Norepinephrine activates store-operated Ca\(^{2+}\) entry coupled to large-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels in rat pinealocytes

So-Young Lee, Bo-Hwa Choi, Eun-Mi Hur, Jong-Hee Lee, Sung-Jin Lee, Chin Ok Lee, and Kyong-Tai Kim

Division of Molecular and Life Science, National Core Research Center for System Bio-Dynamics, Department of Life Science, Pohang University of Science and Technology, Pohang, Republic of Korea

Submitted 11 July 2005; accepted in final form 4 November 2005

The pineal gland is a neuroendocrine organ in which melatonin (5-methoxy-N-acetyltryptamine) is synthesized and released with daily and seasonal rhythms. It has important roles in the regulation of circadian rhythm, seasonal reproduction, and retinal function (11). Pinealocytes in the mammalian pineal gland express the genes encoding the melatonin-synthesizing enzymes. Serotonin N-acetyltransferase, or arylalkylamine N-acetyltransferase (AANAT), a critical enzyme in melatonin production, and is responsible for maintaining the Ca\(^{2+}\) response after repetitive stimulation. Ca\(^{2+}\) entry evoked by NE was dependent on PLC activation. NE evoked a substantial amount of Ca\(^{2+}\) entry even after cells were treated with 1-oleoyl-2-acetyl-sn-glycero (OAG), an analog of diacylglycerol. To the contrary, further OAG treatment after cells had been exposed to OAG did not evoke additional Ca\(^{2+}\) entry. Moreover, NE failed to induce further Ca\(^{2+}\) entry after the development of Ca\(^{2+}\) entry induced by thapsigargin (Tg), suggesting that the pathway of Ca\(^{2+}\) entry induced by NE might be identical to that of Tg. Interestingly, Ca\(^{2+}\) entry evoked by NE or Tg induced membrane hyperpolarization that was reversed by iBerberotoxin (IBTX), a specific inhibitor of large-conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK) channels. Moreover, IBTX-sensitive BK current was observed during application of NE, suggesting that activation of the BK channels was responsible for the hyperpolarization. Furthermore, the activation of BK channels triggered by NE contributed to regulation of the protein level of AANAT. Collectively, these results suggest that NE triggers Ca\(^{2+}\) entry coupled to BK channels and that NE-induced Ca\(^{2+}\) entry is important in the regulation of AANAT.

serotonin N-acetyltransferase; pineal gland

Address for reprint requests and other correspondence: K.-T. Kim, Division of Molecular and Life Science, National Core Research Center for System Bio-Dynamics, Dept. of Life Science, Pohang Univ. of Science and Technology, Pohang, Kyung-buk 790-784, Republic of Korea (e-mail: ktk@postech.ac.kr).

The costs of publication of this article are defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
apoptosis (15, 19). In the present study, we have provided evidence that the Ca\(^{2+}\) entry evoked by NE is attributable mainly to the activation of SOCs. In addition, we show that inhibitors of SOCs suppressed the increase in the protein level of AANAT induced by NE. Furthermore, the SOCs were coupled to large-conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK) channels, thereby inducing membrane hyperpolarization. Taken together, these data suggest that Ca\(^{2+}\) entry triggered by NE plays an important role in pineal gland function (i.e., in modulation of NE signaling and regulation of AANAT).

**MATERIALS AND METHODS**

**Chemicals.** Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated.

**Preparation of cultured pinealocytes.** Sprague-Dawley rats were maintained in a controlled environment (12:12-h light-dark cycle). Pineal glands from male and female rats (aged 7–9 wk) were prepared using methods described in a previous study (9). Briefly, pineal glands removed from rat brains were washed in ice-cold HEPES buffer and transferred to DMEM (GIBCO-BRL, Grand Island, NY) containing papain (20 U/ml; Worthington Biochemical, Lakewood, NJ). After 1-h incubation, the tissue was washed in DMEM, mechanically dispersed in clean medium, and plated onto poly-d-lysine-coated coverslips. The cells were suspended in DMEM containing 10% FBS and maintained at 37°C in a 5% CO\(_2\)-containing atmosphere until use (1–3 days). Animal experiments were performed after being approved by the University Ethics Committee.

