (n = 5). D: inhibitory effect of 0.1 μM diCl-DHAA on spontaneous Ca2+ oscillations was removed by addition of 1 μM paxilline. E and F: amplitude (E) and frequency (F) of spontaneous Ca2+ oscillations after coapplication of diCl-DHAA and paxilline (n = 6). *P < 0.05 or **P < 0.01, statistical significance vs. control or diCl-DHAA, respectively.

Fig. 3. Effect of BKCa channel blocker on Ca2+ oscillations induced by nicotinic acetylcholine receptor (nAChR) stimulation in rat pinealocytes. Effect of BKCa channel blockade on cytosolic Ca2+ mobilization following nAChR stimulation was examined in rat pinealocytes. A: Ca2+ oscillations were often observed following the short application of 100 μM nicotine, which induced a transient [Ca2+]i rise. Effects of 1 μM paxilline on nicotine-induced Ca2+ oscillations are shown in a representative recording. B and C: enhancing effects of paxilline on the amplitude (B) and frequency (C) of nicotine-induced Ca2+ oscillations (n = 7). *P < 0.05, statistical significance vs. control.
Fig. 4. BK$_{Ca}$ channel currents in rat pinealocytes. Electrophysiological and pharmacological characteristics of BK$_{Ca}$ channel currents in rat pinealocytes were analyzed using the whole cell patch-clamp technique. Depolarizing pulses of 150 ms were applied from a holding potential of $-40$ to $+40$ mV in 10-mV increments every 15 s. A: current traces before and after the application of 1 mM tetraethylammonium (TEA). B: current-voltage relationships under control conditions and in the presence of TEA ($n = 5$). C: current recordings in the absence and presence of 1 μM paxilline. D: current-voltage relationships before and after the application of paxilline ($n = 4$). E: when the pCa of the recording pipette solution was fixed at 6.0 with Ca$^{2+}$/EGTA, outward currents were activated by application of 3 μM diCl-DHAA and blocked by further addition of 1 μM paxilline. F: current-voltage relationships under control conditions, in presence of diCl-DHAA, and in presence of diCl-DHAA plus paxilline ($n = 4$). *$P < 0.05$ or **$P < 0.01$, statistical significance.

(100%; Fig. 6). Melatonin secretion evoked by 1 μM NE was reduced by $\sim 50\%$ in the copresence of 100 μM ACh (to 50.5 $\pm$ 5.3%, $N = 13$, $P < 0.01$ vs. NE). The suppression of NE-induced melatonin release by ACh was not significantly affected by 1 μM paxilline (53.0 $\pm$ 5.3%, $N = 11$, $P > 0.05$ vs. NE + ACh). Interestingly, ACh-induced inhibition of NE-induced melatonin secretion was significantly recovered by 3 μM diCl-DHAA (84.9 $\pm$ 9.8%, $N = 12$, $P < 0.01$ vs. NE + ACh). The recovery effect of diCl-DHAA was partly antagonized by the further addition of 1 μM paxilline (60.2 $\pm$ 5.1%, $N = 12$, $P > 0.05$ vs. NE + ACh + diCl-DHAA by Wilcoxon’s rank sum test but $P = 0.036$ by Student’s $t$-test). These data suggested that this inhibitory effect of ACh on NE-evoked melatonin secretion in rat pineal glands was modulated by the activity of BK$_{Ca}$ channels.

DISCUSSION

Our previous report showed that rat pinealocytes have spontaneous Ca$^{2+}$ oscillations evoked by VDCC-mediated Ca$^{2+}$ spikes following membrane depolarization (27). Spontaneous Ca$^{2+}$ oscillations are supposed to contribute to the inhibitory mechanism of melatonin secretion due to parasympathetic nerve activity. The present data show that the α and β3 subunits of BK$_{Ca}$ channels are highly expressed in rat pinealocytes and that the BK$_{Ca}$ channel activity strongly modulates spontaneous Ca$^{2+}$ oscillations and thereby, more importantly, regulates melatonin production.

