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Calcitonin gene-related peptide- and adrenomedullin-induced facilitation of calcium current in submandibular ganglion

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ABSTRACT

Objective: The control of saliva secretion is mainly under parasympathetic control. 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. The aim of this study was to investigate if adrenomedullin (ADM) and/or calcitonin gene-related peptide (CGRP) modulate voltage-dependent calcium channel (VDCCs) current (I_{Ca}) in SMG.

Design: The profile of CGRP and ADM actions in SMG was studied using the whole-cell configuration of the patch-clamp technique.

Results: Both ADM and CGRP facilitated I_{Ca} . These facilitations were attenuated by intracellular dialysis of the anti- $G\alpha_s$ -protein and pretreatment of SQ22536 (an adenylate cyclase inhibitor).

Conclusions: ADM and CGRP facilitates VDCCs mediated by $G\alpha_s$ -protein and adenylate cyclase in SMG.

1. Introduction

Capsaicin-sensitive primary afferents (CSPAs) are the C fibers which are specifically activated by capsaicin, the pungent agent in red hot pepper ¹. The CSPA release transmitters such as CGRP and tachykinins from peripheral endings via local axon reflex ^{1,2}. In most species, the coexistence of CGRP with substance P (SP) in the trigeminal supply of sensory capsaicin-sensitive nerves to the submandibular salivary gland has been demonstrated ^{1,3}. These fibers are seen around blood vessels, secretory ducts, and the acini of submandibular gland ⁴. The majority of CGRP-immunoreactive nerve fibers in the gland originate from neurons of the trigeminal ganglion ⁴. The CSPAs, which are also nociceptive afferent nerves, regulate various peripheral functions by neuropeptides which they release; vasodilation, extravasation of plasma protein and blood cells, chemotaxis and phagocytosis of macrophages and neutrophils, the production of arachidonate metabolites, and the activities of autonomic ganglia ⁵. In the sphenopalatine ganglion, the SP and CGRP positive sensory nerves surround the parasympathetic neuronal somata. The varicose appearance of the neuronal profiles close to the ganglion cells suggests the presence of synapses en passant in the ganglion ¹. The submandibular ganglia contain CGRP-immunoreactive nerve fibers, but the

ganglion cells are not immunoreactive for CGRP ⁴. Since CGRP is a potent vasodilator, it is possible that these fibers have a role in regulation of the glandular blood flow, but their exact functional roles in the parasympathetic ganglia remain to be established.

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.

The calcitonin gene-related peptide (CGRP) is a 37-amino-acid peptide that was initially described to be generated by alternative splicing of calcitonin gene ⁶. It has been demonstrated that CGRP causes depolarization of SMG ⁷. Adrenomedullin (ADM) is a 52-amino-acid peptide originally isolated from a human pheochromocytoma ⁸. It is structurally and functionally related to the CGRP, amylin peptide family ⁹.

Recently, it has been demonstrated that ADM is released in saliva to stimulate oral cell proliferation and antibacterial properties ¹⁰. Moreover, submandibular gland cells express CGRP and ADM receptors ¹¹.

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. Modulation of VDCCs by CGRP and ADM has been described previously in various types of cells¹²⁻¹⁴. However, the effect of CGRP and ADM 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 CGRP and ADM on I_{Ca} in SMG.

2. Materials and methods

2.1. Cell preparation

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 ($45 \times 30 \times 20$ cm) with a 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. The animals were treated in accordance with the principles

approved by the Council of the Physiological Society of Japan and in compliance with the guidelines of the Japanese Government. SMG neurons from hamsters were acutely dissociated using a modified version of the methods described previously¹⁵. 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 Ca²⁺-free Krebs solution of the following composition (in mM), 136 NaCl; 5 KCl; 3 MgCl₂; 10.9 glucose; 11.9 NaHCO₃ and 1.1 NaH₂PO₄. SMG neurons were treated with collagenase type I (3 mg/ml in Ca²⁺-free Krebs solution; Sigma) for 50 min at 37°C, followed by incubation in trypsin type I (1 mg/ml in Ca²⁺-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 technique¹⁶. 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 I_{Ca} carried by Ba²⁺ (I_{Ba}), the extracellular solution was replaced from Krebs solution to a solution containing the following composition (in mM): 151 tetraethylammonium (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 8 (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 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 the access resistance had occurred during the recordings a 5 mV, 10 msec pulses was used before I_{Ba} was evoked.

