Spontaneous and nicotine-induced Ca\(^{2+}\) oscillations mediated by Ca\(^{2+}\) influx in rat pinealocytes

Hiroya Mizutani,1 Hisao Yamamura,1 Makoto Muramatsu,1 Keiko Kiyota,1 Kaori Nishimura,1 Yoshiaki Suzuki,1 Susumu Ohya,1,2 and Yuji Imaizumi1

1Department of Molecular and Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan; and 2Department of Pharmacology, Division of Pathological Sciences, Kyoto Pharmaceutical University, Kyoto, Japan

Submitted 13 January 2014; accepted in final form 31 March 2014

β\(_1\)-Adrenergic receptor stimulation in pinealocytes increases cAMP production and activates arylalkylamine-N-acetyltransferase (AANAT), a melatonin-synthesizing enzyme. cAMP production evoked by β\(_1\)-adrenergic receptor stimulation is thought to be enhanced synergistically by the elevation of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). [Ca\(^{2+}\)]\(_i\) is modulated by inositol 1,4,5-trisphosphate (IP\(_3\))-mediated Ca\(^{2+}\) release via adrenergic α\(_1\)-receptor activation by NE in pineal glands (32).

In addition to this adrenergic pathway, parasympathetic neurons, which originate from the pterygopalatine ganglia, are involved in regulation of melatonin production in pinealocytes (23, 28). The stimulation of nicotinic acetylcholine receptors (nAChRs) elicits membrane depolarization, and this induces Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels (VDCCs) (22). The resulting function as a Ca\(^{2+}\) channel

pineal gland; calcium oscillation; melatonin; nicotinic acetylcholine receptor; voltage-dependent calcium channel

THE PINEAL GLAND is a melatonin-secreting organ. It can play a pivotal role in the generation of circadian rhythm through the synthesis and secretion of melatonin. Melatonin production is strongly regulated melatonin synthesis in pinealocytes. To our knowledge, this is the first observation of such Ca\(^{2+}\) oscillations in pineal glands of lower vertebrates (7, 20, 25). The contribution of Ca\(^{2+}\) influx through VDCCs to the initiation and regulation of Ca\(^{2+}\) oscillations and the synchronization with periodic membrane depolarization was studied in tissue slice preparations from pineal glands and also in primary-cultured single pinealocytes from the rat.

Address for reprint requests and other correspondence: Y. Imaizumi, Dept. of Molecular and Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City Univ., 3-1 Tanabedori Mizuhoku, Nagoya 467-8603, Japan (e-mail: yimaizum@phar.nagoya-cu.ac.jp).

C1008 0363-6143/14 Copyright © 2014 the American Physiological Society http://www.ajpcell.org

First published April 2, 2014; doi:10.1152/ajpcell.00014.2014

Cellular Circadian Rhythms

First published April 2, 2014; doi:10.1152/ajpcell.00014.2014.—The pineal gland regulates circadian rhythm through the synthesis and secretion of melatonin. The rise of intracellular Ca\(^{2+}\) due to nAChR stimulation remain to be clarified. We report here that spontaneous Ca\(^{2+}\) oscillations can be observed in ∼15% of the pinealocytes in slice preparations from rat pineal glands when this dissociation procedure is done within 6 h from a dark-to-light change. The frequency and half-life of [Ca\(^{2+}\)]\(_i\) rise were 0.86 min\(^{-1}\) and 19 s, respectively. Similar spontaneous Ca\(^{2+}\) oscillations were recorded in 17% of rat pinealocytes that were primary cultured for several days. Simultaneous measurement of [Ca\(^{2+}\)]\(_i\) and membrane potential revealed that spontaneous Ca\(^{2+}\) oscillations were triggered by periodic membrane depolarizations. Spontaneous Ca\(^{2+}\) oscillations in cultured pinealocytes were abolished by extracellular Ca\(^{2+}\) removal or application of nifedipine, a blocker of voltage-dependent Ca\(^{2+}\) channel (VDCC). In contrast, blockers of intracellular Ca\(^{2+}\)-release channels, 2-aminoethoxydiphenylborate and ryanodine, have no effect. Our inclusion, both spontaneous and evoked Ca\(^{2+}\) oscillations are due to membrane depolarization following activation of VDCCs. This consists of VDCC α\(_1F\) subunit, and the associated Ca\(^{2+}\) influx can strongly regulate melatonin secretion in pineal glands.

THE PINEAL GLAND is a melatonin-secreting organ. It can play a pivotal role in the generation of circadian rhythm through the synthesis and secretion of melatonin. Melatonin production is considered to be negatively controlled by cAMP production and activates arylalkylamine-N-acetyltransferase (AANAT), a melatonin-synthesizing enzyme. cAMP production evoked by β\(_1\)-adrenergic receptor stimulation is thought to be enhanced synergistically by the elevation of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). [Ca\(^{2+}\)]\(_i\) is modulated by inositol 1,4,5-trisphosphate (IP\(_3\))-mediated Ca\(^{2+}\) release via adrenergic α\(_1\)-receptor activation by NE in pineal glands (32).

