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Neuropeptide Y modulates calcium channels in hamster submandibular ganglion neurons

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ABSTRACT

It is established that neuropeptide Y (NPY) is a transmitter of parasympathetic secretory impulses in submandibular gland. The neuropeptides substance P, vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) are likely mediators of secretory parasympathetic responses of the gland. Previously, we have shown that substance P, VIP and CGRP modulate voltage-dependent Ca\(^{2+}\) channels (VDCCs) in hamster submandibular ganglion (SMG) neurons. In this study, we attempt to characterize the effect of NPY on VDCCs current using Ba\(^{2+}\) (\(I_{Ba}\)) in SMG neurons. Application of NPY caused both facilitation and inhibition of L-type and N/P/Q-type \(I_{Ba}\), respectively. Intracellular dialysis of the G\(\alpha_s\)-protein antibody attenuated the NPY-induced facilitation of \(I_{Ba}\). The adenylate cyclase (AC) inhibitor, as well as protein kinase A (PKA) inhibitor attenuated the NPY-induced facilitation of \(I_{Ba}\). Intracellular dialysis of the G\(\alpha_i\)-protein antibody attenuated the NPY-induced inhibition of \(I_{Ba}\). Application of a strong depolarizing voltage prepulse attenuated the NPY-induced inhibition of \(I_{Ba}\). These results indicate that NPY facilitates L-type VDCCs via G\(\alpha_s\)-protein involving AC and PKA. On the other hand, NPY also inhibits N/P/Q-type VDCCs via G\(\alpha_i\)-protein \(\beta\gamma\) subunits in the SMG neurons.
1. Introduction

The submandibular ganglion (SMG) is a parasympathetic ganglion which receives inputs from preganglionic cholinergic neurons, and innervates the submandibular salivary gland to control saliva secretion. This ganglion receives input from peptidergic afferent neurons and such input provides the physiological pathway for local reflex control of saliva secretion.

Neuropeptide Y (NPY), a 36 amino acid residue polypeptide, is known to act as a neurotransmitter in the central and peripheral nervous system where its actions are mediated by at least six NPY receptor subtypes (NPY₁–NPY₆) that belong to the G-protein-coupled receptor superfamily (Michel et al., 1998). NPY is involved in many brain physiological functions, such as regulation of blood pressure, circadian rhythms, feeding behaviour, anxiety, memory processing, etc. Furthermore, several evidences point to an important role for NPY in the regulation of neuronal activity during
pathological hyperactivity, such as that occurring during seizures (Klapstein and Colmers, 1997; Vezzani et al., 1999).

Voltage-dependent Ca$^{2+}$ channels (VDCCs) serve as crucial mediators of membrane excitability and Ca$^{2+}$-dependent functions such as neurotransmitter release, enzyme activity and gene expression. The modulation of VDCCs is believed to be an important means of regulating Ca$^{2+}$ influx and thus has a direct influence on many Ca$^{2+}$-dependent processes.

The neuropeptides substance P, vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) are likely mediators of secretory parasympathetic responses of the gland. Previously, we have shown that substance P, VIP and CGRP modulate VDCCs in SMG neurons (Yamada et al., 1999; Hayashi et al., 2002; Endoh et al., 2011).Ekström et al. have demonstrated that NPY is a transmitter of parasympathetic secretory impulses in salivary glands. In vitro, tissue of submandibular gland releases protein in response to NPY (Ekström et al., 1996). However, the effect of NPY on VDCCs in SMG has not yet been clarified, and little is known about signal pathways in SMG. Consequently, it is the purpose of this study to investigate the effects of NPY on VDCCs in SMG.
2. Materials and methods