2.3. Materials

CGRP, ADM, CGRP(8-37), ADM(22-52), PD98,059 and nifedipine were purchased from Sigma (Tokyo, Japan). Anti-G α_i antibodies, anti-G α_s antibodies and anti-G $\alpha_{q/11}$ antibodies were purchased from Upstate biotechnology (Lake Placid, NY, U.S.A.). Each antibody was from rabbits immunized with a synthetic peptide corresponding to the COOH-terminal sequence of the human G α_i , G α_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.). ω -Conotoxin G VIA (ω -CgTx GVIA) and ω -agatoxin IVA (ω -Aga IVA) were purchased from Peptide Institute (Osaka, Japan).

2.4. Analysis and statistics

All data analysis were performed using pCLAMP 8.0 acquisition system. Values in text and figures are expressed as mean \pm 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

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

In 24 of 131 neurons tested, both ADM and CGRP facilitated I_{Ba} (Fig. 1A and B). In 61 of 131 neurons tested, only CGRP facilitated I_{Ba} (Fig. 1C and D).

An example of CGRP- and ADM-induced facilitation of I_{Ba} is shown in Fig. 1A and B. Application of 10 μ M ADM facilitated I_{Ba} from -1805 pA to -2492 pA (38.0% facilitation) in this neuron. In other neuron, Application of 10 μ M CGRP facilitated I_{Ba} from -1945 pA to -2575 pA (32.3% facilitation).

The current-voltage relationship measured before and during application of ADM (10 μ M) and CGRP (10 μ 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 -10 mV. Neither ADM (Fig. 1E) nor CGRP (Fig. 1F) altered the current-voltage relationship ($n=5$).

The concentration-response relationship in the ADM- and CGRP-induced facilitation of I_{Ba} is shown in Fig. 2A. Application of 10 nM-100 μ M ADM and CGRP rapidly and reversibly facilitates I_{Ba} . To generate a concentration-response curve, ADM and CGRP concentrations were applied randomly, and each neuron was exposed to only a single concentration. Fig. 2A shows that progressive increases in ADM and CGRP concentrations resulted in a progressively greater facilitation of I_{Ba} .

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

In the next series of experiments, we analyzed the effects of ADM and CGRP on I_{Ba} in neurons treated with selective antagonists. These receptors are characterized by their selective antagonists, namely ADM(22-52) for the ADM receptor and CGRP(8-37) fragment for the CGRP receptor^{17,18}.

As shown in Fig. 2B, selective antagonists were applied prior to ADM and CGRP. Treatment with the selective ADM antagonist, ADM(22-52) (1 μ M), attenuated the ADM-induced facilitation of I_{Ba} . In contrast, treatment with the selective CGRP antagonist, CGRP(8-37) (1 μ M), did not attenuate the ADM-induced facilitation of I_{Ba} . Treatment with ADM(22-52) (1 μ M) did not attenuate the CGRP-induced facilitation of

I_{Ba}. In contrast, treatment with CGRP(8-37) (1 μ M) attenuated the CGRP-induced facilitation of I_{Ba}. These results indicate that CGRP and ADM bind distinct receptors in the SMG.

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

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

As shown in Fig. 3, intracellular dialysis of the anti-G α_s -protein antibody attenuated

the ADM- and CGRP-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 ADM- and CGRP-induced facilitation of I_{Ba} . These results suggest that the $G\alpha_s$ -protein is involved in the ADM- and CGRP-induced facilitation of I_{Ba} in the SMG.

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

To evaluate the possible contribution of adenylate cyclase (AC) to the ADM- and CGRP-induced facilitation of I_{Ba} , the effects of ADM and CGRP on I_{Ba} in neurons treated with SQ22536 (an AC inhibitor) were investigated. Treatment with SQ22536 (10 μ M for 30 min) attenuated the ADM- and CGRP-induced facilitation of I_{Ba} .