The regulation of melatonin production in pinealocytes depends on a balance of activity due to sympathetic and parasympathetic neuronal activities (28, 33). The inhibitory mechanism of melatonin production driven by nAChR stimulation via parasympathetic activity is considered to be closely related to the rise of [Ca$^{2+}$], in mammalian pinealocytes (37, 38). However, it is not well understood how ion channels contribute to cellular Ca$^{2+}$ mobilization for circadian regulation in mammalian pineal glands (19). Our previous work demonstrated spontaneous and nAChR-mediated Ca$^{2+}$ oscillations in tissue slice preparations and single pinealocytes of the rat (27). These Ca$^{2+}$ oscillations are triggered by synchronized periodic membrane depolarizations, and thus induced Ca$^{2+}$ influx mediated by VDCCs (mainly α1F subunit). In addition to nifedipine-sensitive VDCCs in pinealocytes, the involvement of BK$_{Ca}$ channels in the regulation of Ca$^{2+}$ oscillations and the subsequent inhibition of melatonin production were clarified in this study. The parameters of spontaneous and nicotine-induced Ca$^{2+}$ oscillations mediated by Ca$^{2+}$ influx through VDCCs (27) were markedly changed by BK$_{Ca}$ channel openers and blockers. Furthermore, the activity of BK$_{Ca}$ channels completely modified the ACh-induced inhibition of melatonin synthesis by NE, which is known to be regulated by parasympathetic activities in mammalian pineal glands (28). In other types of mammalian glands, the specific role of BK$_{Ca}$ channels on the regulation of exocrine functions has been reported, for example, in salivary glands (6), pituitary glands (34), parathyroid glands (5), lacrimal glands (24), airway submucosal glands (30), and adrenal medulla (10). Based on our present results, pineal BK$_{Ca}$ channels play an obligatory role in the regulation of melatonin secretion.
BK<sub>Ca</sub> channels are highly expressed in excitable cells including neurons, secretory cells, and smooth muscle cells (3, 11). BK<sub>Ca</sub> channels contribute to the regulation of membrane excitability including the suppression of action potential firing and the formation of the action potential repolarizing phase (3). The activity of BK<sub>Ca</sub> channels is triggered by both membrane depolarization and elevated cytosolic Ca<sup>2+</sup>. Membrane hyperpolarization following BK<sub>Ca</sub> channel activation protects cells from excessive excitability and Ca<sup>2+</sup> overload by closing VDCCs (11). Therefore, the BK<sub>Ca</sub> channel is considered a key molecule in the negative-feedback mechanism in [Ca<sup>2+</sup>]<sub>i</sub> regulation (16, 17). Similarly, in pineal glands, activation of the BK<sub>Ca</sub> channel is likely to cause a membrane hyperpolarization and, following a decrease in Ca<sup>2+</sup> influx, to result in the reduction of [Ca<sup>2+</sup>]<sub>i</sub> oscillation parameters and, finally, the recovery of melatonin synthesis. Therefore, pineal BK<sub>Ca</sub> channels may form a negative-feedback mechanism in cytosolic Ca<sup>2+</sup> mobilization during parasympathetic innervation.

The BK<sub>Ca</sub> channel is a tetrameric assembly of pore-forming α subunits with four β subunits (3, 11, 35). The expression pattern of the α subunit is ubiquitous in excitable cells, such as neurons, secretory glands, and smooth muscles, except in the heart. The auxiliary β subunit (β1, β2, β3, and β4) binds with the α subunit in a one-to-one manner and modulates Ca<sup>2+</sup> sensitivity, voltage dependence, and pharmacological characteristics. It has been revealed that genetic deficiency of the α subunits in mice significantly disrupts the regulation of circadian behavioral rhythms (26). The β subunit shows tissue-specific distribution and is responsible for the tissue-specific variation of BK<sub>Ca</sub> channel characteristics (2). Expression anal-

**Fig. 5.** Molecular identification of BK<sub>Ca</sub> channels in rat pinealocytes. Expression pattern for BK<sub>Ca</sub> channels was obtained from rat pinealocytes using quantitative real-time PCR and immunocytochemical techniques. A: expression of mRNA encoding BK<sub>Ca</sub> channel subunits in rat pineal glands was measured using the quantitative real-time PCR method. Expression levels of BK<sub>Ca</sub> channel subunits were normalized by that of endogenous β-actin. Expression of α and β3 genes was clearly identified but β1, β2, and β4 subunits were not. Data were obtained from 3–5 independent experiments. B: immunoreactivity of BK<sub>Ca</sub> channel subunit proteins in rat pinealocytes is shown using specific antibodies. Specific signals of the α and β3 subunits were detected on the plasma membrane in rat pinealocytes. Data were obtained from 5–10 cells.