2.1. Cell preparation

Experiments were conducted according to the guidelines for the treatment of experimental animals at Tokyo Dental College. Golden hamsters (4-6 weeks old) used for all experiments, were purchased from Sankyo Labo Service Corporation, Inc (Tokyo, Japan). Hamsters were housed with their mother in standard Plexiglas cages (45×30×20 cm) with bedding made of wood shavings and placed in an air-conditioned room (22°C) under a constant light-dark cycle (12:12 hr) with lights on at 06:00. SMG neurons from hamsters were acutely dissociated with a modified version of the method described previously (Yamada et al., 2002). In Brief, male hamsters 4-6 weeks old were anesthetized with pentobarbital sodium (30 mg/kg, i.p.) and SMG neurons were isolated. Isolated SMG neurons were maintained in Ca\(^{2+}\)-free Krebs solution of the following composition (in mM), 136 NaCl; 5 KCl; 3 MgCl\(_2\); 1.0 glucose; 11.9 NaHCO\(_3\) and 1.1 NaH\(_2\)PO\(_4\). SMG neurons were treated with collagenase type I (3 mg/ml in Ca\(^{2+}\)-free Krebs solution; Sigma) for 50 min at 37°C, followed by incubation in trypsin type I (1 mg/ml in Ca\(^{2+}\)-free Krebs solution; Sigma) for an additional 10 min. The supernatant
was replaced with normal Krebs solution of the following composition (in mM), 136 NaCl; 5 KCl; 2.5 CaCl₂; 0.5 MgCl₂; 10.9 glucose; 11.9 NaHCO₃ and 1.1 NaH₂PO₄. Neurons were then placed onto poly-l-lysine (Sigma)-coated glass coverslips.

2.2. Whole-cell patch-clamp recordings

Voltage-clamp recordings were conducted using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Fabricated recording pipettes (2-3 MΩ) were filled with the internal solution of the following composition (in mM): 100 CsCl, 1 MgCl₂, 10 HEPES, 10 BAPTA, 3.6 MgATP, 14 Tris₂phosphocreatine (CP), 0.1 GTP, and 50 U/ml creatine phosphokinase (CPK). The pH was adjusted to 7.2 with CsOH. After the formation of a giga seal, in order to record Iₜₐ carried by Ba²⁺ (IₐBa), the extracellular solution was replaced from Krebs solution to a solution containing the following composition (in mM): 151 tetraethyl ammonium (TEA) chloride, 5 BaCl₂, 1 MgCl₂, 10 HEPES and 10 glucose. The pH was adjusted to 7.4 with Tris base. Command voltage protocols were generated with a computer software pCLAMP version 10 (Axon Instruments, Union City, CA, U.S.A.) and transformed to an analogue signal using a DigiData 1200 interface (Axon Instruments, Union City, CA, U.S.A.). The command
pulses were applied to cells through an L/M-EPC7 amplifier (HEKA Elektronik, Lambrecht, Germany). The currents were recorded with the amplifier and a computer software pCLAMP 10 acquisition system. Access resistance ($< 15 \, \text{M} \Omega$) was determined by transient responses to voltage commands. Access resistance compensation was not used. To ascertain that no major changes in the access resistance had occurred during the recordings a 5 mV, 10 msec pulses was used before $I_{Ba}$ was evoked.

2.3. Materials

NPY, nifedipine and PD98,059 were purchased from Sigma (Tokyo, Japan). Anti-$G \alpha_s$ antibodies, anti-$G \alpha_i$ antibodies and anti-$G \alpha_{q/11}$ antibodies were purchased from Upstate biotechnology (Lake Placid, NY, U.S.A.). Each antibodies were from rabbits immunized with a synthetic peptide corresponding to the COOH-terminal sequence of the human $G \alpha_s$, $G \alpha_i$ and $G \alpha_{q/11}$, respectively. U-73122 was purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GF109203X) was purchased from Calbiochem (La Jolla, CA, U.S.A.) SQ22536 and PKI(5-24) were purchased from Biomol Research Laboratories (Plymouth, PA, U.S.A.). $\omega$-Conotoxin G
VIA (ω-CgTx GVI A) and ω-agatoxin IVA (ω-Aga IV A) were purchased from Peptide Institute (Osaka, Japan).

