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

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Norepinephrine activates store-operated Ca\(^{2+}\) entry coupled to large-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels in rat pinealocytes. Am J Physiol Cell Physiol 290: C1060–C1066, 2006. First published November 9, 2005; doi:10.1152/ajpcell.00343.2005.—Norepinephrine (NE) is one of the major neurotransmitters that determine melatonin production in the pineal gland. Although a substantial amount of Ca\(^{2+}\) influx is triggered by NE, the Ca\(^{2+}\) entry pathway and its physiological relevance have not been elucidated adequately. Herein we report that the Ca\(^{2+}\) influx triggered by NE significantly regulates the protein level of 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-glycerol (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 byiberiotoxin (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.

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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).

**Fluorescence measurements of [Ca\(^{2+}\)]\(_i\).** Cells were loaded with 3 \(\mu\)M 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 superimposed 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\(_2\)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).

**Data analysis and statistics.** Each experiment was repeated a minimum of three times, and the results are expressed as means ± SE when appropriate. Numerical data were analyzed using SigmaPlot 2001 for Windows (SPSS, Richmond, CA) and Origin 6.1 software (OriginLab, Northampton, MA). Statistical differences were determined using Student’s t-test. Differences were considered statistically significant at P < 0.05.

**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
inhibitor of L-type Ca\(^{2+}\) channels, which is the major type of VGCC expressed in rat pinealocytes (4). As shown in Fig. 3A, nifedipine did not affect the secondary phase of [Ca\(^{2+}\)]\(_i\) elevation. To the contrary, the secondary phase of the Ca\(^{2+}\) response was abolished by 2-aminoethoxydiphenylborane, a known inhibitor of IP\(_3\) receptors and SOCs (7). Next, we examined the involvement of PLC. In the absence of [Ca\(^{2+}\)]\(_o\), application of NE increased [Ca\(^{2+}\)]\(_i\), because of Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores. When 2 mM [Ca\(^{2+}\)]\(_o\) 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 IP\(_3\), both of which could induce increase in [Ca\(^{2+}\)]\(_i\), (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\(^{2+}\)]\(_i\), PMA, an agonist of PKC that can be activated by DAG, failed to induce an increase in [Ca\(^{2+}\)]\(_i\) (Fig. 3C, middle). Moreover, GF-109203X, an inhibitor of PKC, did not affect the OAG-induced [Ca\(^{2+}\)]\(_i\) increase (data not shown). These results suggest that OAG is able to induce a small amount of [Ca\(^{2+}\)]\(_i\) increase independently of PKC activation. As shown in Fig. 3C, right, NE evoked further Ca\(^{2+}\) influx even after cells were treated with 100 \(\mu\)M OAG, although a higher concentration of OAG (200 \(\mu\)M) did not evoke further Ca\(^{2+}\) influx after treatment with 100 \(\mu\)M 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\(^{2+}\)]\(_i\) 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 evokes NE-dependent melatonin synthesis in rat pinealocytes. 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 (Fig. 5A). 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 Gd3+ 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 L-glutamate, 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 IBTX 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 μM) were applied (data not shown). These results suggest that activation of BK channels triggered by NE participates in the regulation of AANAT.

**Fig. 4.** Activation of SOCE hyperpolarizes membrane potential. A: intracellular Ca2+ concentration ([Ca2+]i) and membrane potential (Vm) were recorded simultaneously. In the presence of 1 μM NE, changes in membrane potential in response to application of 2 mM [Ca2+]o and Ca2+-free extracellular solution were monitored. B and C: effect of 100 nM iberiotoxin (IBTX) on membrane hyperpolarization induced by 1 μM NE (B) or 1 μM Tg (C). D, left, effect of 100 μM Ach on [Ca2+]i, and Vm; right, effect of 3 μM nifedipine (Nif) on [Ca2+]i, increase and depolarization induced by Ach. E: summary of differences in Vm 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 Ca2+-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 mV to +30 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 μM 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 μM NE. Each data point represents a mean ± SE from 7 cells. **P < 0.01, ***P < 0.001. E: I-V relationships showing effect of IBTX on macroscopic outward currents after exposure to 1 μM NE and 10 μM Gd3+. Each data point represents a mean ± SE from 5 cells.
In the presence of prazosin, an antagonist of NE occurs through activation of the subsequent AANAT level (38). Herein we report that activated simultaneously with the stimulation (Fig. 7, adenyl cyclase (35). However, activation of the (Figs. 2 and 3) in rat pinealocytes. We suggest that this Ca2+ entry was associated with membrane hyperpolarization due to the activation of BK channels (Figs. 4 and 5).

In the present study, we have provided evidence that NE triggers Ca2+ entry that is sensitive to inhibitors of SOCE (Figs. 2 and 3) in rat pinealocytes. We suggest that this Ca2+ entry allows for continual Ca2+ transients in response to stimulatory action of NE (Fig. 2) and have shown that NE-induced Ca2+ entry significantly contributes to the upregulation of AANAT (Fig. 1). Interestingly, NE-evoked Ca2+ 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 β1-AR coupled to Gs protein and adenylyl cyclase (35). However, activation of the β1-AR leads to only a 10-fold increase in the cAMP contents. The maximal increase in cAMP is actually reached when the α1-AR is activated simultaneously with the β1-AR (36). Activation of the α1-AR alone has no effect on cAMP, but it potentiates the β1-AR-induced increase (35). Therefore, the role of Ca2+ increased by stimulation of α1-AR has been suggested to potentiate the β1-AR-induced elevation of cAMP contents and the subsequent AANAT level (38). Herein we report that α1-AR is responsible for the Ca2+ transients observed in response to repetitive NE application (Fig. 7). Together with the results suggesting that SOCE might be responsible for continual Ca2+ responses to repetitive stimulation (Fig. 2), the assumed role of SOCE in NE signaling is to refill the Ca2+ stores to enable α1-AR-mediated [Ca2+]i increase, which contributes to the potentiation role of α1-AR.

Outward K+ current plays an important role in determining the membrane potential. In rat pinealocytes, three types of K+ currents (BK current, transient A current, and delayed-rectifier current) have been identified (1, 5). Although activation of BK channels has been reported, its physiological relevance and relationship to Ca2+ entry channels have not yet been elucidated. We have shown that in the absence of NE, BK current is scarcely observed (Fig. 5), a finding that is in agreement with previously published results (1, 3). NE activates Ca2+ and cAMP, called a biochemical “AND” gate to stimulate melatonin production that has been suggested to activate BK current (3). In neurons and neuroendocrine cells, BK channels have been suggested to play an important role in controlling hormone secretion by altering the duration and frequency of action potentials (16, 24, 29). BK channels require both depolarization and elevated [Ca2+], to become activated, and their electrical activity is tightly coupled to changes in [Ca2+]i level. Previous in vivo and in vitro electrophysiological studies showed that pinealocytes exhibit action potentials (22, 26). The firing is suggested to be modulated by NE, which is the primary neurotransmitter regulating melatonin synthesis. Herein we suggest that upon application of NE, although a substantial amount of Ca2+ entered through SOCs, hyperpolarization in membrane potential was detected as a result of the
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.

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