In addition to this adrenergic pathway, parasympathetic neurons, which originate from the pterygopalatine ganglia, are involved in regulation of melatonin production in pinealocytes (23, 28). The stimulation of nicotinic acetylcholine receptors (nAChRs) elicits membrane depolarization, and this induces Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels (VDCCs) (22). The resulting function as a Ca\(^{2+}\) channel mediator of Ca\(^{2+}\) influx through VDCCs to the initiation and regulation of Ca\(^{2+}\) oscillations in rat pinealocytes.
MATERIALS AND METHODS

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

Slice preparation. The pineal glands were removed from male Wistar/ST rats (6–9 wk) and were placed in ice-cold oxygenated solution containing 3 mM KCl, 1 mM CaCl2, 5 mM MgCl2, 25 mM NaHCO3, 1 mM Na2HPO4, 11 mM glucose, and 212.5 mM sucrose (pH 7.35). Coronal slices (150 μm in thickness) were cut from an agarose-fixed block of the pineal glands using a microslicer (DTK-1000; Dosaka EM, Kyoto, Japan) and held in oxygenated Krebs solution at room temperature (24 ± 1°C) for 30 min prior to recording, as described previously (27). The Krebs solution had the following ionic composition: 112 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 1.2 mM MgCl2, 25 mM NaHCO3, 1.2 mM KH2PO4, and 14 mM glucose. The pH was adjusted to 7.4 by gassing with a mixture of 95% O2 and 5% CO2. Pineal glands were dissected within 2–3 h from the scheduled changes between dark and light conditions (dark-to-light at 0°C). Dispersed mechanically, and then cultured on coverslips coated with 4% paraformaldehyde in PBS for 10 min at room temperature. After incubation, these tissues were repeatedly washed in PBS, dispersed mechanically, and centrifuged at 2,000 rpm for 5 min. The supernatant was discarded, and the precipitated pinealocyte population was suspended in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 20 U/ml penicillin, and 20 μg/ml streptomycin (Sigma-Aldrich), dispersed mechanically, and then cultured on coverslips coated with 5 μg/ml poly-t-lysine (Sigma-Aldrich) for 20 min. Experiments were performed following cell culture for 24–96 h.

Ca2+ imaging for slice preparations. Ca2+ imaging of coronal slice preparations of pineal glands was performed using a fluorescent microscope (ECLIPSE TE2000-U; Nikon, Tokyo, Japan) and 0.02% trypsin (Type I; Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. After incubation, these tissues were repeatedly washed in PBS, dispersed mechanically, and centrifuged at 2,000 rpm for 5 min. The supernatant was discarded, and the precipitated pinealocyte population was suspended in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 20 U/ml penicillin, and 20 μg/ml streptomycin (Sigma-Aldrich), dispersed mechanically, and then cultured on coverslips coated with 5 μg/ml poly-t-lysine (Sigma-Aldrich) for 20 min. Experiments were performed following cell culture for 24–96 h.

Ca2+ imaging for slice preparations. Ca2+ imaging of coronal slice preparations of pineal glands was performed using a fluorescent microscope (ECLIPSE TE2000-U; Nikon, Tokyo, Japan) and 0.02% trypsin (Type I; Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. After incubation, these tissues were repeatedly washed in PBS, dispersed mechanically, and centrifuged at 2,000 rpm for 5 min. The supernatant was discarded, and the precipitated pinealocyte population was suspended in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 20 U/ml penicillin, and 20 μg/ml streptomycin (Sigma-Aldrich), dispersed mechanically, and then cultured on coverslips coated with 5 μg/ml poly-t-lysine (Sigma-Aldrich) for 20 min. Experiments were performed following cell culture for 24–96 h.

Electrophysiological recording. Electrophysiological studies were performed using a whole cell voltage-clamp technique with a CEZ-2400 amplifier (Nihon Kohden, Tokyo, Japan) and pCLAMP software (version 10; Molecular Devices/Axon Instruments, Foster City, CA) in single pinealocytes, as described previously (17, 36). The extracellular solution had an ionic composition of 137 mM NaCl, 5.9 mM KCl, 2.2 mM CaCl2, 1.2 mM MgCl2, 14 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with 10 N NaOH. The extracellular solution for VDCC current measurements had the following ionic composition: 109.2 mM NaCl, 5.9 mM KCl, 30 mM BaCl2, 1.2 mM MgCl2, 14 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with 10 N NaOH. The pipette solution for nicotine-sensitive current measurements contained 10 mM NaCl, 130 mM KCl, 4 mM MgCl2, 5 mM Na2ATP, 10 mM HEPES, and 0.05 mM EGTA. The pH was adjusted to 7.2 with 10 N KOH. The pipette solution for VDCC channel currents contained 140 mM KCl, 4 mM MgCl2, 2 mM Na2ATP, 10 mM HEPES, and 5 mM EGTA. The pH was adjusted to 7.2 with 10 N KOH.