2.4. Analysis and statistics

All data analysis were performed using pCLAMP 10 acquisition system. Values in text and figures are expressed as mean ± SEM. Statistical analysis was made by student t-test for comparisons between pairs of groups and by one-way analysis of variance (ANOVA) followed by Dunnett’s test. Probability (p) values of less than 0.05 were considered significant.

3. Results

The effect of NPY on the I_{Ba} of individual neurons was one of three types: solely facilitory, solely inhibitory, or no effect. The data are presented below for each of the two individual effects i.e., facilitation (in 122 of 231 neurons) and inhibition (in 77 of 231 neurons). NPY-induced both facilitation and inhibition in the same neuron (mixed responses) could not be observed in the present study.
3.1. NPY-induced facilitation of $I_{Ba}$

An example of NPY-induced facilitation of $I_{Ba}$ is shown in Figs. 1a and b. Application of 1 $\mu$M NPY facilitated $I_{Ba}$ from $-1010$ pA to $-1334$ pA (32.0% facilitation) in this neuron.

The current-voltage relationships measured before and during application of NPY (1 $\mu$M) are shown in Fig. 1c. From a holding potential of $-80$ mV, $I_{Ba}$ was activated after $-40$ mV with a peak current amplitude at $-10$ mV. NPY did not alter the current-voltage relationship ($n=5$).

The concentration-response relationship in the NPY-induced facilitation of $I_{Ba}$ is shown in Fig. 2. Application of 1 nM-10 $\mu$M NPY rapidly and reversibly facilitates $I_{Ba}$. To generate a concentration-response curve, NPY concentrations were applied randomly, and each neuron was exposed to only a single concentration. Fig. 2 shows that progressive increases in NPY concentrations resulted in a progressively greater facilitation of $I_{Ba}$.
3.2. Characterization of G-protein subtypes in NPY-induced facilitation of $I_{Ba}$

G-proteins are heterotrimeric molecules with $\alpha$, $\beta$ and $\gamma$ subunits. The $\alpha$ subunit can be classified into families, $G\alpha_s$, $G\alpha_i$, or $G\alpha_q/11$. To characterize the G-protein subtypes in NPY-induced facilitation of $I_{Ba}$, selective antibodies cultivated for $G\alpha_s$, $G\alpha_i$, and $G\alpha_q/11$-proteins were used. Experiments were performed using a solution in a pipette containing each G-protein antibody. In these experiments, the G-protein antibody (1:50 dilution; final concentration approximately 0.5 mg/ml) was dissolved in internal solution. The tip of the recording pipette was filled with standard internal solution, and the pipette was then backfilled with solution containing the G-protein antibody. The effect of the antibody was obtained by applying NPY for 7 min after assuming the whole-cell configuration. As shown in Fig. 1, when NPY was applied twice to the same neuron, a rapid desensitization could be observed to its effects. Therefore, it is not possible to determine whether neurons are the type with VDCCs with “facilitation” or “inhibition” before antibodies exert. Using this protocol, we have demonstrated that intracellular dialysis of anti-$G\alpha_q/11$-protein antibodies (0.5 mg/ml) attenuated glutamate receptor agonist-induced facilitation of $I_{Ba}$ (Endoh, 2006). Thus it can be considered that these methods are suitable for the questions posed.
As shown in Fig. 3a, intracellular dialysis of the anti-G\( \alpha_s \)-protein antibody attenuated the NPY-induced facilitation of I\(_{Ba} \). In contrast, intracellular dialysis of the anti-G\( \alpha_i \)-protein and anti-G\( \alpha_{q/11} \)-protein antibody did not attenuate the NPY-induced facilitation of I\(_{Ba} \). These results suggest that the G\( \alpha_s \)-protein is involved in the NPY-induced facilitation of I\(_{Ba} \) in the SMG neurons.

