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Research report

Calcitonin gene-related peptide- and adrenomedullin-induced facilitation of calcium current by different signal pathways in nucleus tractus solitarius

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ABSTRACT

Calcitonin gene-related peptides (CGRP) and adrenomedullin (ADM) belong to the calcitonin family of peptides and are structurally related. Both peptides are found in the neurons of the CNS and play a role in many neuronal functions, including the control of blood pressure. The nucleus tractus solitarius (NTS) is known to play a major role in the regulation of cardiovascular, respiratory, gustatory, hepatic and swallowing functions. Recently, hypotension and bradycardia were observed after CGRP and ADM injection in the NTS. 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 purpose of this study is to investigate the effects of CGRP and ADM on VDCCs currents ($I_{Ca}$) carried by Ba$^{2+}$ ($I_{Ba}$) in the NTS, using patch-clamp recording methods. Application of CGRP and ADM caused facilitation of $I_{Ba}$ in a concentration-dependent manner. Intracellular dialysis of the anti-$G_{\alpha_s}$ protein antibody attenuated CGRP-induced facilitation of $I_{Ba}$. Intracellular dialysis of the anti-$G_{\alpha_i}$ protein antibody attenuated ADM-induced facilitation of $I_{Ba}$. Pretreatment with SQ22536 (an adenylate cyclase inhibitor) and intracellular dialysis of PKI(5-24) (a protein kinase A inhibitor) attenuated CGRP-induced facilitation of $I_{Ba}$. 
In contrast, pretreatment with PD98,059 (a mitogen-activated protein kinas inhibitor) attenuated ADM-induced facilitation of I_{Ba}. Mainly L-type VDCCs were facilitated by both CGRP and ADM. These results indicate that CGRP facilitates L-type VDCCs via G_{\alpha_s}-protein involving adenylate cyclase and protein kinase A. In contrast, ADM facilitates L-type VDCCs via G_{\alpha_i}-protein involving mitogen-activated protein kinase in the NTS.
1. Introduction

The nucleus tractus solitarius (NTS) is known to play a major role in the regulation of cardiovascular, respiratory, gustatory, hepatic and swallowing functions (Jean 2001; Lawrence and Jarrott, 1996). The NTS appears to be not just a simple ‘relay’ nucleus, but performs complex integration of information from multiple synaptic inputs of both peripheral and central origins.

The calcitonin gene-related peptide (CGRP) is a 37-amino-acid peptide that was initially described as being generated by alternative splicing of the calcitonin gene (Amara, et al., 1982). CGRP is found in neurons of the CNS and thought to play a role in many neuronal functions, including the control of blood pressure (Raynaud and Cohen, 1994). It has been described that CGRP mRNA is expressed in neurons of the nodose ganglia and petrosal ganglia (Czyzyk-Krzeska, et al., 1991) and CGRP immunoreactive terminals project from those ganglia to the NTS (Torrealba, 1992). Furthermore, hypotension and bradycardia were observed after CGRP injection in the NTS (Vallejo, et al., 1988), which suggests involvement of the peptide in central cardiovascular control.

Evidence that the level of CGRP decreased in brain areas related to blood pressure control in spontaneously hypertensive rats (SHR) has also been obtained (Lewis et al.,...
It is thought that decreased CGRP in the SHR can be restored after local nerve growth factor administration, which has triggered a fall in blood pressure in test animals (Supowit, et al., 2001). These features further indicate that CGRP plays a role in the central mechanism related to blood pressure control.

Adrenomedullin (ADM) is a 52-amino-acid peptide originally isolated from a human pheochromocytoma (Kitamura, et al., 1993). It is structurally and functionally related to CGRP, belonging to the amylin peptide family (McDonald, et al., 1994). ADM has been shown to be widely involved in the control of fluid and electrolyte homeostasis and cardiovascular function through peripheral and central nervous system (CNS) activity. Voltage-dependent Ca\textsuperscript{2+} channels (VDCCs) serve as crucial mediators of membrane excitability and Ca\textsuperscript{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\textsuperscript{2+} influx and thus has a direct influence on many Ca\textsuperscript{2+} dependent processes. Modulation of VDCCs by CGRP and ADM in various types of cells has been described previously (Nakazawa, et al., 1992; Ono, et al., 1989; Zhang, et al., 2007). However, the effect of CGRP and ADM on VDCCs in the NTS has not yet been clarified, and little is known about signaling pathways in the NTS. Consequently, the purpose of this study is to investigate the effects of CGRP and ADM on VDCC current (I_{Ca}) in the
2. Results