**Fluorescence measurements of [Ca\(^{2+}\)]\(_i\).** Cells were loaded with 3 μM fura-2 AM (Molecular Probes, Eugene, OR) at room temperature (20–23°C) for 50 min and subsequently washed in fura-2-free solution for a minimum of 10 min. Single-cell Ca\(^{2+}\) measurements were performed as described previously by Lee and Lee (17). Briefly, cells loaded with fura-2 were mounted on an experimental chamber and illuminated with UV light (75-W xenon lamp) applied via an epifluorescence microscope (Nikon, Tokyo, Japan). A filter wheel in front of the UV light was rotated continuously at 50 Hz, and excitation filters of 340 and 380 nm were used alternately (Cairn Research, Kent, UK).

**Measurement of membrane potential.** Pinealocytes in the experimental chamber were superfused continuously with Tyrode solution containing (in mM) 140 NaCl, 4 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, and 10 glucose (pH 7.4 with NaOH) at ~37°C. To measure membrane potential, we used conventional microelectrodes pulled from filamented thin-wall glass [1.5-mm outer diameter (OD); TW150F-6; World Precision Instruments, Sarasota, FL]. They were filled with 300 mM KCl and had resistances between 40 and 55 MΩ. The electrode resistance and capacitance were compensated to ~85% of their initial values. Microelectrode potential was measured with an AxoClamp 2A amplifier (0.1-gain headstage; Axon Instruments, Foster City, CA).

**Measurement of BK current.** To record BK current, cells were superfused with HEPES solution containing (in mM) 140 NaCl, 3 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 3 × 10\(^{-7}\) TTX, 1 4-aminopyridine (4-AP), 5 × 10\(^{-7}\) glibenclamide, and 10 glucose (pH adjusted to 7.4 with NaOH). 4-AP (1 mM) was used to reduce voltage-gated K\(^{+}\) currents and unmask the Ca\(^{2+}\) dependence of K\(^{+}\) currents. The concentration of 4-AP was chosen according to the concentration used in a previous study (33) to minimize any possible nonspecific blockade of other K\(^{+}\) currents. Recording pipettes were made of filamented borosilicate capillary glass (1.5-mm OD; TW150F-6; World Precision Instruments), and resistances ranged from 2 to 5 MΩ when the pipettes were filled with the solutions listed below. Patch-pipette solutions for K\(^{+}\) current recording contained (in mM) 110 K\(^{+}\)-gluconate, 10 KCl, 5 NaCl, 2 MgCl\(_2\), 10 HEPES, 0.5 EGTA, 1 ATP, and 0.2 GTP (pH adjusted to 7.3 with KOH). Membrane currents were recorded using an Axopatch 200A amplifier (Axon Instruments). Signals were obtained at sampling rates of 5 kHz. WinWCP software (John Dempster, Strathclyde University, Strathclyde, UK) was used to control the generation of stimuli and to collect data. Capacitance subtraction was performed for all recordings. The series resistances were within 10 MΩ. Experiments were conducted at room temperature (20–23°C).

**Western blot analysis.** Proteins were extracted with lysis buffer containing (in mM) 100 Tris-HCl (pH 7.0), 1 EGTA, 1 MgCl\(_2\), 1 PMSF, 0.1 DTT, 1 Na\(_3\)VO\(_4\), and 1 Triton X-100. Proteins were separated on a 12.5% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk in TBST solution containing 20 mM Tris-HCl (pH 7.5), 140 mM NaCl, and 0.05% Tween 20. Detection of AANAT protein was performed as described previously (6). The immunosignal was detected using the SUpEX detection system (Neuronex, Pohang, Korea).

