Efferent dorsal unpaired median neurons are pacemaker neurosecretory cells. A Ca\(^{2+}\) background current contributing to the pacemaker activity of cockroach dorsal unpaired median neurons is up-regulated by neurohormone D (NHD), an octapeptide belonging to the adipokinetic hormone family. This modulation by neurohormone D (NHD), an octapeptide belonging to the adipokinetic hormone family, is up-regulated and contributes to the pacemaker activity of cockroach dorsal unpaired median neurons.

We investigated the signaling pathway of NHD-induced current modulation. The membrane depolarization produced by NHD was related to the increase in membrane conductance for Ca\(^{2+}\), Ba\(^{2+}\), or Sr\(^{2+}\). This increase was abolished by LOE 908, an inhibitor of noncapacitative Ca\(^{2+}\) entry (NCCE), and it was strongly attenuated by the phospholipase C inhibitor U73122 and the diacylglycerol lipase inhibitor RHC80267. Arachidonic acid and ETYA mimicked the NHD effect on background current. This was abolished by l-NAME and ODQ, inhibitors of NO synthase and NO-sensitive guanylyl cyclase, respectively, but mimicked by the NO donor sodium nitroprusside and 8-bromo-cGMP. Immunocytochemistry using cGMP antibodies indicated that NHD and ETYA increase cGMP. Inhibition of protein kinase G with KT5823 and R\(_{8-8}\)-cPCT-cGMPS had no effect, whereas zaprinast, a cGMP-specific phosphodiesterase 5,6,9 inhibitor, mimicked the NHD effect. Furthermore, inhibition of the cGMP-activated phosphodiesterase 2 by EHNA and trequinsin abolished the effect of NHD. We conclude that the final step of the NHD signal transduction is the phosphodiesterase 2-induced down-regulation of the cAMP level. This removes a depression of NCCE directly attributed to cAMP because inhibition of protein kinase A with KT5720, R\(_{8}\)-cAMPS, and PKI14-22 amide did not mimic the NHD effect. We also demonstrate that any mechanism increasing the cGMP level can induce NCCE.

Calcium is a universal intracellular messenger that controls a variety of cellular activities such as secretion, metabolism, or gene expression. Many neuropeptides and hormones affect the intracellular signaling pathways involved in the modulation of this calcium entry mechanism still remains unknown, particularly in pacemaker neurosecretory cells.

Because the regulation of ionic conductance is a central key in tuning cellular activity according to physiological demands, the present study has been focused on the mechanism by which a neuronal voltage-independent calcium background current, suspected to be related to NCCE, is modulated. Investigations have been performed on identified insect neurosecretory neurons, namely efferent dorsal unpaired median (DUM) neurons (5). These cells that contain the biogenic amine octopamine display a pacemaker activity that involves, for example, a variety of voltage-gated calcium currents (6, 7) as well as a voltage-independent calcium back-
background conductance was permeable to Ca\(^{2+}\) currents were recorded 40 ms after stepping from representing differences between currents recorded before and 2 min after NHD application were normalized by the cell capacitance. Steady-state and PKI from Calbiochem (Bad Soden, Germany); EHNA, L-NAME, 10 nM NHD at a holding potential of SPNWNH\(_2\) known to accelerate spiking by enhancing cal-
lular calcium concentration ([Ca\(^{2+}\)]) physiologically important for: 1) adjusting the free intracellular calcium influx via the activation of this calcium background current inhibited by the NCCE blocker LOE 908 (40 \(\mu\)M). Data are given as mean ± S.D. (n = 7) and were obtained by subtracting the current recorded 2 min after LOE 908 application from control current. The bath solution contained 3 mM Sr\(^{2+}\). The curve represents a Goldman-Hodgkin-Katz fit for Sr\(^{2+}\) conductance. F, the depolarization of the membrane potential induced by 10 nM NHD was strongly reduced by LOE 908 (40 \(\mu\)M). Data are mean ± S.D. (n = 6).

**EXPERIMENTAL PROCEDURES**

**Materials**—AA, 8-Br-cAMP, sodium nitroprusside (SNP), trequinsin, and tetrodotoxin were obtained from Sigma; 8-Br-cGMP, \(R\)-cAMPS, and PKI from Calbiochem (Bad Soden, Germany); EHNA, L-NAME, ODQ, and zaprinast from Toecis (Ellisville, MO), ETYA and OAG from Alexis (Grünberg, Germany); KT8823, rolipram, and \(R\)-8-pCPT-

cGMPs from VWR (Fontenay Sous Bois, France), NHD from Peninsula (Belmont, CA), RHC80267 from Biomol (Hamburg, Germany), and U73122 from RBI (Natick, MA). Finally, LOE 908 was kindly provided by Boehringer Ingelheim (Germany).

**Cell Isolation**—Isolation of adult DUM neuron cell bodies was performed under sterile conditions using enzymatic digestion and mechanical dissociation of the median parts of both fifth and terminal abdominal ganglia of adult male cockroaches (*Periplaneta americana*), as described previously (8, 11). Briefly, ganglia were excised, desheathed, and incubated for 10 min at room temperature in bath solution (BS1) containing (in mM): 190 NaCl, 5 KCl, 5 CaCl\(_2\), 2 MgCl\(_2\), 10 HEPES, pH 7.4 containing 0.5 mg/ml trypsin (type II, Sigma) and 0.5 mg/ml collagenase (type I, Sigma). After thoroughly washing off the enzyme the ganglia were stored in BS1 for at least 1 h. Subsequently isolated DUM neuron cell bodies were immediately used or maintained at 29 °C for 24 h before experiments were carried out.