### 3.3. Characterization of second messengers in NPY-induced facilitation of I\(_{Ba} \)

To evaluate the possible contribution of adenylate cyclase (AC) to the NPY-induced facilitation of I\(_{Ba} \), the effects of NPY on I\(_{Ba} \) in neurons treated with SQ22536 (an AC inhibitor) were investigated. Treatment with SQ22536 (10\( \mu \)M for 30 min) (Mitrta et al., 2006) attenuated the NPY-induced facilitation of I\(_{Ba} \).

To evaluate the possible contribution of protein kinase A (PKA) to the NPY-induced facilitation of I\(_{Ba} \), the effects of NPY on I\(_{Ba} \) in the presence of PKI(5-24) (a selective PKA inhibitor) in the recording pipette were investigated. Intracellular application of PKI(5-24) (20\( \mu \)M for 7 min after assuming the whole-cell configuration) attenuated the NPY-induced facilitation of I\(_{Ba} \).

In contrast to SQ22536 and PKI(5-24), in neurons treated with U-73122 (a
membrane-permeable aminosteroid which blocks phosphatidylinositol-specific phospholipase C; PLC, 10 \( \mu \) M for 15 min) (Bleasdale et al., 1990; Thompson et al., 1991), GF109203X (a selective protein kinase C; PKC inhibitor, 10 \( \mu \) M for 30 min) (Toullec et al., 1991; Pan et al., 2001) and PD98,059 (a mitogen-activated protein kinase; MAPK tyrosine kinase inhibitor, 10 \( \mu \) M for 2 min) (Lei et al., 1998) did not attenuate the NPY-induced facilitation of \( I_{Ba} \). These results suggest that NPY facilitates VDCCs involving AC and PKA pathways in the SMG neurons (Fig. 3b).

3.4. Characterization of VDCC subtypes in NPY-induced facilitation of \( I_{Ba} \)

It has been reported that several different types of VDCCs, such as L\(^-\), N\(^-\), P\(^-\), Q\(^-\) and R\(^-\) type VDCCs exist in the SMG (Endoh, 2004). L\(^-\) type VDCCs are blocked by nifedipine (Nif). N\(^-\) type VDCCs are blocked by \( \omega \)-CgTx GVI A. P/Q\(^-\) types VDCCs are blocked by \( \omega \)-Aga IVA. Despite the addition of all these blockers, a component of current that is resistant still remains and has been termed R\(^-\) type (Endoh and Suzuki, 1998).

Thus, the types of VDCCs which are facilitated by NPY were then investigated. When Nif (10 \( \mu \) M, L\(^-\) type VDCCs blocker) + \( \omega \)-Aga IVA (1 \( \mu \) M, P/Q\(^-\) type VDCCs blocker), Nif + \( \omega \)-CgTx GVIA (1 \( \mu \) M, N\(^-\) type VDCCs blocker) and Nif + \( \omega \)-CgTx GVIA + \( \omega \)-CgTx GVI A...
-Aga IVA were applied first, resistant I_Ba was not significantly facilitated by a subsequent application of NPY. On the other hand, when \( \omega \cdot \text{CgTx GVI A} + \omega \cdot \text{Aga IVA} \)

was applied first, resistant I_Ba were facilitated by the subsequent application of NPY.

These results demonstrate that NPY facilitates L-type VDCCs, without significantly affecting N-, P/Q- and R-types VDCCs in the SMG neurons (Fig. 4).

3.5. NPY-induced inhibition of I_Ba

An example of NPY-induced inhibition of I_Ba is shown in Figs. 5a and b. Application of 1 \( \mu \) M NPY inhibited I_Ba from \(-1372 \text{ pA} \) to \(-900 \text{ pA} \) (34.4% inhibition) in this neuron.