Simultaneous measurement of [Ca2+]i and membrane potential. Simultaneous measurement of [Ca2+]i and membrane potential was performed using an Argus/HiSCA imaging system with fura-2 under whole cell current-clamp mode, as described previously (12). Single pinealocytes achieved in whole cell current-clamp configuration were loaded with 100 μM fura-2 (Invitrogen/Molecular Probes) by diffusion from the recording pipette for a few minutes. Ca2+ images were scanned every 4.6–8.2 s. Analyses of Ca2+ imaging and electrophysiological recording were carried out as described above.

Quantitative real-time polymerase chain reaction. The total RNA was extracted from homogenates of rat pineal glands using ISOGEN (Nippon Gene, Tokyo, Japan). The reverse transcription was performed using oligo(dT)12-18 primer and SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time polymerase chain reaction (PCR) analysis was performed using the SYBR Green assay (Power SYBR Green PCR Master Mix; Applied Biosystems, Foster City, CA) on an ABI PRISM 7000 sequence detection system (Applied Biosystems). Each cDNA sample was tested in triplet. Unknown quantities relative to the standard curve for specific primers were calculated, yielding transcriptional quantitation of gene products relative to β-actin. Specific forward (+) and reverse (−) PCR primers were designed as follows: rat nAChR α1 (NM_052806), (+) ACA GGT GAG CCT CGA TGA ACA; nAChR α3 (NM_052808), (+) GGC TGG GCG GAT CAG TAC; nAChR β3 (NM_052805), (+) AAA AGA TG, (−) AGG CCA ACC GTG ACC TCA; nAChR β4 (NM_052806), (+) TTC TCT CTG CTT GAC GAC GGG GA, (−) GCC GTC CGT ACC AAA GCA; VDCC β1 (NM_053701), (+) CAG TGA CAT TCA ACG CGA CAC T, (−) TGC GTA CCA CCC ACA CAG GGT AGC ATG TAA GA, (−) TGA CCT CAT CTA ACA ATT TCT; VDCC β2 (NM_053873), (+) GCC GCA CAG GGT AGT TAA GA, (−) TGC GTA CGG GGA GGA; β-actin (NM_031144), (+) AGG CCA ACC GTG AAA AGA TG, (−) ACC AGA CGG ATA CAG GA CA.

Immunocytochemistry. Freshly isolated pinealocytes were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature (24 ± 1°C), and excessive paraformaldehyde was removed thoroughly with PBS. These pinealocytes were treated with PBS containing 0.1% collagenase (Amano Enzyme, Nagoya, Japan) and 0.02% trypsin (Type I; Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. After incubation, these tissues were repeatedly washed in PBS, dispersed mechanically, and centrifuged at 2,000 rpm for 5 min. The supernatant was discarded, and the precipitated pinealocyte population was suspended in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 20 U/ml penicillin, and 20 μg/ml streptomycin (Sigma-Aldrich), dispersed mechanically, and then cultured on coverslips coated with 5 μg/ml poly-t-lysine (Sigma-Aldrich) for 20 min. Experiments were performed following cell culture for 24–96 h.

Ca2+ imaging for slice preparations. Ca2+ imaging of coronal slice preparations of pineal glands was performed using a fluorescent microscope (ECLIPSE TE2000-U; Nikon, Tokyo, Japan) and 0.02% trypsin (Type I; Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. After incubation, these tissues were repeatedly washed in PBS, dispersed mechanically, and centrifuged at 2,000 rpm for 5 min. The supernatant was discarded, and the precipitated pinealocyte population was suspended in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 20 U/ml penicillin, and 20 μg/ml streptomycin (Sigma-Aldrich), dispersed mechanically, and then cultured on coverslips coated with 5 μg/ml poly-t-lysine (Sigma-Aldrich) for 20 min. Experiments were performed following cell culture for 24–96 h.

Ca2+ imaging for cultured cells. [Ca2+]i measurements in primary-cultured pinealocytes were performed using an Argus/HiSCA imaging system (Hamamatsu Photonics) equipped with a fluorescent microscope (ECLIPSE TE300; Nikon), an objective lens (Plan Fluor 40× 1.30 NA, oil immersion; Nikon), and Argus/HiSCA software (version 2.60; Hamamatsu Photonics). Slice sections were loaded with 10 μM fluo-4 acetoxymethyl ester (fluo-4/AM; Invitrogen/Molecular Probes, Eugene, OR) for 20 min in oxygenated Krebs solution and excessive fluo-4/AM was removed thoroughly for 10 min. Oxygenated Krebs solution was used as the recording solution. Band-pass filters for excitation and emission wavelengths were 460–500 and 510–560 nm, respectively (Nikon). [Ca2+]i was measured as the ratio of fluorescence intensities (F460/F510). Ca2+ images were scanned every 1.3–2.8 s. The amplitude of Ca2+ changes was evaluated by averaging three consecutive points, including the peak. The amplitude and frequency of Ca2+ transients were normalized by the value before the application of drug.
Ca^{2+} OSCILLATION AND MELATONIN SECRETION IN PINEAL CYTOKES