**Electrophysiology**—Electrophysiological experiments on isolated DUM neuron cell bodies were performed at room temperature using the whole cell patch clamp technique. Pipettes having resistances of 1–2 M\(\Omega\) were pulled with a P-87 micropipette puller (Sutter Instrument Co., Novato, CA) from borosilicate capillary tubes (Hilgenberg, Malsfeld, Germany). Current/voltage measurements and data acquisition were performed using an EPC9 patch clamp amplifier controlled by PULSE software (HEKA Elektronik, Lambrecht, Germany).

Membrane resting potential and spiking of DUM neurons were recorded under current-clamp conditions without current injection (except when otherwise stated). Neurons were bathed in bath solution BS1 (cf. “Cell Isolation”). Patch pipettes (resistance >1.5 M\(\Omega\)) were filled with pipette solution (PS1) composed of 190 mM K-Gluconate, 5 mM

FIG. 1. Modulation of membrane potential and Ca\(^{2+}\) background current in DUM neurons by NHD. A, bath application of NHD (10 nM) depolarized the resting membrane potential of isolated DUM cell bodies held at about −60 mV. The NHD effect observed on membrane potential was measured under current-clamp conditions just before action potentials that were elicited by a 40-ms depolarizing current pulse (0.4 nA). Data are mean ± S.D. B, NHD enhanced the Ca\(^{2+}\) background current density in a concentration-dependent manner. Individual currents representing differences between currents recorded before and 2 min after NHD application were normalized by the cell capacitance. Steady-state currents were recorded 40 ms after stepping from −50 mV to the indicated voltages. Data shown are mean ± S.D. (n = 7). The dotted lines are linear fits to data and solid curves are fits according to Goldman-Hodgkin-Katz models for a pure Ca\(^{2+}\) conductance. C, the NHD-induced background conductance was permeable to Ca\(^{2+}\), Ba\(^{2+}\), and Sr\(^{2+}\). Bar histograms represent mean ± S.D. (n = 5) of current densities evoked by 10 nM NHD at a holding potential of −90 mV. Bath solutions contained 5 mM Ca\(^{2+}\), Ba\(^{2+}\), and Sr\(^{2+}\), respectively. Application of 1 \(\mu\)M thapsigargin (1 min before NHD application) had no significant effect on the response to NHD. D, Sr\(^{2+}\) background current inhibited by the NCCE blocker LOE 908 (40 \(\mu\)M). Data are given as mean ± S.D. (n = 7) and were obtained by subtracting the current recorded 2 min after LOE 908 application from control current. The bath solution contained 3 mM Sr\(^{2+}\). The curve represents a Goldman-Hodgkin-Katz fit for Sr\(^{2+}\) conductance. E, current induced by 10 nM NHD at a holding potential of −90 mV in bath solution containing 3 mM Sr\(^{2+}\). The response to NHD was strongly attenuated in the presence of LOE 908 (40 \(\mu\)M). Data are expressed as mean ± S.D. (n = 6). F, the depolarization of the membrane potential induced by 10 nM NHD was strongly reduced by LOE 908 (40 \(\mu\)M). Data are mean ± S.D. (n = 6).
NaCl, 1 mM CaCl₂, 3 mM EGTA, 2 mM MgATP, and 10 mM HEPES (pH 7.25). Between recordings (duration 1 s) the cells were held under voltage clamp at a holding potential of ~70 mV. Background currents were measured under voltage-clamp with holding potential of ~50 mV. Currents were measured at the end of 40-ms lasting pulses ranging from ~120 to ~50 mV and taken for analysis without leakage correction. The pipette solution (PS2) contained (in mM): 100 choline methyl sulfate (CMS), 60 CaOH, 8 ClCl, 30 tetraethylammonium-Br, 2 MgATP, 1 CaCl₂, 3 EGTA, 10 HEPES. The free Ca²⁺ concentration was calculated to amount to 63 nM (WEBMAXC [12]). The bath solution BS2 contained (in mM): 190 choline methyl sulfate, 5 CaCl₂, 10 HEPES, and 0.5 μM tetrodotoxin. When using Ba²⁺ or Sr²⁺ as charge carrier the 5 mM Ca²⁺ were substituted with 5 mM Ba²⁺ or Sr²⁺, or 3 mM Sr²⁺. The pH value was adjusted to 7.4 and 7.25 for bath and pipette solutions, respectively. Liquid junction potential between pipette and bath solution was taken into account before establishing the seal. This combination of bath and pipette solutions allowed measurements of isolated Ca²⁺ currents ~2 min after breaking into the cell. Application or washout of blocking agents was performed with the bath perfusion system BPS4 (ALA, New York).