To investigate the voltage dependency of inhibition of I_Ba by NPY, we used a double-pulse voltage protocol as shown in Fig. 5. As shown in Fig. 5, the application of a strong depolarizing voltage prepulse attenuated NPY-induced inhibition of I_Ba.

The current-voltage relationships measured before and during application of NPY (1 \( \mu \) M) are shown in Fig. 6a.

The concentration-response relationship in the NPY-induced inhibition of I_Ba is shown in Fig. 6b. Application of 10 nM-10 \( \mu \) M NPY rapidly and reversibly inhibits I_Ba. Fig. 6b shows that progressive increases in NPY concentrations resulted in a progressively
greater inhibition of $I_{Ba}$.

3.6. Characterization of G-protein subtypes in NPY-induced inhibition of $I_{Ba}$

As shown in Fig. 7a, intracellular dialysis of the anti-$G\alpha_i$-protein antibody attenuated the NPY-induced inhibition of $I_{Ba}$. In contrast, intracellular dialysis of the anti-$G\alpha_s$-protein and anti-$G\alpha_q/11$-protein antibody did not attenuate the NPY-induced inhibition of $I_{Ba}$. These results suggest that the $G\alpha_i$-protein is involved in the NPY-induced inhibition of $I_{Ba}$ in the SMG neurons.

3.7. Characterization of second messengers in NPY-induced inhibition of $I_{Ba}$

To further investigate whether $I_{Ba}$ inhibition was mediated by second messengers, the previously mentioned second messenger inhibitors were used. As shown in Fig. 7b, all second messenger inhibitors did not attenuate the NPY-induced inhibition of $I_{Ba}$. These results suggest that NPY inhibits VDCCs without second messenger in the SMG neurons (Fig. 7b).
3.8. Characterization of VDCC subtypes in NPY-induced inhibition of $I_{Ba}$

In next series of experiment, it was investigated about which types of the VDCCs were inhibited by NPY. The effect of NPY on the $I_{Ba,L}$ was investigated using a neuron treated with $\omega$-CgTx GVI (1 $\mu$M) and $\omega$-Aga IV (1 $\mu$M). The effect of NPY on the $I_{Ba,N}$ was investigated using a neuron treated with Nif (10 $\mu$M) and $\omega$-Aga IV (1 $\mu$M). The effect of NPY on the $I_{Ba,P/Q}$ was investigated using a neuron treated with Nif (10 $\mu$M) and $\omega$-CgTx GVI (1 $\mu$M). The effect of NPY on the $I_{Ba,R}$ was investigated using a neuron treated with Nif (10 $\mu$M), $\omega$-CgTx GVI (1 $\mu$M) and $\omega$-Aga IV (1 $\mu$M).

The $I_{Ba}$ sensitive to the inhibitors and the percentage of the inhibition by NPY are summarized in Fig. 8, where $I_{Ba}$ of L-, N-, P/Q- and R-types were 48.0±7.8, 36.1±6.5, 13.5±1.6, 2.6±0.4%, respectively, of the total current, and the $I_{Ba}$ of each type inhibited by NPY were 8.9±5.4, 24.2±7.2, 10.8±2.2, 0.6±0.3%, respectively, of the total current. Results shown in Fig. 8 demonstrate that NPY inhibited $I_{Ba,N}$ and $I_{Ba,P/Q}$ in the SMG neurons.

4. Discussion
This study has shown that NPY facilitates L-type VDCCs via G\(\alpha\)s-protein involving AC and PKA. NPY also inhibits N- and P/Q-type VDCCs via G\(\alpha\)i-protein in SMG.