ing 0.2% Triton X-100, 1% normal goat serum (DakoCytomation, Glostrup, Denmark), and primary antibody (1:100 dilution) for VDCC subunits [anti-VDCCα1C (Cat.1,2) (ACC-003), anti-VDCCα1D (Cat.1,3) (ACC-005), Alomone Lab, Jerusalem, Israel; and anti-VDCCα1F (Cat.1,4) (H0000778-A01), Abnoba, Taipei, Taiwan] for 12 h at 4°C. After washing repeatedly in PBS, these were covered with PBS containing 0.2% Triton X-100, 1% normal goat serum, and Alexa Fluor 488-labeled secondary antibody solution (1:1,000 dilution; Invitrogen/Molecular Probes) for 1 h at room temperature (24 ± 1°C) and then rinsed with PBS. Confocal images were obtained using a laser scanning confocal fluorescent microscope (A1R; Nikon) equipped with a fluorescent microscope (ECLIPSE Ti; Nikon), an objective lens (Plan Apo 60×1.40 NA, oil immersion; Nikon), and NIS Elements software (version 3.10; Nikon).

Melatonin secretion assay. Freshly dissected rat pineal glands were incubated for 1 h at 37°C and then exposed to 1 μM NE or vehicle (control) for 2 h. Test agents, which may affect NE-induced melatonin secretion, 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 (Bender MedSystems, Vienna, Austria; Invitrogen/Molecular Probes) for 1 h at room temperature (24°C) and then rinsed with PBS. Confocal images were obtained using a laser scanning confocal fluorescent microscope (A1R; Nikon) equipped with a fluorescent microscope (ECLIPSE Ti; Nikon), an objective lens (Plan Apo 60×1.40 NA, oil immersion; Nikon), and NIS Elements software (version 3.10; Nikon).

RESULTS

Spontaneous and nicotine-induced Ca^{2+} oscillations in rat pineal glands. Slice preparations (150 μm in thickness) of rat pineal gland were cut, mounted, and then used for image analyses of [Ca^{2+}], using fluo-4 and Ca^{2+} imaging systems. Ca^{2+} images were obtained within 6 h from the dark-to-light conditional change (see MATERIALS AND METHODS). In these slice preparations, spontaneous and oscillatory [Ca^{2+}] i changes were observed in 15% of cells in normal bath solution (16 of 109 cells; Fig. 1, A, B, and E). The kinetics of spontaneous Ca^{2+} oscillations varied among cells. The mean amplitude, frequency, and half duration of spontaneous Ca^{2+} transients were 3.0 ± 0.4 μM (minimum-maximum range, 0.8–8.7 μM), 0.86 ± 0.12 (0.2–2.6) s, and 19 ± 2 (2–52) s, respectively (n = 16). The application of 100 μM nicotine caused a transient [Ca^{2+}] i increase (fluorescent increase of 3.6 ± 0.4%) in half of the cells in pineal gland slices (109 of 220 cells). In addition, Ca^{2+} oscillations were evoked during the application of nicotine or following the withdrawal of nicotine in ~5% of cells (5 of 109 cells; Fig. 1, C–E). Overall, this nicotine-induced increase in [Ca^{2+}] i was observed from most slice preparations (31 of 40 slice sections from 14 rats). However, in slice sections examined within 6 h from the light-to-dark conditional change, spontaneous (6%, 5 of 82 cells) and nicotine-induced Ca^{2+} oscillations (2%, 2 of 82 cells) were observed less frequently (Fig. 1E). Interestingly and in contrast, the percentage of cells susceptible to nicotine (82 of 142 cells) was similar to that in the dark-to-light group.