To evaluate the possible contribution of protein kinase A (PKA) to the ADM- and 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. Intracellular application of PKI(5-24) (20 μ M for 7 min after assuming the whole-cell configuration) attenuated the ADM- and CGRP-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 PLC, 10 μ M for 15 min), GF109203X (a selective PKC inhibitor, 10 μ M for 30 min) and PD98,059 (a MAPK tyrosine kinase inhibitor, 10 μ M for 2 min) did not attenuate the ADM- and CGRP-induced facilitation of I_{Ba} . These results suggest that ADM and CGRP facilitates VDCCs involving AC and PKA pathways in the SMG neurons (Fig. 4).

3.5. Characterization of VDCC subtypes in ADM- and CGRP- 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 SMG¹⁹. L-type VDCCs are blocked by nifedipine (Nif). N-type VDCCs are blocked by ω -CgTx GVIA. P/Q-types VDCCs are blocked by ω -Aga IVA. Despite the addition of all these blockers, a component of current that is resistant still remains and has been termed R-type²⁰.

Thus, the types of VDCCs which are facilitated by ADM and CGRP were then investigated. When Nif (10 μ M, L-type VDCC blocker) + ω -Aga IVA (1 μ M, P/Q-type VDCC blocker) and Nif + ω -CgTx GVIA (1 μ M, N-type VDCC blocker) were applied first, resistant I_{Ba} was not significantly facilitated by a subsequent application of ADM and CGRP. On the other hand, when ω -CgTx GVIA + ω -Aga IVA was

applied first, resistant I_{Ba} were facilitated by the subsequent application of ADM and CGRP. These results demonstrate that ADM and CGRP facilitates L-type VDCCs, without significantly affecting N- and P/Q-types VDCCs in SMG neurons (Fig. 5).

4. Discussion

This study investigated the effects of CGRP and ADM on VDCCs in the SMG. This study has shown that ADM and CGRP facilitates L-type VDCCs via the $G\alpha_s$ -protein involving AC and PKA in the SMG.

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 SMG. 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 receptors²¹. Although ADM is known to bind CGRP receptors with low affinity²², CGRP does not bind ADM receptors^{23,24}.

In addition, we could demonstrate that ADM and CGRP facilitates VDCCs involving AC and PKA. cAMP-PKA pathways are coupled to ADM receptors in various types of

cells ^{8,25,26}, including neural cells ²⁷. This study has shown that ADM and CGRP facilitate L-type VDCCs in the SMG. Similar observation has been demonstrated in smooth muscle cells ¹² and cardiac cells ¹³. There are several mechanisms of VDCCs facilitation ^{28,29}. 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 ³⁰. In contrast, in rat ventricular myocytes, ADM inhibits L-type VDCCs ¹⁴. 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 in SMG? Our results indicate that CGRP facilitate VDCCs and thus provide support for the idea that sensory afferent collaterals containing CGRP can communicate sensory information from the submandibular gland directly to parasympathetic postganglionic neurons within the SMG. It was demonstrated that some neuropeptides are released from collaterals of the sensory nerves and regulate the activity of SMG cells ¹. We have previously reported that substance P inhibits VDCCs currents (I_{Ca}) ³¹, whereas CGRP facilitates in I_{Ca} SMG neurons ³².

CGRP is localized in nerve terminals around blood vessels and ducts, and occasionally around acini. CGRP is present in about 45% in dorsal root ganglion (DRG) ³³. 50% of the

CGRP-immunoreactive nerve (IR) fibers in the rat parotid gland originate in the DRG, 20% are thought to be parasympathetic, while 30% comes from unknown origin ³⁴. CGRP-IR nerve fibers of the rat submandibular gland come mainly from the trigeminal ganglion ^{34,35}. It has not been determined that sensory afferent collaterals containing CGRP reflex modulate SMG. In autonomic neurons, CGRP modulates nicotinic receptor function ³². Future studies are required to fully understand the molecular mechanism of such substance and control saliva secretion.

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Fig.1-A, Typical superimposed I_{Ba} traces according to the time course graph B. 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 ADM- and CGRP-induced facilitation of I_{Ba} . ADM ($10 \mu M$) and CGRP ($10 \mu M$) were bath-applied during the times indicated by the filled bar. C, Typical superimposed I_{Ba} traces according to the time course graph D. D, Typical time course of ADM- and CGRP-induced facilitation of I_{Ba} . ADM ($10 \mu M$) and CGRP ($10 \mu M$) were 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 $10 \mu M$ ADM. 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 $10 \mu M$ CGRP.

Fig. 2-A, Concentration dependence of ADM- and CGRP-induced facilitation of I_{Ba}.