In this study, we acquired two different groups of results: (1) facilitation of I\(_{Ca}\) or (2) inhibition of I\(_{Ca}\). These dual effects may contribute physiological responses in vivo as follows. Entry of Ca\(^{2+}\) through VDCCs opens a class of small-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels (SK channels); these induce a prolonged post-spike hyperpolarization and contribute to the accommodation of spike discharge frequency during sustained depolarization (Davies et al., 1996). In contrast, reduction of Ca\(^{2+}\) influx through VDCCs might inhibit the Ca\(^{2+}\)-activated K\(^{+}\) currents responsible for post-spike hyperpolarization (AHP slow), thereby increasing neuronal excitability.

What determines the relative expression of the two types of I\(_{Ba}\) responses i.e., (1) facilitation of L-type VDCCs or (2) inhibition of N- and P/Q-type VDCCs? NPY-induced facilitation of VDCCs suggests that a principal effect of NPY modulation might be regulation of Ca\(^{2+}\)-dependent enzymes, in particular transcription factors (Alberini et al., 1994). L-type VDCCs have been linked to not only somal action potentials, but also to the activation of Src, the modulation of RTK (Finkbeiner and Greenberg, 1996) and gene expression, including synthesis of ion channels (Murphy et al., 1991; Misra et al., 1994). During differentiation, L-type VDCC-mediated Ca\(^{2+}\) influx is essential for neurite
outgrowth, synapse formation, survival and the shift to the mature action potential profile (Spitzer, 1994). Developmentally, Ca\(^{2+}\) influx would be expected to trigger influx-dependent cellular differentiation (Spitzer, 1994; Gu and Spitzer, 1995) and, because early action potential activity has been linked to establishing neuronal circuits (Katz and Shatz, 1996) potentially could contribute to pattern information. These studies strongly imply that NPY-induced facilitation of L-type VDCCs would lead to a substantial increase in Ca\(^{2+}\) influx. It is well established that N- and P/Q-type VDCCs mediate neurotransmitter release (Hirning et al., 1988). In hippocampal CA1 neuron, NPY inhibits VDCCs and synaptic transmission (Qian et al., 1997).

According to biophysical criteria, the mode of inhibition of I\(_{\text{Ca}}\) can be classified into a voltage-dependent (VD)- and a voltage-independent (VI)-mode. In the VD-mode, inhibition of I\(_{\text{Ca}}\) is relieved at a higher potential or by means of a strong depolarizing voltage prepulse to positive voltages (Bean, 1989; Dolphin, 1996), whereas in the VI-mode, inhibition of I\(_{\text{Ca}}\) is not affected by a strong depolarizing voltage prepulse (Formenti et al., 1993). As mentioned above, G-proteins are heterotrimeric molecules with \(\alpha\), \(\beta\) and \(\gamma\) subunits. The VD-mode inhibition is mediated by a rapid “membrane delimited” pathway, probably involving the interaction of G-protein \(\beta\gamma\) subunits with the VDCCs (Ikeda, 1996; Herlitze et al., 1996; Diversé-Pierluissi et al.,
1995). Whereas, the VI-mode inhibition is generally thought to involve a diffusible second messenger (Bernheim et al., 1991; Hille, 1994). In this study, we demonstrated that NPY produced a typical VD-mode inhibition of $I_{Ba}$ characterized by kinetic slowed and relieved by strong depolarizing voltage prepulse (Fig 5). As relief of inhibition by a prepulse was incomplete, it is possible that other mechanisms are also operative, as has been suggested by Hille (1994). NPY inhibited $I_{Ba}$ via a voltage-sensitive, G-protein-dependent mechanism. NPY-induced $I_{Ba}$ inhibition was dose-dependent (Fig. 5), associated with the slowing of activation kinetics and exhibited voltage dependence and prepulse facilitation (Fig. 5A) Moreover, we found that $I_{Ba}$ inhibition by NPY via G $\alpha_i$-protein (Fig. 7A). Such an effect is usually interpreted as an indication that VDCCs inhibition is mediated by a rapid membrane delimited pathway, possibly involving an interaction between the G-protein $\beta \gamma$ subunits with the VDCCs $\alpha_1$-subunit (Zamponi and Snutch, 1998).