Functional expression of nAChR in rat pinealocytes. The pineal gland is known to be composed of several different types of cells. We identified pinealocytes in primary-cultured cells prepared from rat pineal gland by their functional expression of nAChR. The application of 100 μM nicotine caused a transient [Ca^{2+}] i increase (A_F340/F_380 ratio of fura-2: 0.80 ± 0.05, n = 22; Fig. 2, A and B) in ~90% of these cells that were examined. Nicotine induced a [Ca^{2+}] i rise in a concentration-dependent manner with an EC_{50} value of 7.0 μM and a Hill coefficient of 1.6 (n = 22; Fig. 2C). A similar [Ca^{2+}] i increase was strongly inhibited by pretreatment with a nAChR blocker, 100 μM d-tubocurarine (0.05 ± 0.01, n = 22, P < 0.01), and this inhibitory effect was eliminated by removing d-tubocurarine (0.81 ± 0.07, n = 22). Application of 100 μM atropine did not affect the ACh-induced [Ca^{2+}] i rise in pinealocytes (data not shown). The application of 1 μM NE also elicited a [Ca^{2+}] i increase in pinealocytes, but this response was relatively small (0.13 ± 0.02, n = 22). In this investigation, round cells, which responded to nicotine and NE, were identified as pinealocytes (~90% of isolated cells). These cells were morphologically and pharmacologically distant from cells originating from glia (nicotine-insensitive, NE-insensitive, 8%) and blood vessels (nicotine-insensitive, NE-sensitive, 2%). Furthermore, the expression of nAChR in pinealocytes was confirmed by quantitative real-time PCR and the whole cell patch-clamp technique. In pineal glands, molecular expression of mRNAs encoding α3 and β4 subunits of nAChR was detected by real-time PCR, and the signal for the α7 subunit was absent (n = 3; Fig. 2D), as reported previously (15). At a holding potential of −40 mV, a transient inward current was evoked by the application of 50 μM nicotine (~100 pA at peak, n = 5) in pinealocytes (Fig. 2E). Current-voltage relationships in the absence and presence of 100 μM nicotine were generated using a ramp voltage-clamp protocol from −100 to 40 mV for 500 ms. Figure 2F shows the current-voltage relationship of nicotine-induced current recorded in pinealocytes. Note that the reversal potential is −11.4 ± 4.6 mV (n = 5).

Spontaneous Ca^{2+} oscillations and membrane depolarization in rat pinealocytes. To gain further information concerning the way in which electrical activity is involved in the generation of spontaneous Ca^{2+} oscillations, the membrane potential was measured from rat pinealocytes using whole cell current-clamp mode (Fig. 3A). Of 40 pinealocytes, 6 cells (15%) exhibited spontaneous electrical activity; specifically, periodic membrane depolarizations from the resting membrane potential of −59.6 ± 6.1 mV to −28.5 ± 5.4 mV (31.3 ± 5.6 mV depolarization; n = 6) were observed. The characteristic frequency of these depolarizations varied significantly among cells (1.42 ± 0.71 min−1, 0.2–4.6 min−1, n = 6).

We succeeded in challenging simultaneous measurements of changes in [Ca^{2+}] i and membrane potential in a primary-cultured rat pinealocyte. After a pinealocyte was impaled in the whole cell current-clamp configuration, Ca^{2+} imaging with fura-2 was carried out with monitoring the membrane potential. As shown in Fig. 3B, spontaneous [Ca^{2+}] i increases were well synchronized with transient membrane depolarization.
(n = 18 from 3 pinealocytes). The extent of membrane depolarization was positively correlated with that to \([Ca^{2+}]_i\), increases (correlation coefficient of 0.90, 3 pinealocytes).

**Spontaneous Ca\(^{2+}\) oscillation and Ca\(^{2+}\) source for oscillation in rat pinealocytes.** When changes in \([Ca^{2+}]_i\) under resting conditions were measured in primary cultured rat pinealocytes, a subset of cells showed spontaneous and oscillatory \([Ca^{2+}]_i\), changes when superfused with normal bath solution (17%, 365 of 2,137 cells; Fig. 4). The amplitude and frequency of spontaneous \(Ca^{2+}\) oscillations varied somewhat among cells. Spontaneous \(Ca^{2+}\) events usually continued for \(\sim 30\) min during \([Ca^{2+}]_i\) measurements. The frequency of spontaneous \(Ca^{2+}\) transients ranged from 0.5 to 2.0 min\(^{-1}\) (n = 365).