Fluorescence Optic Measurement of Intracellular Calcium—Cells were loaded with fura-2 by incubation in BS1 (cf. “Cell Isolation”) containing 2 μM fura-2/acetomethylester for 20 min. Free [Ca²⁺], was determined with the fluorescence ratio method according to Ref. 13. Light exciting fura-2 at 340 and 380 nm, provided by Polychrome II (T.I.L.L. Photonics, Graefelfing, Germany), was coupled via an epifluorescence condenser into an Axioskop FS (Carl Zeiss, Jena, Germany) equipped with a water immersion objective (LUMPLFL 40X/WIR/0.8; Olympus, Hamburg, Germany). Emitted light was separated by a 40-nm dichroic mirror and filtered with a 420-nm long-pass filter. The free [Ca²⁺], was calculated according to the equation [Ca²⁺], = Rₐ[R - Rₚ] / (Rₛₚ - R). The Rₛₚ, Rₚ, and Rₛₚ were determined using DUM neurons permeabilized with 2 μM ionomycin and three solutions with different calcium concentrations (Ca²⁺ free, 5 mM Ca²⁺, and 500 mM Ca²⁺). The 500 nM calcium concentration solution was calculated with WEBMAXC [12] and measured with calcium-sensitive electrodes (KWIK tips; WPI, Berlin, Germany). The values of Kₛₚ, Rₚ, and Rₛₚ were 3.72, 0.38, and 5.9 μM, respectively.

Photomultiplier-based Fluorescence Detection and Fluorescence Imaging—The fluorescence detection area of interest (~200 μm²) amounted to about 10% of the cross-section of a DUM neuron. A cooled CCD camera (Imago, T.I.L.L. Photonics) controlled by TILLVision 4.0 (T.I.L.L. Photonics) was equipped with a “view finder” system (T.I.L.L. Photonics). Image pairs were obtained by excitation for 100 ms at 340 and 380 nm, with a 500 nM calcium concentration solution as charge carrier.

Immunocytochemistry—For light microscope immunocytochemistry, isolated DUM neuron cell bodies were fixed for 1 h in 4% paraformaldehyde containing 5% (w/v) sucrose in phosphate-buffered saline (PBS). After fixation, cells were washed 3 times for 5 min each in PBS and 5 min in PBS containing 0.2% Triton X-100 (PBS-T). To block nonspecific binding of the primary antibody, cell bodies were preincubated with 4% bovine serum albumin in PBS-T for 1 h. Primary antiserum (sheep anti-formaldehyde fixed cGMP, generous gift of Dr. J. De Vente), diluted 1/2000 in PBS-T was applied overnight at 4 °C. After repeated washing in PBS-T, the secondary antibody (fluorescein isothiocyanate-labeled rabbit anti-sheep IgG (Euromedex, France), diluted 1/150 in PBS-T containing 1% bovine serum albumin was applied for 3 h at 20 °C in the dark. Isolated DUM neuron cell bodies were then washed in 4% bovine serum albumin in PBS and mounted on glass slides in glycerol/PBS. The fluorescence detection area was 300 μm on an Axioskop microscope. Images were digitized with Axiovision and stored as TIF format files for later analysis.

Data Analysis—Results were given as mean ± S.D. or S.E., n = number of cells. The evaluation of statistical significance of differences was performed with Student’s t test. For data analysis including fitting procedures, the software IGOR (WaveMetrics, Lake Oswego, OR) was used.

RESULTS

Peptidergic Regulation of Background Current—Bath application of the adipokinetic hormone, NHD (10 nM), depolarized the resting membrane potential of DUM neurons from ~60.1 ± 1.8 mV (n = 9) to ~45.7 ± 2.4 mV (n = 12; Fig. 1A). Under
experimental conditions that suppressed Na\(^+\) and K\(^+\) currents in DUM neurons and only allowed Ca\(^{2+}\) currents, NHD generated a voltage-independent current in a concentration-dependent manner (Fig. 1B). Linear approximation of the reversal potential of the NHD-induced current gave figures around 0 mV. Alternatively, the currents could be reasonably well fitted by a conductance showing Goldman rectification because of the Ca\(^{2+}\) gradient across the membrane (Fig. 1B). Whereas CCE channels show higher Ca\(^{2+}\) selectivity, NCCE channels conduct Ca\(^{2+}\), Ba\(^{2+}\), and Sr\(^{2+}\) equally well (1). We therefore tested the NHD effect when Ca\(^{2+}\) was substituted with Ba\(^{2+}\) or Sr\(^{2+}\) as charge carrier did not significantly differ from that of Ca\(^{2+}\) currents (Fig. 1C). It was previously shown that NHD up-regulated a constitutively active background current rather than a receptor-activated or store-operated current (9, 10). The latter was confirmed with experiments using thapsigargin. As indicated in Fig. 1C, the size of the Sr\(^{2+}\) current generated by 10 nM NHD was not significantly changed by prior store depletion with thapsigargin (1 \mu M). Furthermore, LOE 908, a blocker of NCCE (4), reduced a voltage-independent Sr\(^{2+}\) current. This LOE908-sensitive current (LOE – control) is shown in Fig. 1D and represents a current that is constitutively active. In the presence of LOE 908 (40 \mu M), NHD failed to increase the Sr\(^{2+}\) current in the voltage range between −60 and −120 mV (shown for −90 mV in Fig. 1E). In the same way, as illustrated in Fig. 1F, the depolarization of membrane potential induced by NHD was strongly reduced by LOE 908.