**RESULTS**

Ca\(^{2+}\) influx triggered by NE regulates the protein level of AANAT. NE stimulates transcription of AANAT through activation of adenyl cyclase-cAMP pathway (11). Because NE activates the Ca\(^{2+}\) pathway as well as the cAMP pathway, we investigated the possible role of Ca\(^{2+}\) influx triggered by NE in the modulation of AANAT. For this purpose, we examined whether low (0.2 mM) extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)) would affect the protein level of AANAT elevated by NE. As shown in Fig. 1, when Ca\(^{2+}\) entry was limited by lowering [Ca\(^{2+}\)]\(_o\), the protein level of AANAT was significantly reduced. In addition, Gd\(^{3+}\) and SKF-96365, both of which are general inhibitors of Ca\(^{2+}\) entry channels, also caused a reduction in the level of AANAT upregulated by NE.

We next examined the Ca\(^{2+}\) response to repetitive NE stimulation, focusing on Ca\(^{2+}\) entry. To this end, we first compared the Ca\(^{2+}\) response in the absence and presence of 2 mM [Ca\(^{2+}\)]\(_o\). As shown in Fig. 2A, when NE was applied five times, the amplitude of the NE-evoked Ca\(^{2+}\) peak gradually decreased and the amplitude of the fifth peak was 64 ± 4% (n = 7) of the first one. However, the biphasic Ca\(^{2+}\) response trace maintained its shape. On the other hand, removal of extracellular Ca\(^{2+}\) dramatically decreased the fifth NE-evoked Ca\(^{2+}\) peak to 3 ± 1% (n = 4) of the first one, and the secondary sustained phase was not detected. Similarly, in the presence of Gd\(^{3+}\) or La\(^{3+}\), inhibitors of Ca\(^{2+}\) entry, the amplitude of the fifth NE-evoked Ca\(^{2+}\) peak was reduced 6 ± 1% (n = 4) and the secondary sustained phase was not detectable. These results suggest that Ca\(^{2+}\) entry evoked by NE is necessary to maintain the Ca\(^{2+}\) response by repetitive stimulation and seems to contribute to potentiation of the NE-induced increase in the AANAT protein level.

Ca\(^{2+}\) influx evoked by NE is SOCE. The secondary plateau phase in the NE-induced Ca\(^{2+}\) response reflected Ca\(^{2+}\) entry after receptor activation, because it was completely inhibited by removal of extracellular Ca\(^{2+}\) as well as by Gd\(^{3+}\) (Fig. 3A). To test whether the activation of voltage-gated Ca\(^{2+}\) channels (VGCCs) was involved in the secondary phase of the Ca\(^{2+}\) response, we examined the effect of nifedipine, a specific
response was abolished by 2-aminoethoxydiphenylborane, a known inhibitor of IP3 receptors and SOCs (7). Next, we examined the involvement of PLC. In the absence of [Ca\(^{2+}\)], application of NE increased [Ca\(^{2+}\)], because of Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores. When 2 mM [Ca\(^{2+}\)], was added to the medium, we observed a substantial amount of Ca\(^{2+}\) entry in response to NE (Fig. 3B, left). In the presence of U-73122, a selective inhibitor of PLC, NE did not evoke Ca\(^{2+}\) release and subsequent Ca\(^{2+}\) entry (Fig. 3B, middle), whereas its inactive analog U-73343 had no effect (Fig. 3B, right), suggesting that NE-induced Ca\(^{2+}\) entry is dependent on PLC signaling. PLC hydrolyzes PIP\(_2\) into DAG and IP3, both of which could induce increase in [Ca\(^{2+}\)], (20, 34). To investigate whether DAG was involved in the NE-induced Ca\(^{2+}\) entry, we used a membrane-permeable DAG analog, 1-oleoyl-2-acetyl-sn-glycerol (OAG). Figure 3C shows that OAG alone increased a small amount of [Ca\(^{2+}\)], PMA, an agonist of PKC that can be activated by DAG, failed to induce an increase in [Ca\(^{2+}\)], (Fig. 3C, middle). Moreover, GF-109203X, an inhibitor of PKC, did not affect the OAG-induced [Ca\(^{2+}\)], increase (data not shown). These results suggest that OAG is able to induce a small amount of [Ca\(^{2+}\)], increase independently of PKC activation. As shown in Fig. 3C, right, NE evoked further Ca\(^{2+}\) influx even after cells were treated with 100 mM OAG, although a higher concentration of OAG (200 mM) did not evoke further Ca\(^{2+}\) influx after treatment with 100 mM OAG (Fig. 3C, left). These results suggest that NE evokes Ca\(^{2+}\) entry, probably through a pathway other than OAG.