In this study, NPY inhibits N- and P/Q-type VDCCs via G $\alpha_i$-protein. These results are consistent with several previous studies. NPY receptors are coupled to pertussis toxin (PTX)-sensitive G-proteins (G $\alpha_1$-protein) (Vezzami et al., 1999; Sun and Miller, 1999). In intracardiac neuron, NPY inhibits VDCCs via PTX-sensitive G-proteins (Jeong et al., 1999).
NPY also has been implicated in a variety of disease states including depression, anxiety, pain, seizures, intestinal dysfunction, and cardiovascular disease (Gehlert, 1998; Balasubramaniam, 2002; Redrobe et al., 2002; Thorsell and Heilig, 2002). The present study also provided that NPY may modulate saliva secretion in these diseases.
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Fig. 1. (a) Typical superimposed $I_{Ba}$ traces according to the time course graph. $I_{Ba}$ was evoked from a holding potential of $-80$ mV by a 100 msec voltage step to $-10$ mV at 20 sec intervals. (b) Typical time course of NPY-induced facilitation of $I_{Ba}$. NPY (1 $\mu$M) was bath-applied during the times indicated by the filled bar. (c) Current-voltage relationship of $I_{Ba}$ evoked by a series of voltage steps from a holding potential of $-80$ mV to test potentials between $-80$ and $+40$ mV in $+10$ mV increments in the absence (opened circles) and presence (filled circles) of 1 $\mu$M NPY.
Fig. 2. Concentration dependence of NPY-induced facilitation of $I_{Ba}$. $I_{Ba}$ were evoked from a holding potential of $-80$ mV by a 100 ms voltage step to $-10$ mV at 20 s intervals. Each neuron was tested with only one drug concentration and only the first application of drug was analyzed. The curve was obtained from fitting to a single-site binding isotherm with least-squares nonlinear regression. Numbers in parentheses indicate the number of neurons tested.
Fig. 3. (a) Histogram showing the degree of $I_{Ba}$ facilitation by 1 $\mu$M NPY (control, recording pipette was filled with GTP), after intracellular dialysis with anti-$G_{\alpha_s}$-protein antibodies, boiled anti-$G_{\alpha_s}$-protein antibodies (90°C for 30 min), anti-$G_{\alpha_i}$-protein antibodies, and anti-$G_{\alpha_q/11}$-protein antibodies. (b) The histogram shows the degree of $I_{Ba}$ facilitation by 1 $\mu$M NPY (control), after U-73122 (a PLC inhibitor), after GF109203X (a PKC inhibitor), after SQ22536 (an AC inhibitor), intracellular dialysis with PKI(5-24) (a PKA inhibitor) and after PD98,059 (a MAPK tyrosine kinase inhibitor). Numbers in parentheses indicate the number of neurons tested. *P < 0.05 compared with control, ANOVA.
Fig. 4. Histogram showing the degree of $I_{Ba}$ facilitation by 1 $\mu$ M NPY on L + R types (after treatment with $\omega$-CgTx GVI A + $\omega$-Aga IVA), N + R types (after treatment with Nif + $\omega$-Aga IVA), P/Q + R types (after treatment with Nif + $\omega$-CgTx GVI A) and R types (after treatment with Nif + $\omega$-CgTx GVI A + $\omega$-Aga IVA) VDCCs. Numbers in parentheses indicate the number of neurons tested.
Fig. 5. (a) Typical superimposed $I_{Ba}$ traces recorded using a double-pulse voltage protocol at the times indicated in the time course graph b. Paired $I_{Ba}$ were evoked from a holding potential of $-80$ mV by a 100 msec voltage step to $-10$ mV at 20 sec intervals. An intervening strong depolarizing prepulse (100 mV, 30 msec) ended 5 msec prior to the second $I_{Ba}$ activation. (b) Typical time course of NPY-induced $I_{Ba}$ inhibition. Opened circle and triangles in the graph indicate $I_{Ba}$ without prepulse and $I_{Ba}$ with prepulse, respectively. NPY (1 μM) was bath-applied during the time indicated by the filled bar.
Fig. 6. (a) Current-voltage relationship of $I_{Ba}$ evoked by a series of voltage steps from a holding potential of $-80 \text{ mV}$ to test potentials between $-80$ and $+40 \text{ mV}$ in $+10 \text{ mV}$ increments in the absence (opened circles) and presence (filled circles) of $1 \mu \text{ M NPY}$. (b) Concentration dependence of NPY-induced inhibition of $I_{Ba}$. Numbers in parentheses indicate the number of neurons tested.
Fig. 7. (a) Histogram showing the degree of $I_{\text{Ba}}$ inhibition by 1 $\mu$M NPY (control, recording pipette was filled with GTP), after intracellular dialysis with anti-$G \alpha_s$-protein antibodies, anti-$G \alpha_i$-protein antibodies, boiled anti-$G \alpha_i$-protein antibodies (90°C for 30 min), and anti-$G \alpha_{q/11}$-protein antibodies. (b) The histogram shows the degree of $I_{\text{Ba}}$ inhibition by 1 $\mu$M NPY (control), after U-73122 (a PLC inhibitor), after GF109203X (a PKC inhibitor), after SQ22536 (an AC inhibitor), intracellular dialysis with PKI(5-24) (a PKA inhibitor) and after PD98,059 (a MAPK tyrosine kinase inhibitor). Numbers in parentheses indicate the number of neurons tested. *P < 0.05 compared with control, ANOVA.
Fig. 8. Fractional components of L-, N-, P/Q- and R-types $I_{Ba}$ and those inhibited by NPY (■). The total height of the bars (open and black) represents the mean ± SEM contribution of the indicated VDCCs type to the total $I_{Ba}$. The black bars represent the mean ± SEM inhibition by NPY of the corresponding VDCCs type. Numbers in parentheses indicate the number of neurons tested.
Fig. 1