B, Histogram showing the degree of I_{Ba} facilitation by 10 μ M ADM alone, ADM(22-25)

+ ADM and CGRP(8-37) + ADM. 10 μ M CGRP alone, ADM(22-25) + CGRP,

CGRP(8-37) +ADM receptor. Numbers in parentheses indicate the number of neurons

tested. *P < 0.05 compared with control, ANOVA.

Fig. 3- A, Histogram showing the degree of I_{Ba} facilitation by 10 μ M ADM (recording pipette was filled with GTP), after intracellular dialysis with anti-G α_i -protein antibodies, anti-G α_s -protein antibodies, boiled anti-G α_s -protein antibodies (90°C for 30 min) and anti-G $\alpha_{q/11}$ -protein antibodies. B, Histogram showing the degree of I_{Ba} facilitation by 10 μ M CGRP (recording pipette was filled with GTP), after intracellular dialysis with anti-G α_i -protein antibodies, anti-G α_s -protein antibodies, boiled anti-G α_s -protein antibodies (90°C for 30 min) and anti-G $\alpha_{q/11}$ -protein antibodies. Numbers in parentheses indicate the number of neurons tested. *P < 0.05 compared with control, ANOVA.

Fig. 4-A, The histogram shows the degree of I_{Ba} facilitation by 10 μ M ADM (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 10 μ M CGRP (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. 5- A, Histogram showing the degree of I_{Ba} facilitation by $10 \mu M$ ADM on L + R types (after treatment with ω -CgTx GVIA + ω -Aga IVA), N + R types (after treatment with Nif + ω -Aga IVA), and P/Q + R types (after treatment with Nif + ω -CgTx GVIA) VDCCs. Numbers in parentheses indicate the number of neurons tested.

6-B, Histogram showing the degree of I_{Ba} facilitation by $10 \mu M$ CGRP on L + R types (after treatment with ω -CgTx GVIA + ω -Aga IVA), N + R types (after treatment with Nif + ω -Aga IVA), and P/Q + R types (after treatment with Nif + ω -CgTx GVI A) VDCCs. Numbers in parentheses indicate the number of neurons tested.