2.1. CGRP- and ADM-induced facilitation of $I_{Ba}$

An example of CGRP- and ADM-induced facilitation of $I_{Ba}$ is shown in Fig. 1. $I_{Ba}$ was emitted every 20 sec with a 100 msec depolarizing voltage step, up to 0 mV from a holding potential of $-80$ mV. As shown in Fig. 1A-D, application of 1 $\mu$M CGRP and ADM rapidly and reversibly facilitated $I_{Ba}$. The current-voltage relationship measured before and during application of CGRP (1 $\mu$M) and ADM (1 $\mu$M) are shown in Fig. 1E and F, respectively. From a holding potential of $-80$ mV, $I_{Ba}$ was activated after $-40$ mV with a peak current amplitude at 0 mV. Neither CGRP nor ADM altered the current-voltage relationship ($n=5$).

The concentration-response relationship in the CGRP- and ADM-induced facilitation of $I_{Ba}$ is shown in Fig. 2A. Application of 1 nM-10 $\mu$M CGRP and ADM rapidly and reversibly facilitates $I_{Ba}$ without changing current kinetics. To generate a concentration-response curve, CGRP and ADM concentrations were applied randomly,
and each neuron was exposed to only a single concentration. Fig. 2A shows that progressive increases in CGRP and ADM concentrations resulted in a progressively greater facilitation of $I_{Ba}$.

Whether or not a superimposed effect of CGRP- and ADM-induced facilitation of $I_{Ba}$ exists was investigated by the co-application of CGRP and ADM. A concentration of 1 $\mu$M CGRP facilitated $I_{Ba}$ by 26.2 $\pm$ 3.3% (n=7), 1 $\mu$M ADM facilitated $I_{Ba}$ by 22.3 $\pm$ 3.0% (n=6), and co-application of both 1 $\mu$M CGRP and 1 $\mu$M ADM facilitated $I_{Ba}$ by 42.1 $\pm$ 4.1% (n=5). A superimposed effect after co-application of CGRP and ADM was observed. These results indicate that CGRP and ADM facilitate VDCCs using distinct intracellular pathways in the NTS.

2.2. Pharmacological characterization of CGRP- and ADM-induced facilitation of $I_{Ba}$

In the next series of experiments, we analyzed the effects of CGRP and ADM on $I_{Ba}$ in neurons treated with selective antagonists. These receptors are characterized by their selective antagonists, namely CGRP(8-37) fragment for the CGRP receptor and ADM(22-52) for the ADM receptor (Chiba, et al., 1989; Eguchi, et al., 1994). As shown in Fig.3B, selective antagonists were applied prior to CGRP and ADM. Treatment with the
selective CGRP antagonist, CGRP(8-37) (1 μ M), attenuated the CGRP-induced facilitation of I_{Ba}. In contrast, treatment with the selective ADM antagonist, ADM(22-52) (1 μ M), did not attenuate the CGRP-induced facilitation of I_{Ba}. Treatment with CGRP(8-37) (1 μ M) did not attenuate the ADM-induced facilitation of I_{Ba}. In contrast, treatment with ADM(22-52) (1 μ M) attenuated the ADM-induced facilitation of I_{Ba}. These results indicate that CGRP and ADM bind distinct receptors in the NTS.

2.3. Characterization of G-protein subtypes in CGRP- and ADM-induced facilitation of I_{Ba}.

G-proteins are heterotrimERIC molecules with α, β and γ subunits. The α subunit can be classified into families, Gα_i, Gα_s, or Gα_q/11. To characterize the G-protein subtypes in CGRP- and ADM-induced facilitation of I_{Ba}, selective antibodies cultivated for Gα_i, Gα_s, and Gα_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 CGRP and ADM for 7 min after assuming the whole-cell configuration.

As shown in Fig. 4, intracellular dialysis of the anti-Gαs-protein antibody attenuated the CGRP-induced facilitation of I_{Ba}. In contrast, intracellular dialysis of the anti-Gαi-protein antibody attenuated the ADM-induced facilitation of I_{Ba}. These results suggest that the Gαs-protein is involved in the CGRP-induced facilitation of I_{Ba}. The Gαi-protein is involved in the ADM-induced facilitation of I_{Ba} in the NTS.

