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Muscarinic receptor-mediated excitation of rat intracardiac ganglion neurons

Michiko Hirayama [a, b], Masanori Ogata [a], Tomoyuki Kawamata [b], Hitoshi Ishibashi [a, *]

[a] Department of Physiology, School of Allied Health Sciences, Kitasato University, Sagamihara 252-0373, Japan
[b] Department of Anesthesiology, Wakayama Medical University, Wakayama 641-0012, Japan

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ABSTRACT

Modulation of the membrane excitability of rat parasympathetic intracardiac ganglion neurons by muscarinic receptors was studied using an amphotericin B-perforated patch-clamp recording configuration. Activation of muscarinic receptors by oxotremorine-M (OxoM) depolarized the membrane, accompanied by repetitive action potentials. OxoM evoked inward currents under voltage-clamp conditions at a holding potential of −60 mV. Removal of extracellular Ca^{2+} markedly increased the OxoM-induced current (I_{OxoM}). The inward I_{OxoM} in the absence of extracellular Ca^{2+} was fully inhibited by removal of extracellular Na^{+}, indicating the involvement of non-selective cation channels. The I_{OxoM} was inhibited by organic cation channel antagonists including SKF-96365 and ML-204. The I_{OxoM} was antagonized by muscarinic receptor antagonists with the following potency: 4-DAMP > pirenzepine = darifenacin > methoctramine. Muscarinic toxin 7 (MT-7), a highly selective inhibitor for M_1 receptor, produced partial inhibition of the I_{OxoM}. In the presence of MT-7, concentration −inhibition curve of the M_3-preferring antagonist darifenacin was shifted to the left. These results suggest the contribution of M_1 and M_3 receptors to the OxoM response. The I_{OxoM} was inhibited by U-73122, a phospholipase C inhibitor. The membrane-permeable IP_3 receptor blocker xestospongin C also inhibited the I_{OxoM}. Furthermore, pretreatment with thapsigargin and BAPTA-AM inhibited the I_{OxoM}, while KN-62, a blocker of Ca^{2+}/calmodulin-dependent protein kinase II, had no effect. These results suggest that the activation mechanism involves a PLC pathway, release of Ca^{2+} from intracellular Ca^{2+} stores and calmodulin.

The cation channels activated by muscarinic receptors may play an important role in neuronal membrane depolarization in rat intracardiac ganglion neurons.

1. Introduction

Neural regulation of the heart is under the control of the sympathetic and parasympathetic divisions of the autonomic nervous system. Activation of the parasympathetic post-ganglionic neurons of intracardiac ganglia (ICG) produces negative chronotropic, dromotropic and inotropic actions (Tsuboi et al., 2000). The ICG were long thought to be simple relay stations for parasympathetic inputs from vagal pre-ganglionic neurons in the brainstem to the cardiac end-effectors. However, there is increasing evidence indicating that the ICG neurons are innervated by various inputs from neural networks, including parasympathetic pre-ganglionic, sympathetic post-ganglionic and sensory fibers (Konopka et al., 1992; Parsons et al., 1987; Calupca et al., 2000). In addition, the membrane excitability of the ICG neurons is modulated by various neurotransmitter and neuromodulators. Substance P inhibits acetylcholine (ACh)-evoked currents in the ICG neurons of rats (Cuevas and Adams, 2000) and guinea pigs (Zhang et al., 2001a). Substance P also produces slow depolarization in guinea-pig ICG neurons through the NK_3 and NK_1 receptors (Zhang et al., 2005). The pituitary adenylate cyclase-activating polypeptide, which is co-localized with ACh in preganglionic parasympathetic fibers innervating guinea pig ICG, causes an increase in the membrane excitability of these neurons

Abbreviations: ACh, acetylcholine; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; ICG, intracardiac ganglion; mAChRs, muscarinic ACh receptors; OxoM, oxotremorine-M; PLC, phospholipase C; TRP, transient receptor potential; TTX, tetrodotoxin; XeC, xestospongin-C.

* Corresponding author. Department of Physiology, School of Allied Health Sciences, Kitasato University, Sagamihara-City, Kanagawa 252-0373, Japan. Tel./fax: +81 42 778 8153.
E-mail address: isibasih@kitasato-u.ac.jp (H. Ishibashi).

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(Merriam et al., 2004). The vasoactive intestinal polypeptide also increases the excitability of intracardiac neurons (DeHaven and Cuevas, 2004). These observations suggest that ICG are important sites of neuronal integration of various inputs to the heart.