Fig. 1

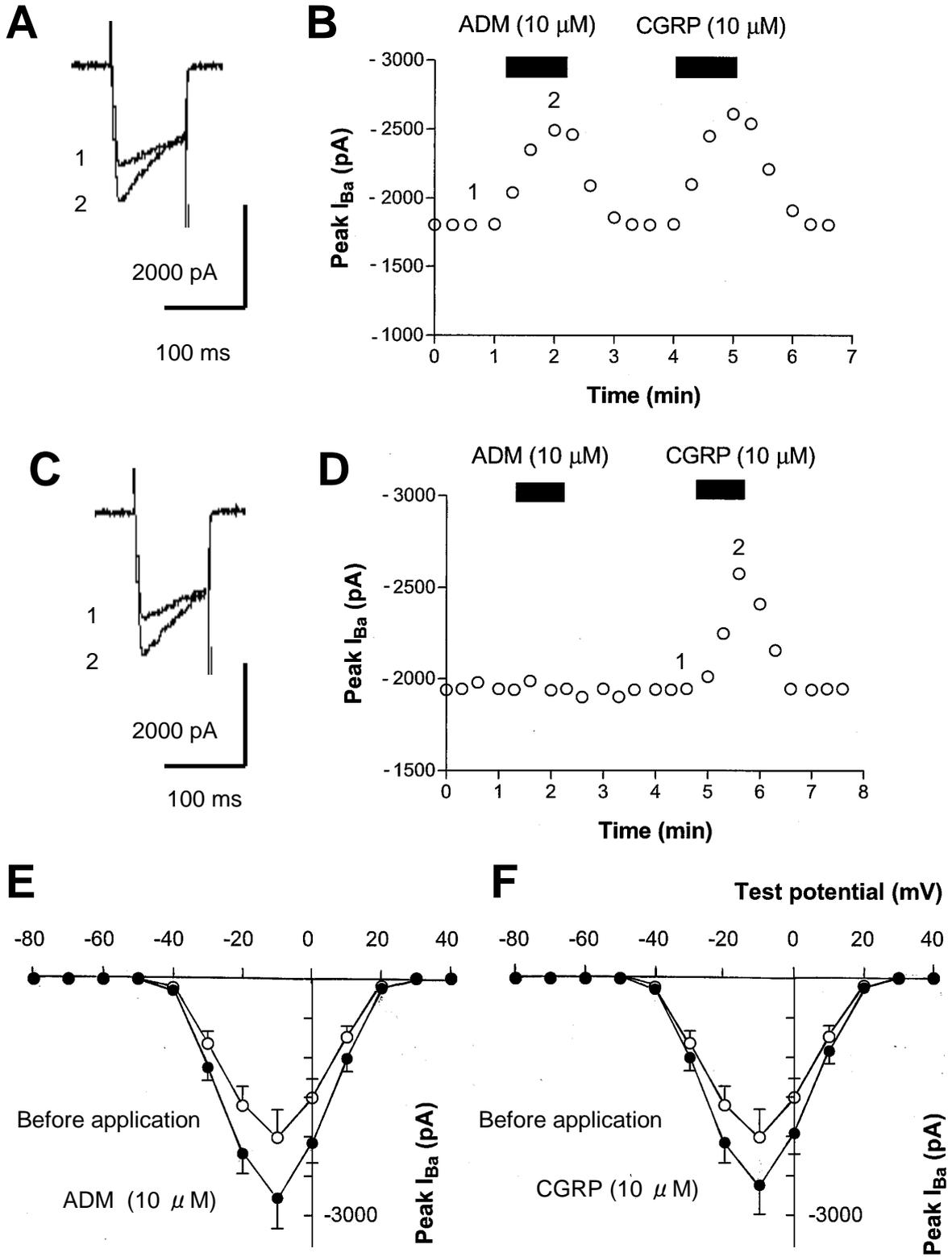
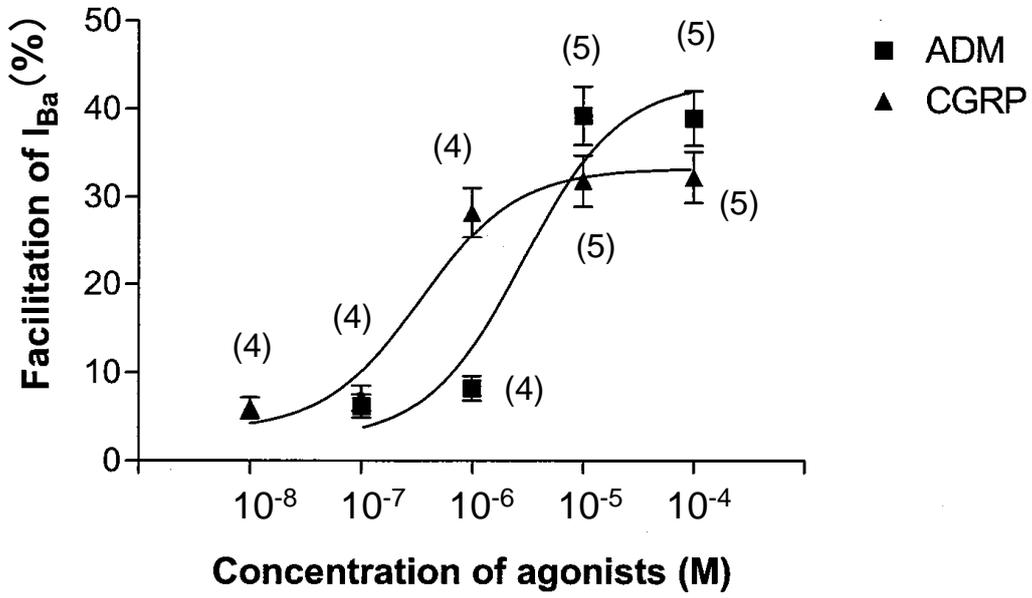