2.4. Characterization of second messengers in CGRP- and ADM-induced facilitation of I_{Ba}

To evaluate the possible contribution of adenylate cyclase (AC) to the CGRP-induced facilitation of I_{Ba}, the effects of CGRP on I_{Ba} in neurons treated with SQ22536 (an AC inhibitor) were investigated. In six neurons tested, treatment with SQ22536 (10 μM for 30 min) attenuated the CGRP-induced facilitation of I_{Ba}.

To evaluate the possible contribution of protein kinase A (PKA) to the CGRP-induced facilitation of I_{Ba}, the effects of CGRP on I_{Ba} in the presence of PKI(5-24) (a selective PKA inhibitor) in the recording pipette were investigated. In six neurons tested,
intracellular application of PKI(5-24) (20 μM for 7 min after assuming the whole-cell configuration) attenuated the CGRP-induced facilitation of IbA.

In contrast to SQ22536 and PKI(5-24), in neurons treated with U-73122 (a membrane-permeable aminosteroid which blocks phosphatidylinositol-specific PLC, 10 μM for 15 min) (5, 39), GF109203X (a selective PKC inhibitor, 10 μM for 30 min) and PD98,059 (a MAPK tyrosine kinase inhibitor, 10 μM for 2 min) did not attenuate the CGRP-induced facilitation of IbA. These results suggest that CGRP facilitates VDCCs involving AC and PKA pathways in the NTS neurons (Fig. 5).

To evaluate the possible contribution of MAPK tyrosine kinase to the ADM-induced facilitation of IbA, the effects of ADM on IbA in neurons treated with PD98,059 were investigated. In six neurons tested, treatment with PD98,059 (10 μM for 2 min) attenuated the ADM-induced facilitation of IbA.

In contrast to PD98,059, in neurons treated with U-73122, GF109203X, SQ22536 and PKI(5-24) in the recording pipette did not attenuate the ADM-induced facilitation of IbA. These results suggest that ADM facilitates VDCCs involving MAPK pathways in NTS neurons (Fig. 5).

2.5. Characterization of VDCC subtypes in CGRP- and ADM-induced facilitation of IbA
It has been reported that several different types of VDCCs, such as L-, N-, P-, Q- and R-type VDCCs exist in the NTS (Rhim and Miller, 1994). We previously demonstrated in the NTS that the mean percentages of $I_{Ba-L}$, $I_{Ba-N}$, $I_{Ba-P/Q}$ and $I_{Ba-R}$ of total $I_{Ba}$ were 35.7, 28.7, 19.3 and 16.0%, respectively (Endoh, 2006). L-type VDCCs are blocked by nifedipine. 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, 2004).

Thus, the types of HVA VDCCs which are facilitated by CGRP and ADM were then investigated. When nifedipine (10 $\mu$ M, Nif; L-type VDCC blocker) + $\omega$-Aga IVA (1 $\mu$ M, P/Q-type VDCC blocker) and Nif + $\omega$-CgTx GVI A (1 $\mu$ M, N-type VDCC blocker) were applied first, resistant $I_{Ba}$ was not significantly facilitated by a subsequent application of CGRP and ADM. On the other hand, when $\omega$-CgTx GVI A + $\omega$-Aga IVA was applied first, resistant $I_{Ba}$ were facilitated by the subsequent application of CGRP and ADM. These results demonstrate that CGRP and ADM facilitates L-type VDCCs, without significantly affecting N- and P/Q-types VDCCs in NTS neurons (Fig. 6).

3. Discussion
This study investigated the effects of CGRP and ADM on VDCCs in the NTS. This study has shown that CGRP facilitates L-type VDCCs via the Gαs-protein involving AC and PKA. On the other hand, ADM facilitates L-type VDCCs via the Gαi-protein involving MAPK in the NTS.

In this study, a CGRP selective antagonist did not attenuate the ADM-induced inhibition of I_Ba, whereas an ADM selective antagonist attenuated the ADM-induced inhibition of I_Ba, suggesting that an ADM effect is mediated by ADM selective receptor in the NTS. It has been demonstrated that an ADM receptor belongs to the CGRP receptor family. Many biological actions of ADM are mediated by selective ADM receptors or CGRP type 1 receptors (Smith, et al., 2002). Although ADM is known to bind CGRP receptors with low affinity (Aiyar, et al., 1996), CGRP does not bind ADM receptors (Owji, et al., 1996; Owji, et al., 1995).