To identify the source and learn more about the intracellular signaling pathways for this \([Ca^{2+}]_i\), increase and spontaneous \([Ca^{2+}]_i\), oscillations in rat pinealocytes, components of the transmembrane \(Ca^{2+}\) influx pathway were first examined. Removal of extracellular \(Ca^{2+}\) and the addition of 1 mM EGTA (\(Ca^{2+}\) free) almost completely abolished spontaneous \(Ca^{2+}\) oscillations (0.13 ± 0.05 and 0.09 ± 0.09 in relative amplitude and frequency, respectively, n = 3, P < 0.01). This response was recovered after the addition of 2.2 mM \(Ca^{2+}\).
Fig. 2. Functional expression of nicotinic acetylcholine receptor (nAChR) in rat pinealocytes, confirmed by Ca\(^{2+}\) imaging, quantitative real-time PCR, and electrophysiological recordings. A: transient [Ca\(^{2+}\)]\(_i\) rises were evoked by the application of 100 μM nicotine or 100 μM ACh to primary cultured pinealocytes, which were loaded with 10 μM fura-2/AM. Note that the ACh-induced [Ca\(^{2+}\)]\(_i\) rise can be blocked by 100 μM d-tubocurarine (dTC). NE, norepinephrine. B: changes in [Ca\(^{2+}\)]\(_i\) (monitored by changes in F\(_{340}/F_{380}\)) following application of drugs as summarized in histograms. Data were obtained from 22 cells that responded to nicotine. Statistical significance vs. ACh alone is indicated as **P < 0.01. C: nicotine-induced [Ca\(^{2+}\)]\(_i\) rise, which was monitored by F\(_{340}/F_{380}\) change, exhibited a classical concentration-dependent relationship. The fitted curve was obtained as described in MATERIALS AND METHODS. The EC\(_{50}\) value was 7.0 μM, and the Hill coefficient was 1.6 (n = 22). D: the relative expressions of mRNAs encoding nAChRβ3, α7, and β4 subunits in pineal glands, based on quantitative real-time PCR measurements. These expression levels were normalized to endogenous β-actin signals. E: at a holding potential of −40 mV, a transient inward current was elicited by 50 μM nicotine in a pinealocyte (inset). Current-voltage (I-V) relationships in the absence (black) and presence (gray) of 100 μM nicotine were obtained using a voltage-ramp protocol from −100 to +40 mV, for 500 ms. F: nicotine-sensitive current obtained by subtraction of the 2 records in E and replotted as difference current. Note that the average reversal potential is near −11.4 ± 4.6 mV (n = 5).

(Fig. 4, A, D, and E). In addition, spontaneous Ca\(^{2+}\) events were effectively inhibited by the application of a blocker of VDCC, 1 μM nifedipine (0.09 ± 0.02 and 0.02 ± 0.02, n = 6, P < 0.01). Significant recovery followed the removal of nifedipine (Fig. 4, B, D, and E). Next, the effects of 2-aminoethoxydiphenylborate (2-APB, a blocker of IP\(_3\) receptor) and ryanodine (an inhibitor of ryanodine receptor) were examined. Neither 30 μM 2-APB (0.95 ± 0.08 and 1.24 ± 0.19 in relative amplitude and frequency, respectively, n = 12, P > 0.05) nor 30 μM ryanodine (0.92 ± 0.15 and 1.04 ± 0.16, n = 7, P > 0.05) changed the parameters of spontaneous Ca\(^{2+}\) oscillations significantly (Fig. 4, C–E). These results strongly suggest that the spontaneous Ca\(^{2+}\) oscillations in cultured pinealocytes are mediated by Ca\(^{2+}\) influx through VDCC but are not strongly dependent on intracellular Ca\(^{2+}\)-release channels.

Nicotine-evoked Ca\(^{2+}\) oscillations in cultured pinealocytes. Cytosolic Ca\(^{2+}\) mobilization during stimulation of nAChR was also examined in primary cultured pinealocytes. In some pinealocytes (23%, 18 of 327 cells), Ca\(^{2+}\) oscillations were observed following a brief application of nicotine, which induced a transient [Ca\(^{2+}\)]\(_i\) rise (Fig. 5A). Occurrence of Ca\(^{2+}\) oscillations after withdrawal of nicotine was dependent on its concentration (Fig. 5B). Nicotine-induced Ca\(^{2+}\) oscillations were blocked strongly by 1 μM nifedipine (0.15 ± 0.07 and 0.13 ± 0.06 in relative amplitude and frequency, respectively, P < 0.01, n = 5). These observations demonstrate that the pharmacological features of nicotine-induced Ca\(^{2+}\) oscillations are very similar to those of spontaneous oscillations.

VDCC currents and their molecular basis in rat pinealocytes. The functional expression of VDCC in rat pinealocytes was demonstrated by the whole cell patch-clamp technique. The activity of VDCC was measured in extracellular solution containing 30 mM Ba\(^{2+}\) and assessed as inward currents sensitive to 100 μM Cd\(^{2+}\). When pinealocytes were depolarized from a holding potential of −50 mV in 10-mV steps, the maximum peak amplitude of inward currents was recorded at 0 mV (7.5 ± 1.5 pA/pF, n = 5; Fig. 6A). These inward currents were significantly reduced by the addition of 1 μM nifedipine (2.3 ± 0.7 pA/pF at 0 mV, n = 5, P < 0.05). The averaged cell capacitance of pinealocytes was 19.4 ± 0.9 pF (n = 5).