Because NCCE is evoked by activation of phospholipase C (PLC) in many preparations (e.g. Ref. 3), we tested whether inhibition of this enzyme would impair the NHD effect. Pretreatment of DUM neurons with U73122 (3 \mu M), an inhibitor of PLC, caused a dramatic reduction of the response to 10 nM NHD (Fig. 2A). PLC activation leads to the production of IP\(_3\) and diacylglycerol (DAG), which both could potentially mediate the NHD effect on background current. An earlier study (9) has shown that inhibition of the IP\(_3\) receptor with heparin had no effect on intracellular Ca\(^{2+}\) signals evoked by the NHD-induced increase of Ca\(^{2+}\) background current. In addition, and as indicated above, depletion of Ca\(^{2+}\) stores by pretreatment of cells with 1 \mu M thapsigargin (an event expected as a result of IP\(_3\) production) did not affect the NHD-induced potentiation of background current (10). Thus IP\(_3\) does not seem to play a significant role in controlling the Ca\(^{2+}\) background current. Therefore we tried to mimic the effect of NHD with the DAG analog OAG. As shown in Fig. 2B, 1 \mu M OAG induced a voltage-independent Sr\(^{2+}\) current within 2 min after application. Subsequent application of NHD (even at 20 nM) did not lead to a remarkable further current increase (Fig. 2B). This supports the conclusion that indeed DAG mediates, either directly or indirectly, the peptide effect on background current. DAG, for example, activates certain members of the TRP channel family (14) and it is not excluded that a Trp channel might be responsible for the background current. On the other hand, DAG is known to be metabolized by DAG lipase to AA, which also activates NCCE (15). To test whether DAG modulates the background channel in DUM neurons directly or via generation of AA (or other AA derivatives) we blocked DAG lipase with RHC80267 (3). In the presence of RHC80267 (50 \mu M), there was no significant response to 10 nM NHD (Fig. 2A). Thus DAG itself is not capable of acting on the background channel but it has to be metabolized to AA to up-regulate the background current. If this was true, AA should mimic the DAG effect. Indeed, AA (10 \mu M) evoked a Sr\(^{2+}\) background current very similar in size to that of evoked by 1 \mu M OAG (Fig. 2C). To exclude the possibility that AA metabolites were involved in up-regulating the background current, ETYA (10 \mu M), an acety-
A leucine AA analog that blocks all AA-metabolizing enzymes by acting as a false substrate and mimics the response to AA, was tested. As shown in Fig. 2C, the ETYA-induced increase in Sr2+ background current was very similar to that produced by AA (Fig. 2C).

Role of NO-cGMP Pathway in the Regulation of the Background Calcium Current—The results described above do not necessarily imply that AA would directly act on the background channel and it remains open whether AA might affect further intracellular signaling pathways. Among possible candidates, NO and cGMP are involved in the regulation of various Ca2+ entry pathways. For example, in vascular smooth muscle cells AA stimulates NCCE via the NO system (16). We, therefore, tested the putative involvement of NO and cGMP in the intracellular regulation of NCCE by NHD in DUM neurons.

As previously indicated and illustrated in Fig. 1A, a control experiment showed that NHD (10 nM) produced membrane depolarization of DUM neurons. An involvement of the NO-GC/cGMP cascade in the NHD-induced signal transduction process was studied by comparing the amplitude of the NHD-induced depolarization (normalized to the maximum effect of 10 nM NHD) before and after application of ODQ (10 μM), a selective inhibitor of NO-GC. As shown in Fig. 3A, ODQ strongly reduced the membrane depolarization produced by 10 nM NHD (by 81.9 ± 3.8%, n = 5). In line with this, ODQ abolished the NHD-induced rise in Sr2+ background current (Fig. 3B). This suggests that NHD-induced depolarization is mediated by activation of NO-GC, which in turn raises the intracellular cGMP.

![Fig. 5. A, inhibition of PKG by KT5823 (1 μM, n = 6) and R,R,S-PT-CPT-cGMPS (cGMPS, 10 μM, n = 5) did not affect the depolarizing effect induced by NHD. B, zaprinast (ZAP, 20 μM, n = 6) alone, an inhibitor of PDE 5/6/9, mimicked the effect of NHD. In the presence of ZAP, NHD did not produce any further depolarization (n = 6). C, inhibition of cAMP-dependent PDE by rolipram (ROL, 20 μM, n = 5) did not affect the depolarizing effect induced by NHD. D, the PDE2 inhibitor EHNA (20 μM, n = 5) produced a slight hyperpolarization and it abolished the depolarizing effect of NHD (10 nM, n = 7). E, the PDE2 inhibiting trequinsin (TRE, 10 μM, n = 3) also produced a slight hyperpolarization and very strongly depressed the depolarizing effect of NHD (10 nM, n = 4). F, effects of EHNA (10 μM, n = 7) and trequinsin (10 μM, n = 5) on the background current density induced by 10 nM NHD. Data were obtained at a holding potential of −90 mV in bath solution containing 3 mM Sr2+. The response to NHD was abolished by EHNA and strongly reduced by trequinsin. G, effect of trequinsin (10 μM) on the stimulation of background current by 8-Br-cGMP (cGMPS, 10 μM, n = 4) and SNP (1 μM, n = 4). Trequisin completely abolished cGMPS-induced stimulation and strongly reduced the SNP effect on background current. H, effect of PKA inhibitors KT5720 (10 μM, n = 4), Rp-cAMPS (cAMPS, 100 μM, n = 7), and PKI (1 μM, n = 5) on the NHD-induced stimulation of the background current. The PKA inhibitors were applied 3 min before NHD application (in continuous presence of PKA inhibitors). Data were obtained 2 min after NHD application and represent the mean ± S.E. Pre-treatment with PKA inhibitors had no statistically significant effect on NHD action. Data shown from A–G represents mean ± S.D.
level. According to this, enhancement of the cGMP level is expected to produce the NHD effect on background current. Indeed, application of the membrane-permeable cGMP analog 8-Br-cGMP (10 μM) caused an increase in Sr2+ background current density (Fig. 3C). Furthermore, production of NO should stimulate the NO-GC in the absence of NHD. We thus tested the effect of the NO donor SNP. In this case, SNP (1 μM) produced an increase in Sr2+ background current (Fig. 3C).