In addition, we could demonstrate that CGRP facilitates VDCCs involving AC and PKA. ADM facilitates VDCCs involving MAPK in the NTS. cAMP-PKA pathways are coupled to ADM receptors in various types of cells (Kitamura, et al., 1993; Shimekake, et al., 1995; Ross and Yallampalli, 2006), including neural cells (Xu and Krukoff, 2005). Recently, it has been demonstrated that ADM enhances baroreceptor reflex response via cAMP-PKA in the NTS (Ho, et al., 2008). We could previously demonstrate that
MAPK-induced VDCCs facilitation is an important signaling in the NTS (Endoh, 2005; Endoh, et al., 2008). Although the mechanism by which MAPK signaling facilitates VDCCs is not known, there are several potential routes. The most direct model would involve extracellular signal-regulated kinases (ERK) or their activated downstream kinases phosphorylating the VDCCs. The phosphorylation could facilitate VDCCs by triggering a shift in the voltage-dependence of channel activation. In the dorsal root ganglion, CGRP facilitates sodium current ($I_{Na}$) involving PKA and PKC (Natura et al., 2005).

This study has shown that CGRP and ADM facilitate L-type VDCCs in the NTS. Similar observation has been demonstrated in smooth muscle cells (Nakazawa, et al., 1992) and cardiac cells (Ono, et al., 1989). There are several mechanisms of VDCCs facilitation (Dolphin, 1996). L-type VDCCs facilitation can result from a strong conditioning depolarization that recruits ‘silent channels’ (Artelejo, et al., 1990). Alternatively, L-type VDCCs can be facilitated by protein kinases. L-type VDCCs possess several consensus PKA and PKC phosphorylation sites and physiological studies have demonstrated channel facilitation by these enzymes (McDonald, et al., 1994). In contrast, in rat ventricular myocytes, ADM inhibits L-type VDCCs (Zhang, et al., 2007). These differences in effect of ADM on VDCCs may depend on the cell type.
What is the physiological relevance of CGRP and ADM-induced facilitation of VDCCs? Microinjection of CGRP into the NTS area induces hypotension and bradycardia (Vallejo, et al., 1988). In contrast, microinjection of ADM into the NTS area elevates arterial pressure and heart rate (Cui, et al., 2008). In this study, we could demonstrate that both CGRP and ADM facilitate L-type VDCCs in the NTS. However, their effects on cardiovascular parameters were opposite. The exact mechanisms by which CGRP and ADM produce increasing or decreasing responses in the NTS are unknown. It should be noted that neuronal networks exist within the NTS. NTS neurons can be divided into two groups, GABAergic and glutamatergic (Mifflin and Felder, 1990; Brooks, et al. 1992). As mentioned above, microinjection of CGRP into the NTS decreases cardiovascular function. CGRP-induced facilitation of VDCCs demonstrated in this study may lead to depolarization of the NTS and as a result increase cardiovascular responses. Facilitation of VDCCs on GABAergic neurons may enhance GABA release and therefore decrease cardiovascular function.

In the case of glutamatergic neurons, the effect would be opposite. Microinjection of ADM into the NTS increases cardiovascular function. It can be considered that facilitation of VDCCs on glutamatergic neurons can enhance glutamate release and therefore increase cardiovascular function. In addition, we could not rule out the fact
that the NTS expresses Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (Tell and Bradley, 1994), which can hyperpolarize the membrane (Halliwell, 1990; Hille, 1981). In this study, identification of distinct NTS subgroups was not possible.

In conclusion, the present data shows that CGRP facilitates L-type VDCCs via the G\textsubscript{\alpha}s-protein involving AC and PKA. On the other hand, ADM facilitates L-type VDCCs via the G\textsubscript{\alpha}i-protein involving MAPK in the NTS.