**Fig. 6.** Contribution of BK<sub>Ca</sub> channels to the regulation of melatonin secretion by neurotransmitters. Effects of ACh and BK<sub>Ca</sub> channel modulators on melatonin secretion elicited by 1 μM norepinephrine (NE) were examined in freshly dissected rat pineal glands. Measurement of melatonin secretion from 1 pineal gland was quantitatively determined using a melatonin ELISA kit and then normalized by that of NE-induced melatonin release (100%). The NE (1 μM)-induced melatonin secretion was reduced by 100 μM ACh. Opening of BK<sub>Ca</sub> channels by 3 μM diCl-DHAA clearly reduced inhibition of NE-induced melatonin secretion by ACh. This effect of diCl-DHAA was partly compensated for by addition of 1 μM paxilline. Data were obtained from 7–28 pineal glands. Statistical significance: **p < 0.01 vs. vehicle, ***p < 0.01 vs. NE alone, or $^p < 0.01$ vs. NE + ACh.

**Fig. 7.** Diagram of mechanism underlying melatonin production modulated by activities of voltage-dependent Ca<sup>2+</sup> channel (VDCC) and BK<sub>Ca</sub> channel (BK) in pinealocytes. In pinealocytes, ACh is released from nerve terminal of parasympathetic axons in the pterygopalatine ganglion (PPG) and activates nAChRs (α3β4 subunits). Stimulation of nAChRs causes a membrane depolarization (Depo.) followed by Ca<sup>2+</sup> influx (Ca<sup>2+</sup> oscillation) through VDCCs (mainly α1F subunit). [Ca<sup>2+</sup>]<sub>i</sub> increase is likely to elicit the release of glutamic acid (L-glutamate; Glu) from pinealocytes. The released glutamic acid is supposed to activate G<sub>i</sub> protein-coupled metabotropic glutamate receptor type 3 (mGluR3), and results in inhibitory regulation of melatonin synthesis. Therefore, parasympathetic innervation is considered to regulate negatively the NE-dependent melatonin synthesis in pineal glands. Activation of BK<sub>Ca</sub> channels (α and β3 subunits) controls negatively the activity of VDCCs and thus induces recovery of melatonin production.
yses at the mRNA and protein levels in the present study revealed that the BKCa channel was predominantly composed of a combination of α and β3 subunits. It is known that the β3 subunits mainly distribute in the spleen, placenta, pancreas, testis, and prostate, but that the β4 subunits are predominant in neuronal tissues (2, 11). Some reports, however, indicate that β subunits other than β4 are also localized in the brain and may contribute to these neuronal functions (2, 20, 23, 36). One of the characteristic modulations of BKCa channel currents by β4 subunits is a marked reduction of sensitivity to IbTx and charybdotoxin (25). In the present study, spontaneous Ca2+ oscillations were highly susceptible to IbTx. In addition, electrophysiological results suggested that BKCa channel currents in rat pinealocytes are completely blocked by IbTx and charybdotoxin (7, 8, 21, 22). Therefore, β3 rather than β4 is the predominant β subunit of BKCa channel functionally expressed in rat pineal glands.

One of the most important findings in this study is that melatonin secretion from whole pineal gland following nAChR stimulation is strongly modulated by the activity of BKCa channels. The parasympathetic signals mediated by nAChR is considered to regulate negatively the NE-dependent melatonin synthesis due to sympathetic activity in pineal glands (28). The negative-feedback model for melatonin secretion is summarized in Fig. 7. Spontaneous and nAChR (α3β4)-mediated Ca2+ oscillations in pinealocytes are triggered by synchronized periodic membrane depolarizations, and thus induce Ca2+-influx though VDCCs (mainly α1F subunits). The VDCC-mediated [Ca2+]i rise is supposed to elicit the release of glutamic acid from pinealocytes. Consequently, the released glutamate stimulates mGluR3, by both autocrine and paracrine mechanisms, and thus suppresses adenylyl cyclase via Gi protein activation (37, 38). Finally, the activation of this pathway leads to the reduction of melatonin secretion. Because the BKCa channel opener abolished the suppression of NE-induced melatonin secretion by ACh, it is strongly suggested that membrane hyperpolarization via the activation of BKCa channels (α and β3 subunits) is essential for this inhibitory regulation of melatonin secretion following nAChR stimulation. The finding that nAChR-induced Ca2+ oscillation was strongly suppressed by the BKCa channel opener supports the hypothesis that Ca2+ oscillations are involved in glutatione release.

In conclusion, we found that spontaneous and nAChR-mediated Ca2+ oscillations were completely modified by the activity of the BKCa channel that was composed of α and β3 subunits. Changes in membrane potential modulated by BKCa channel activity are involved in the inhibitory mechanism of melatonin secretion, which is known to be regulated by parasympathetic activities in mammalian pineal glands.

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DISCLOSURES

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