Conversely, inhibition of the NO synthase with L-NAME (10 μM) abolished the effect of 10 nM NHD on this current (Fig. 3D). Also the response to 10 μM ETYA is strongly reduced (compare Figs. 2C with 3D). Finally, although L-NAME itself caused a slight current reduction that may reflect a constitutive NO synthase activity (Fig. 3D), the effect of 8-Br-cGMP remained unaffected by L-NAME (Fig. 3D). These results indicate that NHD triggers the activation of NO-GC, which catalyzes the conversion of GTP to cGMP. This was further confirmed more directly by studying the effect of NHD before and after application of ODQ (10 μM) on the cGMP level using antibody raised against formaldehyde-fixed cGMP (17). Compared with control experiments (Fig. 4, A1) pretreatment with NHD (10 nM) increased cGMP immunoreactivity in DUM neurons (Fig. 4, A2). By contrast, the presence of 10 μM ODQ during incubation of DUM neurons dramatically reduced the intensity of fluorescent cGMP immunostaining observed with NHD (Fig. 4, A3). As also expected, application of 10 μM ETYA under the same experimental conditions also increased cGMP immunoreactivity (Fig. 4, B2), an effect that was reduced by ODQ (10 μM) (Fig. 4, B3). These results confirmed electrophysiological data presented above and indicated that ETYA, the analog of AA, was able to directly activate the NO-sensitive guanylyl cyclase.

The resulting accumulation of cGMP is known to regulate complex signaling cascades through immediate downstream effectors including cGMP-dependent protein kinase (PKG) and/or cGMP-regulated phosphodiesterases (PDE). To study whether PKG was involved in such an effect we applied KT5823, an inhibitor of PKG. As illustrated in Fig. 5A, 1 μM KT5823, and also Rp,-8-pCPT-cGMP (10 μM), did not significantly affect NHD-induced depolarization, indicating that PKG does not play a role in the NHD signal transduction process. We next focused on a possible effect of cGMP in the regulation of PDEs. Among different mechanisms reported in the literature (18), cGMP may regulate PDEs by increasing the activity of PDE isozymes (e.g., PDE5), known to convert cGMP to 5’-nucleoside phosphate or by altering the rate of hydrolysis of cAMP through competition at the catalytic site (e.g., PDE2). In this way, zaprinast, which inhibits cGMP-specific PDE, like PDE5, was first tested. As indicated in Fig. 5B, zaprinast (20 μM) alone was able to produce membrane depolarization of the DUM neuron very similar in amplitude to that induced by NHD. This indicates that elevation in the intracellular cGMP level resulting from zaprinast-induced inhibition of PDE mimics the effect of NHD. In addition, no significant change of membrane potential was observed when 10 nM NHD was applied in the presence of zaprinast. It should be noted that it is unlikely that NHD acted through activation of cAMP-dependent PDE because 20 μM rolipram (an inhibitor of cAMP-dependent PDE) did not significantly affect the NHD effect (Fig. 5C).

To examine whether NHD-induced depolarization was modulated by a cGMP-stimulated PDE with cAMP hydrolytic activity we inhibited PDE2 with EHNA. Fig. 5D indicates that 20 μM EHNA alone produced a slight hyperpolarization of DUM cells. When NHD (10 nM) was applied in the presence of EHNA, the usually observed depolarization did not appear. Instead of this we observed a hyperpolarization similar to that obtained with EHNA alone (Fig. 5D). p > 0.05; n = 7). Trequinsin, also known to inhibit PDE2, strongly attenuated the effect of NHD on membrane potential (Fig. 5E). These results were confirmed under voltage-clamp conditions. In this case, both PDE2 inhibitors completely abolished the NHD-induced rise in the Sr2+ background current (Fig. 5F). Moreover, trequinsin also abolished the stimulating effect of 8-Br-cGMP on background current and it largely reduced the current induced by SNP (Fig. 5G).