4. Experimental procedures

4.1. Cell preparation

Young Wistar rats (7-18 days old) were purchased from Sankyo Labo Service Corporation, Inc (Tokyo, Japan) and used for all experiments. Rats were housed with their mothers in standard Plexiglas cages (45×30×20 cm) with a bedding of wood shavings in an air-conditioned room (22°C) under a constant light-dark cycle (12:12 hr) with lights turned on at 06:00. Animal treatment complied with the principles regarding research ethics by the Physiological Society of Japan and the guidelines of the Japanese Government.
NTS neurons in neonatal rats were acutely dissociated as previously reported (Endoh, 2005). Young Wistar rats (7-18 days old) were decapitated and their brains were quickly removed and submerged in ice cold artificial cerebrospinal fluid (aCSF) of the following composition (in mM): NaCl 126, NaHCO$_3$ 26.2, NaH$_2$PO$_4$ 1, KCl 3, MgSO$_4$ 1.5, CaCl$_2$ 1.5 and glucose 30; pH 7.4 and saturated with 95% O$_2$ and 5% CO$_2$. Thin transverse slices from brainstems, 400 $\mu$m in thickness, were prepared by a tissue slicer (DTK-1000; Dosaka EM Co., Ltd, Kyoto, Japan). After being sectioned, 3-5 slices obtained from a single brain were transferred to a holding chamber and stored in oxygenated aCSF at room temperature for at least 40 min before use. Slices were then transferred to a conical tube containing gently aerated aCSF at 36 $^\circ$C to which 1.8 U/ml dispase (grade I; 0.75 ml/slice) was added. After incubation for 60 min, slices were rinsed with enzyme-free aCSF. Under a dissecting microscope, the NTS region was micropunched and placed on a poly-l-lysine-coated coverslip. The cells were then dissociated by trituration using progressively smaller diameter pipettes and allowed to settle on a coverslip for 20 min.

4.2. 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 an 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). pH was adjusted to 7.2 with CsOH. After formation of a giga seal, in order to record I_{Ca} carried by Ba²⁺ (I_{Ba}), the extracellular solution was replaced, from Krebs solution to a solution of the following composition (in mM): 151 tetraethylammonium (TEA) chloride, 5 BaCl₂, 1 MgCl₂, 10 HEPES and 10 glucose. pH was adjusted to 7.4 with Tris base. Command voltage protocols were generated by computer software, pCLAMP version 8 (Axon Instruments, Union City, CA, U.S.A.) and converted to an analogue signal using a DigiData 1200 interface (Axon Instruments, Union City, CA, U.S.A.). Command pulses were applied to cells through an L/M-EPC7 amplifier (HEKA Elektronik, Lambrecht, Germany). The currents were recorded with the amplifier and the computer software pCLAMP 8 acquisition system. Access resistance (< 15 MΩ) was determined by transient responses to voltage commands. Access resistance compensation was not used. To ascertain that no major changes in access resistance had occurred during the recordings, 5 mV, 10 msec pulses were used before I_{Ba} was evoked.
4.3. Materials

CGRP, ADM, CGRP(8-37), ADM(22-52), PD98,059 and nifedipine were purchased from Sigma (Tokyo, Japan). Anti-\(G_\alpha_i\) antibodies, anti-\(G_\alpha_s\) antibodies and anti-\(G_\alpha_{q/11}\) antibodies were purchased from Upstate biotechnology (Lake Placid, NY, U.S.A.). All antibodies were derived from rabbits immunized with a synthetic peptide corresponding to the COOH-terminal sequence of the human \(G_\alpha_i\), \(G_\alpha_s\) 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 (\(\omega\)-CgTx G VIA) and \(\omega\)-agatoxin IVA (\(\omega\)-Aga IVA) were purchased from Peptide Institute (Osaka, Japan).

4.4. Analysis and statistics

All data analysis was performed using the pCLAMP 8.0 acquisition system. Values in
text and figures are expressed as mean ± SEM. Statistical analysis was performed by student t-test for comparison between pairs of groups and by one-way analysis of variance (ANOVA) followed by a Dunnett's test. Probability (p) values of less than 0.05 were considered significant.

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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 $0$ mV at 20 sec intervals. B, Typical time course of CGPR-induced facilitation of $I_{Ba}$. CGPR (1 $\mu$M) was bath-applied during the times indicated by the filled bar. C, Typical superimposed $I_{Ba}$ traces according to the time course graph. D, Typical time course of ADM-induced facilitation of $I_{Ba}$. ADM (1 $\mu$M) was bath-applied during the times indicated by the filled bar. E, 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 CGRP. F, 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 ADM.
Fig. 2. A, Concentration dependence of CGRP- and ADM-induced facilitation of $I_{Ba}$.

B. Summary of CGRP- and ADM-induced facilitation of $I_{Ba}$. The histogram shows the degree of $I_{Ba}$ facilitation by 1 $\mu$M CGRP alone, 1 $\mu$M ADM alone and in co-application of 1 $\mu$M CGRP and 1 $\mu$M ADM. Numbers in parentheses indicate the number of neurons tested.
Fig. 3-A, Typical superimposed I_Ra traces according to the time course graph B.