(a) 
-10 mV
-80 mV

(b) 
Peak $I_{Ba}$ (pA)

(c) 
Test potential (mV)

Before application
NPY (1 μM)
-1500 pA

-80 -60 -40 -20 0 20 40

-80
-60
-40
-20
0
20
40

-1500 pA
Fig. 2

Concentration of NPY (M)

Facilitation of $I_{Ba}$ (%)
Fig. 4

Facilitation of \( I_{Ba} \) by NPY

- **L + R types**
- **N + R types**
- **P/Q + R types**
- **R type**

Experiments:
- \( \omega \)-CgTX GVIA + \( \omega \)-Aga IVA
- Nif + \( \omega \)-Aga IVA
- Nif + \( \omega \)-CgTX GVIA
- Nif + \( \omega \)-CgTX GVIA + \( \omega \)-Aga IVA
Fig. 5

(a) Voltage protocols and current traces for different voltages. The voltage is cycled between -10 and -80 mV with a 600 ms duration.

(b) Time course of peak inward Ba currents (peak IBa) with and without NPY (1 μM). The plot shows the relationship between time and peak IBa for different experimental conditions.
Before application

**Fig. 6**

**a**

Test potential (mV)

-80 -60 -40 -20 0 20 40

NPY (1 μM)

Peak $I_{Ba}$ (pA)

-1500 pA

**b**

Inhibition of $I_{Ba}$ (%)

0 10 20 30 40

Concentration of NPY (M)

$10^{-8}$ $10^{-7}$ $10^{-6}$ $10^{-5}$

(3) (4) (5)
Fig. 8

![Bar chart showing contribution of total $I_{Ba}$ by L-, N-, P/Q-, and R-types. The chart indicates percentages with error bars and includes numerical values in parentheses next to each bar.](image-url)