Taken together, the above results support the view that AA stimulates a NO-sensitive guanylyl cyclase that in turn increases the cGMP level. This activates a cGMP-specific PDE with cAMP hydrolytic activity. Finally, the resulting decrease in cAMP level potentiates the background current. When the drop in cAMP level would cause a reduced level of background channel phosphorylation by PKA, application of PKA inhibitors...
should mimic this effect and subsequent NHD application should have no further effect. We thus tested the effect of three inhibitors of PKA, KT5720, Rp-cAMPS, and myristoylated PKA inhibiting peptide 14–22 amide (PKI) on the NHD-induced stimulation of Sr\(^{2+}\) background current. For example, KT5720 has previously been shown to abolish the effects of NHD on voltage-gated Na\(^+\) currents (19) and Ca\(^{2+}\) currents (20). Preincubation of cells with these PKA inhibitors for 3 min did not significantly enhance the Sr\(^{2+}\) background current and did not change the effect of 10 nM NHD on this current (Fig. 5H). Thus, because no PKA inhibitor seemed to mimic the NHD effect, dephosphorylation of background channels via the reduction of Ca\(^{2+}\) level was reached 2–6 min after the start of peptide application, whereas the relaxation of such signals took between 5 and 15 min (Fig. 8, A5). The maximum [Ca\(^{2+}\)]\(_{i}\) level was reached 2–6 min after the start of peptide application, whereas the relaxation of such signals took between 5 and 15 min (Fig. 8, A6). As demonstrated earlier, these large Ca\(^{2+}\) signals were accompanied by intracellular Ca\(^{2+}\) release via ryanodine receptor activation (9, 10). Pre-treatment of cells with a low peptide concentration as shown in the experiments in Figs. 6A and 8, A2–A4, contributes to filling of Ca\(^{2+}\) stores from which Ca\(^{2+}\) is released by the next stimulus, i.e. by increasing [Ca\(^{2+}\)]\(_{i}\), up to the activation threshold for ryanodine receptors (10).

These results demonstrate the role of the Ca\(^{2+}\) background channel in controlling [Ca\(^{2+}\)]\(_{i}\). In light of previous experiments (see above), block of this background channel by LOE 908 should reduce [Ca\(^{2+}\)]\(_{i}\). After acute isolation of DUM neuron somata, [Ca\(^{2+}\)]\(_{i}\), at the apical pole was sometimes enhanced (e.g. Fig. 6C, time 0). Application of 40 nM LOE 908 in such cells caused an immediate reduction of [Ca\(^{2+}\)]\(_{i}\), in this region (Fig. 6C, solid line). For the mean initial [Ca\(^{2+}\)]\(_{i}\) of 168 ± 41 nM, LOE 908 caused a reduction of 50 ± 13 nM (n = 6, ±S.E.). Interestingly, there was also a slight reduction of [Ca\(^{2+}\)]\(_{i}\), by LOE 908 in other regions of the cells, even at the basal pole where the reduction amounted to 29 ± 12 nM (apparent in Fig. 6C, dotted line).

The electrophysiological experiments revealed that OAG, AA, and ETYA mimic the effect of NHD on Ca\(^{2+}\) background current, i.e. by acting on or as elements of the NHD signal transduction pathway. Thus, these agents are also expected to mimic the effects of NHD on [Ca\(^{2+}\)]\(_{i}\). OAG (1 μM) produced
conclusion that AA plays a role within the transduction cascade of NO-GC by ODQ (10 μM) prevented the stimulating effect of NHD on the Sr2+ current (Fig. 3B). ODQ also abolished the induction of Ca2+ signals by ETYA (Fig. 7D). On the other hand, application of 8-Br-cGMP, an analog of the product of NO-GC, mimicked the effect of NHD and led to a rise in [Ca2+]i at the apical pole of cells (Fig. 8, C3). Furthermore, stimulation of NO-GC with SNP also enhanced [Ca2+]i in this region. Applied at a concentration of 1 μM, SNP produced a gradual increase in [Ca2+]i at the apical pole of cells (by 125 ± 54 nM, n = 4, ± S.D., not shown). 10 μM SNP caused a rise by 500 ± 169 nM, n = 4, ± S.D., Fig. 8, C5). The effects of 8-bromo-cGMP and SNP were reversible, after washing off the agents [Ca2+]i recovered to the resting level. Further application of each compound again produced a Ca2+ signal.

Our above results have supported the view that one of the final steps of the NHD-induced signal transduction cascade is the down-regulation of the intracellular cAMP level. When ETYA in turn leads to a reduction of [cAMP], an artificial increase in [cAMP], e.g. by use of the membrane permeable analog of cAMP, 8-Br-cAMP, should prevent the ETYA-induced rise in [Ca2+]i. Indeed, application of 500 nM 8-Br-cAMP 2 min before application of ETYA completely abolished any Ca2+ signal (Fig. 7D). Furthermore, when 1 μM 8-Br-cAMP was applied 10 min after SNP (10 μM), the enhanced [Ca2+]i level dropped within 15 min (Fig. 8, C6). Even after washing off cAMP a further application of SNP failed to enhance [Ca2+]i again (n = 3, data not shown).

Regulation of Background Current and Spiking—NHD has complex effects on spiking of DUM neurons that includes an enhancement of: 1) the action potential discharge frequency by accelerating the pacemaker depolarization, and 2) both overshoot and undershoot amplitudes (21). The Ca2+ background current solely affects pacemaking and pharmacological manipulation of this current only changes pacemaker depolarization. Reduction of the Ca2+ background current by LOE 908 (10 μM) prolonged the interspike interval from 155 ± 24 to 312 ± 55 ms (n = 6, ± S.E., Fig. 9, A1 and A2). The shape of action potential remained unaffected (Fig. 9, A1). Because we previously indicated that AA might affect NCCE, we also tested this compound on spontaneous electrical activity. Potentiation of this background current by AA (10 μM) shortened the interspike interval from 86 ± 13 (S.E.) to 78 ± 12 (S.E.) ms (n = 6, + S.E., Fig. 9, B1 and B2), again without affecting the action potential (Fig. 9, B1). For comparison, 10 nM NHD for 2 min accelerates the spike frequency (Fig. 9, C1 and C2) but it also slightly increased action potential overshoot and hyperpolarization (Fig. 9, C1).