Fig. 2

A



B

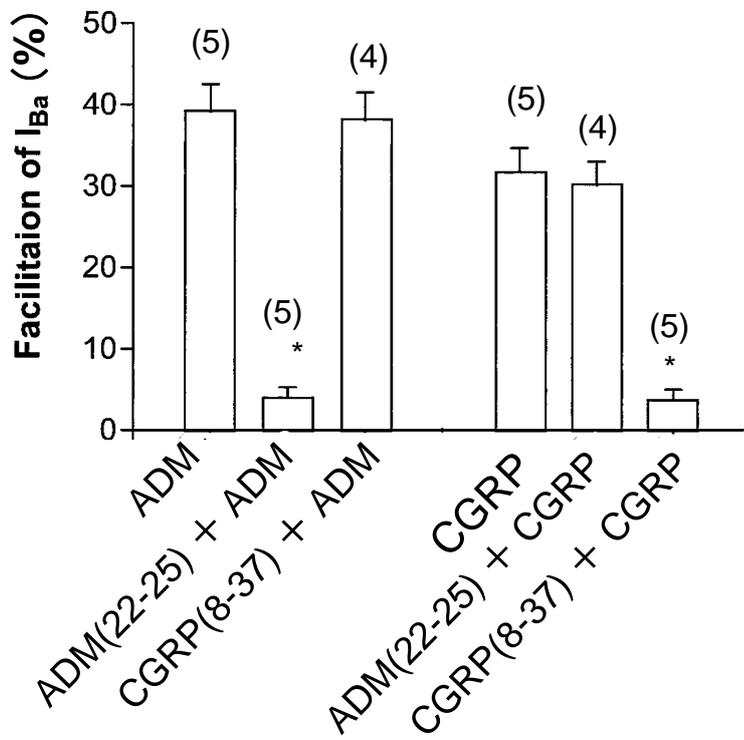
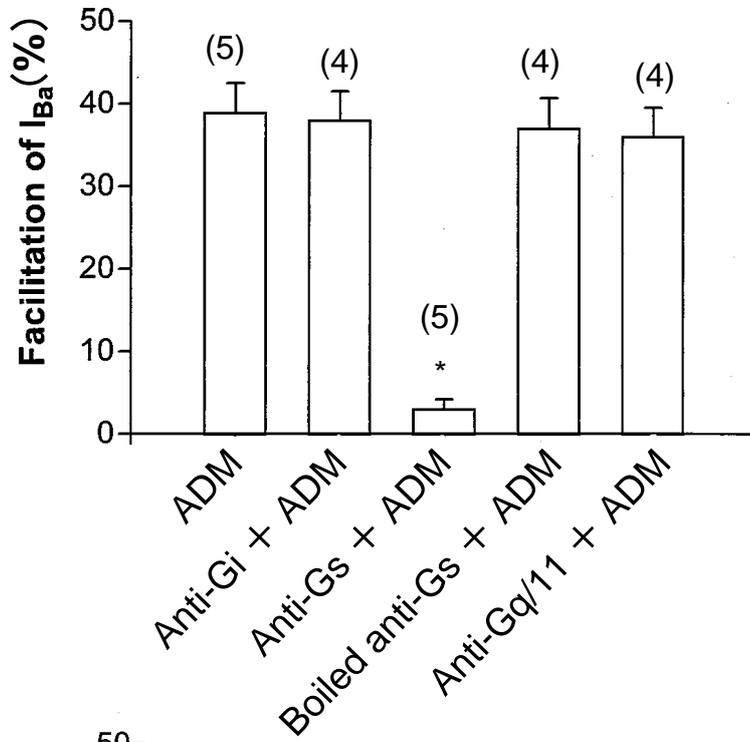