Muscarinic ACh receptors (mAChRs) are divided into five subtypes, i.e., the M_1 to M_5 receptors that have been shown to have distinct G-protein linked pharmacological properties and second-messenger signaling pathways (Bonner, 1989; Caulfield, 1993). The M_1, M_3 and M_5 muscarinic receptors couple to $G_{q/11}$ proteins, while M_2 and M_4 muscarinic receptors couple to the pertussis-toxin sensitive $G_{i/o}$ proteins (Caulfield and Birdsall, 1998). The mammalian intracardiac ganglion neurons express mAChRs (Hoover et al., 1994; Hassall et al., 1993). In cultured guinea pig ICG neurons, stimulation of mAChRs produces both depolarization by a reduction of potassium conductance, and hyperpolarization by an increase in potassium conductance (Allen and Burnstock, 1990). In rat ICG neurons, activation of M_4 receptors inhibits high-voltage-activated Ca^{2+} channels (Cuevas and Adams, 1997). It has also been reported that stimulation of mAChR causes the activation of phospholipase C (PLC) and increases the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of rat ICG neurons through generation of IP_3 and subsequent Ca^{2+} release from IP_3-sensitive intracellular Ca^{2+} stores (Beker et al., 2003). In most vertebrate cells, on the other hand, stimulation of the PLC-coupled receptors and its downstream second messenger cascades play important roles in Ca^{2+} entry through Ca^{2+}-permeable cationic channels on the plasma membrane, thereby causing membrane depolarization (Plant and Schaefer, 2003). However, it remains unclear how muscarinic receptors modulate the excitability of ICG neurons. In the present study, therefore, the effect of the muscarinic agonist OxoM was investigated in acutely isolated rat ICG neurons using an amphotericin B perforated patch-clamp recording configuration.

2. Materials and methods

All experiments were conducted under the 'Guiding Principles for the Care and Use of Laboratory Animals' approved by the Japanese Pharmacological Society and were approved by the Animal Experimentation Ethics Committee of the Kitasato University School of Allied Health Sciences.

2.1. Isolation of neurons

The experiments were performed on cardiac parasympathetic ganglion neurons freshly dissociated from 8- to 15-day-old Wistar rats. The procedure for obtaining dissociated ganglion neurons was similar to that used in previous studies (Fieber and Adams, 1991; Ishibashi et al., 2003). Briefly, rats were killed by decapitation under pentobarbital sodium anesthesia (100 mg/kg, i.p.) and the ganglia located at the surface of the atria (Batulevicius et al., 2003; Pardini et al., 1987) were rapidly removed. The isolated ganglia were treated with the normal external solution containing 0.4% collagenase and 0.4% trypsin for 60 min at 37 °C. Following this enzyme treatment, the neurons were dissociated mechanically by gentle triturating using a fire-polished Pasteur pipette in a culture dish (Primaria 3801, Becton Dickinson, Rutherford, NJ, USA). The dissociated neurons adhered to the bottom of the dish within 20 min. We used the isolated neurons 1–8 h after preparation.

2.2. Solution and chemicals

The ionic composition of the standard external solution was (mM): NaCl 150, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10 and glucose 10. The pH was adjusted to 7.4 with tris (hydroxymethyl) aminomethane (Tris-OH). The nominally Ca^{2+}-free solution was made by simply omitting Ca^{2+} from the standard external solution. The composition of the patch pipette (internal) solution was (mM): cesium methanesulfonate 80, cesium chloride 70, and HEPES 10. The initial current-clamp experiment was performed by using an internal solution containing (mM): NaCl 10, KCl 60, potassium methanesulfonate 80, and HEPES 10. The pH of these patch-pipette solution was adjusted to 7.3 with Tris-OH. Amphotericin B was dissolved in dimethyl sulfoxide (DMSO), resulting in a 100 mg/ml stock solution, and added to the internal solution to give a final concentration of 7 μg/ml, just before use. Drugs were topically applied with the 'Y-tube' solution exchange device (Murase et al., 1989).