**FIG. 8.** A, effect of NHD on free intracellular Ca2+ concentration revealed by the imaging technique. A1, transmission image of an isolated DUM cell. Bar, 20 μM. After loading with fura-2/acetomethyl-ester, the fluorescence ratio calculated after excitation at 540 and 380 nm (100 ms) was recorded before (A2) and 5 min after continuous application of 10 μM NHD (A3). Note that NHD caused a rise in [Ca2+]i, restricted to the apical pole of the cell. The signal declined within 5 min after washing off the peptide (A4). A5, 5 min after application of 10 μM NHD, there was a massive rise in [Ca2+]i throughout the cell. The largest increase also appeared at the apical pole of the neuronal cell body. A6, [Ca2+]i measured 15 min after washing off NHD. B, effect of 10 μM ETYA on free [Ca2+]i, B1, transmission image of an isolated DUM cell. Bar, 20 μM. [Ca2+]i was measured before (B2) and 5 min after application of ETYA (B4). Again, the largest rise in [Ca2+]i appeared at the apical pole, whereas there was hardly a change at the basal pole. B3 represents the ETYA-induced changes in [Ca2+]i (in regions indicated in B2) plotted as a function of time of application. C, effect of 2 μM 8-Br-cGMP and 10 μM SNP on free [Ca2+]i, C1, transmission image of a DUM cell. Bar, 25 μM. [Ca2+]i was measured before (C2) and 3 min after application of 8-Br-cGMP (C3). 25 min after washing off 8-Br-cGMP [Ca2+]i attained the resting level (C4). C5, SNP (10 μM for 3 min) produced a rise in [Ca2+]i. C6, application of 8-Br-cAMP (1 μM) in the continuous presence of SNP dramatically reduced [Ca2+]i. Note that the [Ca2+]i level attained after 15 min incubation was lower than under control conditions (C2 and C4).

mostlly large Ca2+ rises (by 243 ± 214 nM, n = 8, ± S.D.) as shown in Fig. 7A. This corresponds to the rather large effect of 1 μM OAG on the resting current (Fig. 2B). A gradual increase in [Ca2+]i, by OAG was observed when it was applied after decline of the initial Ca2+ signal. This rise amounted to 54 ± 23 nM (n = 4, not shown). AA increased [Ca2+]i, in a concentration-dependent manner (Fig. 7, B and C). When NHD (10 nM) was applied in the presence of 10 μM AA, there was no significant further increase in [Ca2+]i (Fig. 7C). This again supports the conclusion that AA plays a role within the transduction cascade of the NHD signal. Similarly, 10 μM ETYA produced gradual responses (not shown) as well as large [Ca2+]i rises (Fig. 8, B3).

Furthermore, as shown in Fig. 8D, the Ca2+ signal evoked by ETYA was more pronounced at the apical pole (Fig. 8, B4), whereas there was no signal at the basal pole (Fig. 8, B3).

Discussion

In the present study we investigated the mechanism and the physiological effects of the potentiation of a NCCE current in DUM neurons by NHD. There are two main physiological consequences for the pacemaker neurons. First, there is an elevation of [Ca2+]i and second, there is an increase in spike frequency. The second aspect shows a new role of NCCE, namely the control of pacemaker neuronal activity. The mechanism by which NHD stimulates NCCE has been shown to be complex and to involve more than one of the classical signal transduction cascades. From the results of this work we suggest the following briefly outlined cascade of molecular events underlying this intracellular regulation (Fig. 10). Stimulation of adi-
plays a crucial role in that it is the substrate for AA production by DAG lipase. AA activates the NO-sensitive guanylyl cyclase thus leading to an elevated intracellular cGMP level. Increased cGMP concentration results in activation of the cGMP-dependent PDE2 that is characterized by cAMP hydrolytic activity. Finally, the decrease of the cAMP level produced by PDE2 stimulates NCCE.

Some of the mechanisms proposed above have to be now considered in more detail. The up-regulation of a constitutive Ca$^{2+}$ conductance in the neurosecretory DUM neurons by NHD was found to critically depend on stimulation of PLC. The two products of PLC activity, IP$_3$ and DAG, can activate different Ca$^{2+}$ entry pathways. Whereas IP$_3$, via triggering intracellular Ca$^{2+}$ release resulting in store depletion, can activate CCE, DAG can trigger NCCE by various mechanisms. It was shown to activate certain members of the short TRP channel family.

![Figure 9](attachment:image9.png)

**Fig. 9.** Spontaneous electrical activity of DUM neuron cell bodies recorded under various conditions (A1, B1, and C1) together with the corresponding interspike interval histograms (A2, B2, and C2). Cells were held under voltage-clamp at −90 mV. Prior to switching to current clamp mode the holding potential was reduced to −50 mV, which corresponds to the physiological membrane potential. A1, LOE 908 (10 μM) prolonged the interspike interval (A2) by slowing down the pacemaker depolarization but it did not affect the action potential shape. B1, AA (10 μM) shortened the interspike interval (B2) by accelerating pacemaker depolarization. Again, action potential shape remained unchanged. For comparison, NHD (10 nM) applied for 2 min, displayed more complex effects (C1 and C2). It accelerated pacemaking and enhanced both overshoot and hyperpolarization. Asterisks above each histogram indicate that the differences are statistically significant according to the paired t test (p < 0.05).