Fig. 3

A



B

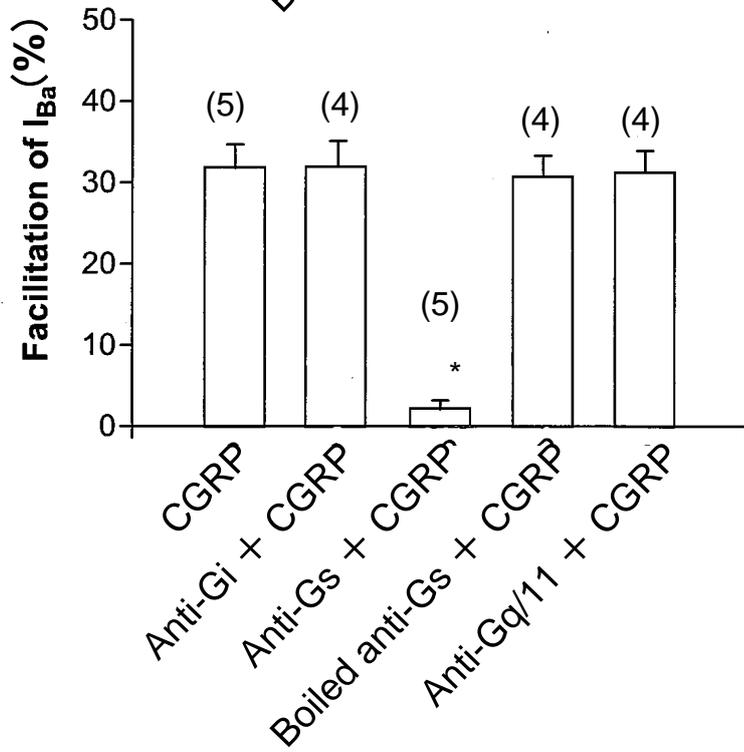
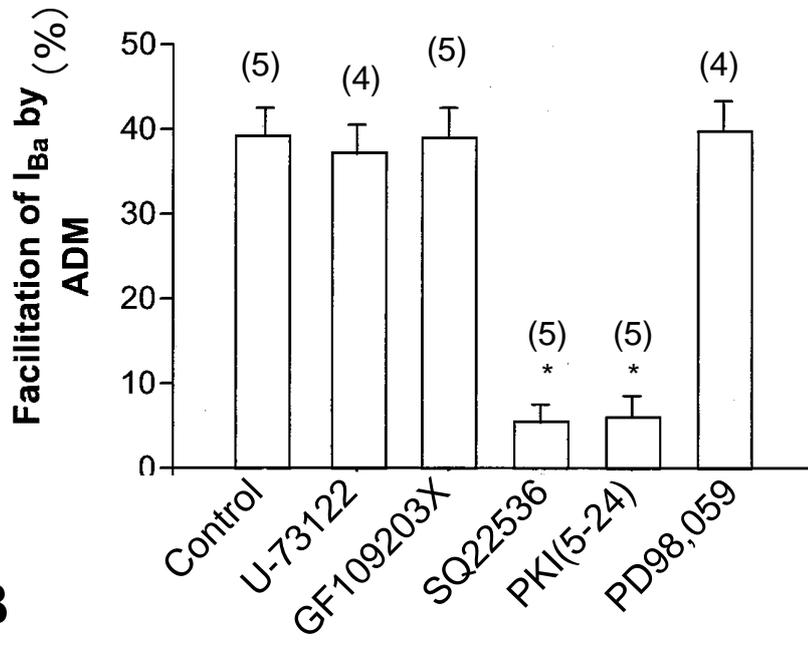


Fig. 4

A



B

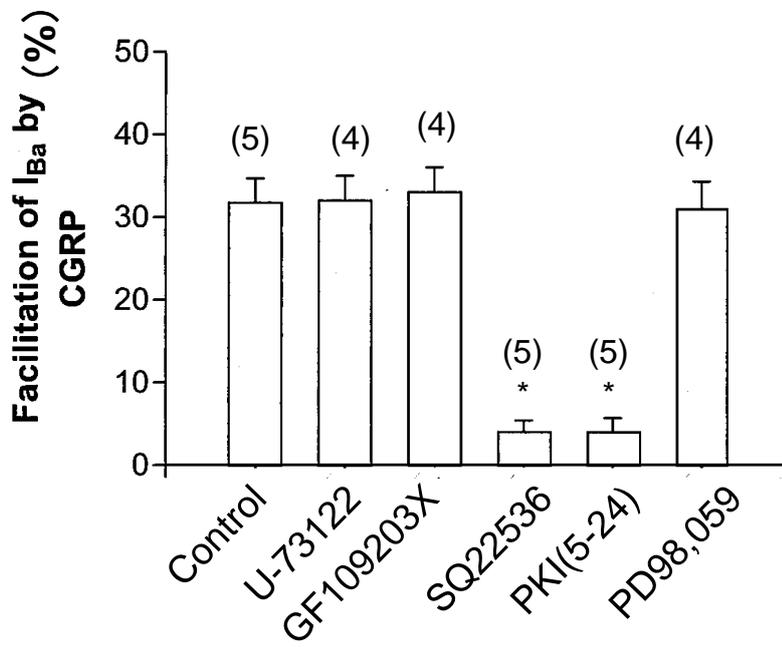


Fig. 5