The drugs used in the present experiments were tetrodotoxin (TTX), xestospongin-C (XeC), (S)-5-isoquinolinesulfonic acid 4-[2-[(5-isoquinolinylsulfonyl) methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl] phenyl ester (KN-62), N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) (Wako, Tokyo, Japan), N-(6-Aminohexyl)-1-naphthalenesulfonamide hydrochloride (W-5), BAPTA-AM, wortmannin (Tokyo Kasei, Tokyo, Japan), acetylcholine (ACh), amphotericin B, collagenase, (4-Hydroxy-2-butynyl)-1-trimethylammonium-3-chlorocarbanilate chloride (McN-A-343), 4-Methyl-2-(1-piperidinyl)quinoline (ML-204), 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), methoctramine, nicotine, oxotremorine-M (OxoM), pirenzepine, 1-[β-(3-(4-Methoxyphenyl) propoxy)- 4-methoxyphenethyl]-1Himidazolehydrochloride (SKF-96365), trypsin, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U-73122), 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5- pyrrolidinedione (U-73343) (Sigma, St Louis, MO, USA), darifenacin (LKT Laboratories, Inc, Saint Paul, MN, USA) muscarinic toxin 7 (MT-7) (Peptide Institute, Osaka, Japan) and pentobarbital sodium (Dainippon Seiyaku, Tokyo, Japan).

Drugs that are not soluble in water were first dissolved in dimethyl sulfoxide (DMSO) and then diluted in standard external solution. The final concentration of DMSO never exceed 0.1%.

2.3. Electrophysiological recordings

Membrane currents were monitored with the amphotericin B perforated-patch recording mode (Akaike and Harata, 1994; Ishibashi et al., 2012). Patch pipettes were made from borosilicate glass tubes in two stages on a vertical pipette puller (PC-10, Narishige, Tokyo, Japan). The resistance between the recording electrode filled with the internal solution and the reference electrode in the normal external solution was 4–8 MΩ. The intracardiac ganglion neurons were visualized on an inverted microscope with phase-contrast equipment (DMIRB, Leica, Nussloch, Germany). The current signals were amplified by a patch-clamp amplifier (AXOPATCH 200B, Molecular Devices, Sunnyvale City, California, USA). After stable perforated-patch formation, the series resistance ranged from 10 to 22 MΩ and was compensated in the same manner as previously described (Murai et al., 1998). Before digitization (sampling rate 10 kHz), the signals were filtered at 2 kHz with a three-pole low-pass Bessel-type filter. Data were stored on a microcomputer hard disk for subsequent analysis with the pClamp system (Axon Instruments, Foster City, CA, USA). The membrane potential was given without a liquid junction potential correction of −3 mV. All experiments were carried out at room temperature (21–24 °C).

2.4. Data analysis

Current responses were quantified using the Clampfit program (Axon Instruments). The concentration–response curves were drawn with the Michaelis–Menten equation, $I = I_{max}C^n/(C^n + K^n)$ where I is the observed agonist-induced current, I_{max} is the maximum current, C is the agonist concentration, n is the Hill coefficient, and K is the agonist concentration that evoked the half-maximal response (EC_{50}). Similarly, concentration–inhibition curve was drawn with the following equation: $I = 1 - C^n/(C^n + Ki^n)$ where Ki is the antagonist concentration that produced the half-maximum inhibition (IC_{50}).

Data are expressed as mean ± S.E.M and n values refer to the number of cells. Data were analyzed statistically using Student's unpaired t-test or one-way ANOVA followed by Tukey's test with the level of significance being taken as $^*P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$.

3. Results

3.1. Effects of cholinergic agonists on membrane potential

Acutely isolated parasympathetic neurons from rat intracardiac ganglia (ICG) were studied using whole-cell recording with an amphotericine-B perforated-patch recording configuration. Under current-clamp conditions, the neurons exhibited no spontaneous action potentials, and had a resting membrane potential of −55.9 ± 0.5 mV (n = 56) measured with a K^+-based pipette solution. This value was consistent with those previously reported in rat ICG neurons (Xu and Adams, 1992; Xi-Moy and Dun, 1995). Application of 3 μM ACh, 3 μM nicotine and 1 μM OxoM depolarized the membrane by 18.2 ± 2.7 (n = 8), 17.8 ± 2.6 (n = 4) and 9.1 ± 1.2 mV (n = 17), respectively, accompanied by repetitive firing of action potentials in all neurons tested (Fig. 1). These results suggest that not only nicotinic but also muscarinic receptors mediate excitation of rat ICG neurons.

3.2. Inward currents evoked by cholinergic agents

To reveal the mechanisms underlying the muscarinic receptor-mediated excitation, voltage-clamp experiments were performed