![Figure 10](attachment:image10.png)

**Fig. 10.** Hypothetic pattern of regulation of NCCE. This scheme summarizes the essential components of the intracellular signaling pathways that may regulate NCCE via the effect of NHD (see text for details). AKH-R, adipokinetic hormone receptor.
(14). Formation of AA from DAG because of DAG lipase activity is another way to activate NCCE (22). In our case inhibition of DAG lipase abolishes AA/EYTA mimics the NHD effect, i.e. DAG has to be metabolized to AA to facilitate NCCE. However, whereas, for example, in HEK293 cells AA directly activates non-capacitative Ca\(^{2+}\) entry channels (23, 24) this seems not to be the case in DUM cells. Our both electrophysiological and immunocytochemical studies favor the view that AA directly stimulates in a presently unknown way a NO-sensitive guanylyl cyclase (e.g. Ref. 25). An example for such a process in the nervous system is the moulting regulation of the tobacco hornworm, Manduca sexta. The ecdysis-triggering eclosion hormone was found to stimulate cGMP production via AA (26). In DUM neurons, the enhanced cGMP level stimulates cGMP-sensitive PDE2 that seems to reduce the cAMP level near the Ca\(^{2+}\) background channels. In a previous study (27) we showed that application of the membrane-permeable cAMP analog 8-Br-cAMP reduces the Ca\(^{2+}\) background current. This was also confirmed in this present study (Fig. 7D). Thus cAMP seems to exert an inhibiting control on Ca\(^{2+}\) background channels and the resting cAMP level is obviously sufficient to partially inhibit these background channels.

Taken together, the transduction cascade of the NHD signal in DUM neurons involves cooperation of various signal transduction systems, such as the PLC, NO, and cAMP systems. A cooperative effect of the NO-sensitive pathway and the PLC system in the control of NCCE channels has been recently reported in vascular smooth muscle cells (16) but never in pacemaker neurosecretory cells. Such tight regulation of a signal transduction process allows the integration of various signal inputs that thereby could influence the neurosecretory function via a modulation of spontaneous activity. On the other hand, the experiments with the NO donor SNP have also shown that NO-GC plays a central role in the regulation of NCCE channels in DUM neurons and that, independent of inputs stimulating PLC, NO synthesis may be sufficient to enhance Ca\(^{2+}\) entry.

The imaging experiments performed with the blocker of NCCE channels, LOE 908, have indicated that the most pronounced reduction in [Ca\(^{2+}\)]
i occurred mainly at the apical pole of cells. This could be explained by an enhanced expression level of NCCE channels particularly at the apical pole. The adiopokinetic hormone receptors that recognizes NHD and the downstream signal transduction machinery including G proteins may also be spatially restricted to the apical pole. Within the ganglion, the apical pole of DUM cell somata is located at the dorsal surface. The peptide NHD is produced in the corpora cardiaca and in insects, the pars intercerebralis-corpora cardiaca system is the endocrinological equivalent of the vertebrate hypothalamus-pituitary system. From corpora cardiaca, NHD is released into circulation. To attend the DUM neurons, NHD has to cross the blood-brain barrier, presumably by an active transport mechanism. Because the apical pole of DUM neurons is situated at the surface of the ganglion, it is the best accessible part of the neuron for approaching membrane NHD sites.

Finally, from an electrophysiological point of view, localization of the machinery transducing the NHD signal to the target Ca\(^{2+}\) background channel, a major player in setting pacemaker depolarization, is interesting in that it is situated opposite to the spike generating zone. The latter zone near the primary neurite corresponds to the axon hillock in vertebrate neurons and is equipped with the highest density of voltage-gated Na\(^{+}\) channels (28, 29). NHD also acts on Na\(^{+}\) channels that require the presence of adipokinetic hormone receptors, but it enhances the cAMP level to modulate these channels (21). A spatial distance between these two distinct signal transduction machineries seems to be crucial for proper function of NHD, which raises [cAMP] to accelerate the inactivation of Na\(^{+}\) channels (19) and depresses [cAMP] to up-regulate NCCE channels.

From these results, we conclude that in pacemaker neurosecretory cells identified as DUM neurons the calcium entry evoked by low concentrations of NHD is mediated largely by a NCCE pathway regulated by the complex combination of intracellular molecular events including: 1) AA produced by the sequential activities of PLC and DAG lipase, 2) NO-guanylyl cyclase/cGMP cascade, phosphodiesterases, and finally cAMP, which directly regulates the functional property of NCCE. We bring new insights on the understanding of the ionic mechanisms underlying neuronal pacemaker activity by involving this NCCE in shaping the firing pattern.

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A New Regulation of Non-capacitative Calcium Entry in Insect Pacemaker Neurosecretory Neurons: INVOLVEMENT OF ARACHIDONIC ACID, NO-GUANYLYL CYCLASE/cGMP, AND cAMP
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