Next, we applied Tg, which is widely used to induce depletion of Ca\(^{2+}\) stores and to activate subsequent SOCE. As shown in Fig. 3D, OAG evoked an additional [Ca\(^{2+}\)], increase even after Tg had induced SOCE, whereas NE failed to induce further Ca\(^{2+}\) influx. Therefore, we concluded that the NE-induced Ca\(^{2+}\) entry would be included in SOCE induced by Tg.
SOCE hyperpolarizes membrane potential. In excitable cells, activation of SOC current not only may be the path for Ca\(^{2+}\) to enter the cell but also may provide a depolarizing trigger (21). To elucidate whether activation of SOC induces changes in membrane potential, we recorded single-cell [Ca\(^{2+}\)]\(_i\) level and membrane potential simultaneously. When the pinealocytes were penetrated using conventional microelectrodes, the mean membrane potential was \(-65 \pm 5\) mV (\(n = 45\)), which is in agreement with the findings reported previously (10). Unexpectedly, the addition of 2 mM [Ca\(^{2+}\)]\(_o\) to the superfusion solution containing NE resulted in the hyperpolarization of the membrane potential and removal of extracellular Ca\(^{2+}\) depolarized the membrane potential (Fig. 4A), suggesting that Ca\(^{2+}\) influx has an important role in the regulation of membrane potential. BK channels require both depolarization and elevated [Ca\(^{2+}\)]\(_i\), to become activated, which results in hyperpolarization of membrane potential coupled with increased [Ca\(^{2+}\)]\(_i\) (16). To test the possible involvement of BK channels in the hyperpolarization induced by Ca\(^{2+}\) influx, we examined the effect of iberiotoxin (IBTX), a specific inhibitor of BK channels. Ca\(^{2+}\) entry evoked by NE and Tg induced \(-17 \pm 8\) mV (\(n = 7\)) and \(-18 \pm 8\) mV (\(n = 5\)) membrane hyperpolarization, respectively, which was completely reversed by application of IBTX (Fig. 4, B and C). To test whether the membrane hyperpolarization accompanied by Ca\(^{2+}\) entry induced by NE and Tg could be mimicked by similar amounts of Ca\(^{2+}\) influx, we treated the cells with Ach, an activator of nicotinic Ach receptors. Ach treatment, however, resulted in a \(29 \pm 4\) mV (\(n = 10\)) increase in membrane potential (i.e., depolarization) (Fig. 4D), which was reduced by nifedipine (48 ± 7%; \(n = 5\)). Nifedipine significantly inhibited the Ca\(^{2+}\) influx induced by Ach (49 ± 5%; \(n = 5\)), which presents a striking contrast to its effect on Ca\(^{2+}\) influx evoked by NE (Fig. 3A), excluding the involvement of VGCCs in NE-induced Ca\(^{2+}\) influx. Taken together, these results suggest that SOCE induces membrane hyperpolarization because of the coupling of BK channels.

IBTX-sensitive BK current is developed by NE. To confirm that the BK channels were activated in response to NE stimulation, IBTX-sensitive BK current was measured before and during application of NE. Figure 5A shows the macroscopic outward currents from a pinealocyte to which NE was not applied. The depolarization-induced BK current is defined as the outward current sensitive to blockage by IBTX. In the absence of NE, outward currents were not sensitive to IBTX (Fig. 5A). However, IBTX reduced outward current by 40 ± 7% at +30 mV (\(n = 7\)) during application of NE (Fig. 5B), suggesting that BK current is developed upon stimulation by NE. When Gd\(^{3+}\) was applied to inhibit SOCE, NE did not induce IBTX-sensitive BK current (Fig. 5E). When Tg was used as an alternative activator of SOCE, IBTX-sensitive BK current was also detected (data not shown).