B, Typical time course of CGPR- and ADM-induced facilitation of I_Ra. CGPR (1 μM), ADM (1 μM), and CGPR(8-37) (1 μM, CGRP receptor antagonist) were bath-applied during the time indicated by the filled and open bar, respectively. C, Histogram showing the degree of I_Ra facilitation by 1 μM CGRP alone, CGRP(8-37) + CGRP, ADM(22-25) (1 μM, ADM receptor antagonist) + CGRP, 1 μM ADM alone, CGRP(8-37) + ADM and ADM(22-25) + ADM. Numbers in parentheses indicate the number of neurons tested. *P < 0.05 compared with control, ANOVA.
Fig. 4. A, Histogram showing the degree of $I_{Ba}$ facilitation by 1 $\mu$ M CGRP (recording pipette was filled with GTP), after intracellular dialysis with anti-$G \alpha_i$-protein antibodies, anti-$G \alpha_s$-protein antibodies, boiled anti-$G \alpha_s$-protein antibodies (90$^\circ$C for 30 min) and anti-$G \alpha_{q11}$-protein antibodies. B, Histogram showing the degree of $I_{Ba}$ facilitation by 1 $\mu$ M ADM (recording pipette was filled with GTP), after intracellular dialysis with anti-$G \alpha_i$-protein antibodies, boiled anti-$G \alpha_i$-protein antibodies (90$^\circ$C for 30 min), anti-$G \alpha_s$-protein antibodies and anti-$G \alpha_{q11}$-protein antibodies. Numbers in parentheses indicate the number of neurons tested. *$P < 0.05$ compared with control, ANOVA.
Fig. 5-A. The histogram shows the degree of $I_{Ba}$ facilitation by 1 $\mu$M CGRP (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). B, Histogram showing the degree of $I_{Ba}$ facilitation by 1 $\mu$M ADM (control), after U-73122, after GF109203X, after SQ22536, intracellular dialysis with PKI(5-24) and after PD98,059. Numbers in parentheses indicate the number of neurons tested. *$P < 0.05$ compared with control, ANOVA.
Fig. 6·A, Histogram showing the degree of $I_{Ba}$ facilitation by 1 $\mu$M CGRP on L + R types (after treatment with $\omega$-CgTx GVI A + $\omega$-Aga IVA), N + R types (after treatment with Nif + $\omega$-Aga IVA), and P/Q + R types (after treatment with Nif + $\omega$-CgTx GVI A) VDCCs. Numbers in parentheses indicate the number of neurons tested.

6·B, Histogram showing the degree of $I_{Ba}$ facilitation by 1 $\mu$M ADM on L + R types (after treatment with $\omega$-CgTx GVI A + $\omega$-Aga IVA), N + R types (after treatment with Nif + $\omega$-Aga IVA), and P/Q + R types (after treatment with Nif + $\omega$-CgTx GVI A) VDCCs. Numbers in parentheses indicate the number of neurons tested.
Before application of CGRP (1 μM)

Before application of ADM (1 μM)
Fig. 2

**A**

Facilitation of I_{Ba} (%) against Concentration of agonists (M)

- CGRP
- ADM

**B**

Facilitation of I_{Ba} (%) for CGRP, ADM, CGRP + ADM
Fig. 4

A

Facilitation of I_Ba (%) vs treatments:
- CGRP
- Anti-Gi + CGRP
- Anti-Gs + CGRP
- Boiled anti-Gs + CGRP
- Anti-Gi/11 + CGRP

B

Facilitation of I_Ba (%) vs treatments:
- ADM
- Anti-Gi + ADM
- Anti-Gs + ADM
- Boiled anti-Gi + ADM
- Anti-Gi/11 + ADM

Legend:
- (n) number of experiments
- * indicates statistical significance
Fig. 5

A

Facilitation of $I_{Ba}$ by CGRP (%)

- Control
- U-73122
- GF109203X
- SQ22536
- PK(5-24)
- PD98059

B

Facilitation of $I_{Ba}$ by ADM (%)

- Control
- U-73122
- GF109203X
- SQ22536
- PK(5-24)
- PD98059

Note: * indicates statistical significance.
Fig. 6

A

Facilitation of $I_{ab}$ by CGRP

L + R types

N + R types

P/Q + R types

B

Facilitation of $I_{ab}$ by ADM

L + R types

N + R types

P/Q + R types