\(\text{AJP-Cell Physiol} \cdot \text{doi:10.1152/ajpcell.00014.2014} \cdot \text{www.ajpcell.org}\)
The expressions of mRNAs encoding nifedipine-sensitive VDCC genes (α1C, α1D, α1F, and α1S) in rat pineal glands were identified by real-time PCR. Quantitative real-time PCR analysis showed the clear expression of α1F transcript (0.075 ± 0.014 of β-actin, n = 3, Fig. 6C). Weak signals for α1C and α1D mRNAs were also detected (0.003 ± 0.001, n = 3 and 0.015 ± 0.004, n = 3, respectively). Further evidence of VDCC expression in pinealocytes was provided by an immunocytochemical approach using specific polyclonal antibody for these subunits. Immunoreactivity to α1F (positive signal, 8 of 12 inspected cells) and α1C (6 of 10 cells) proteins was clearly observed at the plasma membrane of pinealocytes (α1D, all negative of 13 cells; Fig. 6D).

Modulation of melatonin secretion by the activity of VDCC. Melatonin secretion from rat pineal glands evoked by neurotransmitters and its regulation by the activity of VDCCs were analyzed quantitatively using a melatonin ELISA kit (Fig. 7). Melatonin secretion from whole pineal glands was observed following treatment with 1 μM NE for 1 h at 37°C (26.0 ± 3.5 ng/ml, n = 6, P < 0.01 vs. vehicle of 4.2 ± 0.9 ng/ml, n = 8). Melatonin secretion evoked by 1 μM NE was reduced by ~50% when 100 μM ACh was applied simultaneously (14.7 ± 1.3 ng/ml, n = 9, P < 0.01). This ACh-induced inhibition recovered almost completely following the addition of 1 μM nifedipine (25.2 ± 3.7 ng/ml, n = 6, P < 0.01 vs. NE + ACh).

In the absence of ACh, nifedipine did not affect NE-evoked melatonin release (25.8 ± 2.8, n = 4, P > 0.05). This pattern of response suggests that the inhibitory effect of ACh on NE-evoked melatonin secretion in pineal glands is modulated by VDCC mediated Ca²⁺ influx.

**DISCUSSION**

The pineal gland is an endocrine organ in the brain. It regulates circadian rhythm through the synthesis and secretion of melatonin. In mammalian pinealocytes, the regulation of melatonin production depends upon a balance of activity due to sympathetic and parasympathetic innervation (2, 23, 28, 32). This modulation is very different from that in nonmammals (9). The inhibitory mechanism for melatonin production driven...
following nAChR stimulation via parasympathetic activity is considered to be closely related to the rise of \([\text{Ca}^{2+}]_i\) in mammalian pinealocytes (34). However, essential details of the underlying cellular \([\text{Ca}^{2+}]_i\) dynamics and identification of the involvement of ion channels that are responsible for its regulation are lacking. The present study shows that \([\text{Ca}^{2+}]_i\) increase, e.g., following nAChR stimulation, is often accompanied by \([\text{Ca}^{2+}]_i\) oscillations in rat pinealocytes, in both slice preparations and primary-cultured cells. An essential role for nifedipine-sensitive VDCCs in the initiation and regulation of \([\text{Ca}^{2+}]_i\) oscillations and in the subsequent inhibition of melatonin secretion was demonstrated by our results.

Oscillatory \([\text{Ca}^{2+}]_i\) events have been detected in pinealocytes dissociated from birds and fish, and these events have been suggested to be associated with melatonin secretion (7, 20, 25). Spontaneous \([\text{Ca}^{2+}]_i\) transients were observed in 10–30% of pinealocytes isolated from birds and fish, and in ~15% of pinealocytes in this study. We note that pineal glands in lower vertebrates are directly regulated by photoreceptors, as well as by the autonomic nervous system. In fact, the pineal gland response in these species is believed to oscillate as a secondary consequence of photoreceptor-mediated responses (9). Our results reveal that \([\text{Ca}^{2+}]_i\) oscillations are more frequent in slice preparations studied within 6 h after dark-to-light conditioning changes, and that a light-to-dark change was a less effective stimulus than from the converse. The expression of AANAT, the rate-limiting enzyme of melatonin production, highly depends upon the circadian cycle in rodents (19). Although the underlying molecular mechanisms of \([\text{Ca}^{2+}]_i\) oscillation related to the dark/light cycle remain to be determined, the regulation of ion channel expression might be involved.

Spontaneous \([\text{Ca}^{2+}]_i\) oscillations observed in pinealocytes were predominantly due to \([\text{Ca}^{2+}]_i\) influx from extracellular spaces through VDCCs. It has been reported that candidates for pore subunits of VDCCs are \(\alpha1D (\text{Ca}_{1.3})\) in rat pineal glands (6) and \(\alpha1F\) in human pineal glands (14). Expression analyses in the present study revealed that nifedipine-sensitive VDCCs were predominantly composed of \(\alpha1F (\text{Ca}_{1,4})\) subunits, which were originally identified in the retina (10), and also with \(\alpha1C (\text{Ca}_{1,2})\) subunit. We expected that blockers of \([\text{Ca}^{2+}]_i\)-releasing channels (IP3 and ryanodine receptors) would affect \([\text{Ca}^{2+}]_i\) oscillation, but our results showed the opposite. These findings suggest that the source of \([\text{Ca}^{2+}]_i\), for \([\text{Ca}^{2+}]_i\),
oscillation in rat pinealocytes is similar to that in lower creatures (7, 20, 25). In many other Ca2+ oscillations detected in various cell types, Ca2+ released from intracellular Ca2+ store sites has essential roles in the mechanism underlying their generation and regulation (16); therefore, the lack of dependency on Ca2+ release may be characteristic of Ca2+ oscillations in pinealocytes regardless of species.