Inhibition of BK channels reduces the protein level of AANAT. NE-dependent melatonin synthesis is negatively regulated by parasympathetic stimulation, such as that induced by nicotinic Ach receptors. Ach depolarizes membrane potential, which leads to the secretion of \(\gamma\)-glutamyl, thereby inhibiting the synthesis of AANAT (28). Because activation of BK channels is responsible for the membrane hyperpolarization induced by NE (Fig. 4), it is possible that BK channels might
contribute to the regulation of the level of AANAT, the synthesis of which is influenced by the status of membrane potential. To test this possibility, we investigated whether the protein level of AANAT elevated by NE would be affected by the inhibition of BK channels. As shown in Fig. 6, 100 nM Ach partially decreased the protein level of AANAT upregulated by NE, as expected. Importantly, IBTX alone caused a reduction in AANAT protein level in the presence of NE. Furthermore, application of IBTX resulted in a further reduction in the AANAT protein level elevated by NE in the presence of Ach compared with the level inhibited by Ach. Similar results were obtained when lower concentrations of Ach (10 and 50 nM) were applied (data not shown). These results suggest that activation of BK channels triggered by NE participates in the regulation of AANAT.

SOCE evoked by NE occurs through activation of \( \alpha_1 \)-AR. Because NE stimulates both \( \alpha_1 \)- and \( \beta_1 \)-AR, we examined the effect of phenylephrine (PE) and isoproterenol (Iso), specific agonists of \( \alpha_1 \) and \( \beta_1 \)-AR, respectively, to determine which receptor is responsible for \( \text{Ca}^{2+} \) influx. Stimulation of \( \alpha_1 \)-AR with PE in the presence of propranolol (Prop), an antagonist of \( \beta_1 \)-AR, caused a biphasic \( \text{Ca}^{2+} \) increase and maintained the

Fig. 4. Activation of SOCE hyperpolarizes membrane potential. A: intracellular \( \text{Ca}^{2+} \) concentration ([\( \text{Ca}^{2+} \)]\text{_i}) and membrane potential \( V_m \) were recorded simultaneously. In the presence of 1 \( \mu \text{M} \) NE, changes in membrane potential in response to application of 2 mM [\( \text{Ca}^{2+} \)]\text{_o} and \( \text{Ca}^{2+} \)-free extracellular solution were monitored. B and C: effect of 100 nM iberiotoxin (IBTX) on membrane hyperpolarization induced by 1 \( \mu \text{M} \) NE (B) or 1 \( \mu \text{M} \) Tg (C). D, left, effect of 100 \( \mu \text{M} \) Ach on [\( \text{Ca}^{2+} \)]\text{_i} and \( V_m \); right, effect of 3 \( \mu \text{M} \) nifedipine (Nif) on [\( \text{Ca}^{2+} \)]\text{_i} increase and depolarization induced by Ach. E: summary of differences in \( V_m \) induced by NE, Tg, and IBTX in presence of NE, IBTX in presence of Tg, and Ach in presence or absence of Nif. Typical recordings from single pinealocytes are presented. Each data point represents a mean ± SE from 5–10 cells. * \( P < 0.05 \).

Fig. 5. IBTX-sensitive, large-conductance \( \text{Ca}^{2+} \)-activated K\(^+\) (BK) channel current is developed by NE. A: superimposed whole cell outward currents in response to voltage steps from holding potential of \(-60 \text{ mV}\) to \(+30 \text{ mV}\) for 250 ms in 10-mV steps before (left) and during (middle) application of 100 nM IBTX. IBTX-sensitive currents (control minus IBTX) are shown at right. Typical recordings are presented. B: superimposed whole cell outward currents before (left) and during (middle) application of 100 nM IBTX in presence of 1 \( \mu \text{M} \) NE. After application of NE for 4 min, IBTX was added to perfusion solution. IBTX-sensitive currents (control minus IBTX) are shown at right. Typical recordings are presented. C: current-voltage (I-V) relationships showing effect of IBTX on macroscopic outward currents. Each data point represents a mean ± SE from 5 cells. D: I-V relationships showing effect of IBTX on macroscopic outward currents after exposure to 1 \( \mu \text{M} \) NE. Each data point represents a mean ± SE from 7 cells. * \( P < 0.05 \), ** \( P < 0.01 \). E: I-V relationships showing effect of IBTX on macroscopic outward currents after exposure to 1 \( \mu \text{M} \) NE and 10 \( \mu \text{M} \) Gd\(^{3+}\). Each data point represents a mean ± SE from 5 cells.
Ca$^{2+}$ response after repetitive stimulation as observed in NE stimulation (Fig. 7, top). However, stimulation of β$_1$-AR with ISO in the presence of prazosin, an antagonist of α$_1$-AR, failed to evoke a detectable Ca$^{2+}$ increase (Fig. 7, middle). Taken together, these results suggest that Ca$^{2+}$ entry stimulated by NE occurs through activation of α$_1$-AR.

**DISCUSSION**

In the present study, we have provided evidence that NE triggers Ca$^{2+}$ entry that is sensitive to inhibitors of SOCE (Figs. 2 and 3) in rat pinealocytes. We suggest that this Ca$^{2+}$ entry allows for continual Ca$^{2+}$ transients in response to repetitive stimulation of NE (Fig. 2) and have shown that NE-induced Ca$^{2+}$ entry significantly contributes to the upregulation of AANAT (Fig. 1). Interestingly, NE-evoked Ca$^{2+}$ entry was associated with membrane hyperpolarization due to the activation of BK channels (Figs. 4 and 5).

Upon noradrenergic stimulation of the rat pineal gland, intracellular cAMP contents increase ~100-fold compared with the basal level (30), leading to the induction of AANAT gene expression and elevation of AANAT activity. This action of NE is initiated by the β$_1$-AR coupled to G$_s$ protein and adenyl cyclase (35). However, activation of the β$_1$-AR leads to only a 10-fold increase in the cAMP contents. The maximal increase in cAMP is actually reached when the isoproterenol (ISO) for 50 s repeated 5 times every 3 min in presence of 3 μM prazosin. C: application of 10 μM ISO for 30 s repeated 5 times every 3 min in presence of 3 μM prazosin.
activation of the BK channel (Fig. 4). This hypothesis is in line with a previous finding that NE decreased the firing rate in an ex vivo pineal gland (26). Thus it is plausible to suggest that the BK channel coupled to NE-induced Ca\(^{2+}\) entry would reduce excitability by hyperpolarizing the membrane potential.

In the rat pineal gland, parasympathetic activation during daylight hours induces depolarization followed by activation of L-type Ca\(^{2+}\) channels, which results in the secretion of L-glutamate and thus eventually decreases melatonin synthesis (37). Because the longer depolarization could induce the secretion of L-glutamate, coupling of BK channels to NE-induced SOCE might contribute to maintenance of the state of melatonin synthesis by preventing depolarization.

REFERENCES

This work was supported by the Brain Neurobiology Research Program Grant M10412000088-04N1200-08810, System Bio-Dynamics National Research Center of the Ministry of Science and Technology Grant R15-2004-033-03001-0, the Brain Korea 21 Program of the Ministry of Education, and Korea Research Foundation Grant KRF-2003-015-C00513.

17. Lee SY and Lee CO. Inhibition of Na\(^+-K\) pump and L-type Ca\(^{2+}\) channel by glibenclamide in guinea pig ventricular myocytes. J Pharmacol Exp Ther 312: 61–68, 2005.