Spontaneous Ca2+ oscillations coupled to ion channel activities and their physiological functions have been studied in detail in many types of mammalian cells, for example, in pacemaker sinoatrial node cells (24), interstitial cells of Cajal in the gastrointestinal tract for gut pacemaker activity (30), pancreatic β cells for insulin secretion (13), pituitary cells for the release of neuroendocrine hormones (33), and glial cells for neuronal activities (26, 31). Our findings provide novel evidence supporting that Ca2+ oscillations in mammalian pinealocytes contribute to the synthesis and secretion of melatonin.

Functional expression of VDCCs (1, 6, 22), voltage-gated K+ channels (1, 3–5), nonselective cation channels (8), store-operated Ca2+ channels (21), and Ca2+-activated K+ channels (5, 21) have been suggested in pineal cells, mainly based on electrophysiological methods and pharmacological tools. In addition, tetrodotoxin-insensitive spontaneous electrical activity (action potentials) have been detected using extracellular recordings in pineal glands (1, 11, 29, 37). In the present study, periodic membrane depolarization of ∼30 mV in amplitude from the resting potential near −60 mV was observed in 15% of cultured pinealocytes under whole cell current-clamp mode. Although their frequency was quite varied (0.2–4.6 min−1), the value was close to those of spontaneous Ca2+ oscillations in slice preparation of pineal glands (0.2–2.6 min−1) and primary cultured pinealocytes (0.5–2.0 min−1), respectively. Simultaneous measurement of [Ca2+]i and membrane potential clearly demonstrated that Ca2+ oscillations were triggered by a transient membrane depolarization and were coupled in a 1:1 manner in rat pinealocytes. In addition, essential details of the functional coupling between Ca2+ oscillations and VDCCs have been identified in this study. The stimulation of nAChR via parasympathetic nerve activity induced spontaneous membrane depolarizations followed by a [Ca2+]i increase. Presumably these complexes can induce glutamate release from pinealocytes, and this subsequently reduces melatonin secretion. Specifically, the released glutamate can stimulate glutamate receptors, by both autocrine and paracrine mechanisms, and thus suppress adenylate cyclase via Gi protein activation (18, 34, 35). Our results show that nAChR activation often caused Ca2+ oscillations in isolated pinealocytes (23%) and that the pharmacological properties of evoked Ca2+ oscillations were similar to those of spontaneous Ca2+ oscillations. Overall, 40% of pinealocytes showed spontaneous or nicotine-evoked Ca2+ oscillations; therefore, Ca2+ signaling underlying glutamate release may occur, at least in part, as Ca2+ oscillations.

One of the most important findings from this study is that melatonin secretion from whole pineal gland following nAChR stimulation is strongly modulated by ion channel activities, particularly VDCC activation. Moreover, melatonin production during sympathetic stimulation is depressed by parasympathetic signals mediated by defined nAChR subtypes as has been reported previously (32). Since nifedipine abolished the suppression of NE-induced melatonin secretion by ACh, it is clear that Ca2+ influx through VDCCs is essential for this inhibitory regulation of melatonin secretion via nAChR stimulation. These results also provide evidence to support the hypothesis that Ca2+ oscillations are involved in glutamate release, which subsequently reduces melatonin secretion.

In conclusion, we found that a substantial subset of pinealocytes generated spontaneous Ca2+ oscillations that are modulated by synchronized periodic membrane depolarizations, under both normal physiological conditions and following nAChR stimulation. Spontaneous and evoked [Ca2+]i oscillations were strongly dependent on Ca2+ influx through VDCCs. More importantly, these spontaneous and evoked [Ca2+]i oscillations are importantly involved in the inhibitory mechanism of melatonin secretion, which is known to be regulated by parasympathetic activities in mammalian pineal glands.

ACKNOWLEDGMENTS

We thank Dr. Wayne R. Giles (Univ. of Calgary, Calgary, Canada) for reading of this manuscript.

GRANTS

This investigation was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology (23136512 and 25136717; to Y. Imaizumi), and Grant-in-Aids for Scientific Research (B) (23390020; to Y. Imaizumi) and Scientific Research (C) (25460104; to H. Yamamura) from the Japan Society for the Promotion of Science. This work was also supported by a Grant-in-Aid from the Smoking Research Foundation (to Y. Imaizumi).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


