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Identification of ATP-sensitive K^+ Conductances in Male Rat Major Pelvic Ganglion Neurons

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Major pelvic ganglia (MPG) neurons are classified into sympathetic and parasympathetic neurons according to the electrophysiological properties; membrane capacitance (Cm), expression of T-type Ca^{2+} channels, and the firing patterns during depolarization. In the present study, function and molecular expression of ATP-sensitive K^+ (K_{ATP}) channels was investigated in MPG neurons of male rats. Only in parasympathetic MPG neurons showing phasic firing patterns, hyperpolarizing changes were elicited by the application of diazoxide, an activator of K_{ATP} channels. Glibenclamide (10 μM), a K_{ATP} channel blocker, completely abolished the diazoxide-induced hyperpolarization. Diazoxide increased inward currents at high K^+ (90 mM) external solution, which was also blocked by glibenclamide. The metabolic inhibition by the treatment with mitochondrial respiratory chain inhibitors (rotenone and antimycin) hyperpolarized the resting membrane potential of parasympathetic neurons, which was not observed in sympathetic neurons. The hyperpolarizing response to metabolic inhibition was partially blocked by glibenclamide. RT-PCR analysis revealed that MPG neurons mainly expressed the K_{ATP} channel subunits of Kir6.2 and SUR1. Our results suggest that MPG neurons have K_{ATP} channels, mainly formed by Kir6.2 and SUR1, with phenotype-specificity, and that the conductance through this channel in parasympathetic neurons may contribute to the changes in excitability during hypoxia and/or metabolic inhibition.

Key Words: ATP-sensitive K^+ channel, Major pelvic ganglia, Phenotype-specific, Metabolic inhibition

INTRODUCTION

ATP-sensitive K^+ (K_{ATP}) channels play an important role of sensing cellular metabolic activity. Through this channel, information concerning intracellular energy status is transduced into electrical information (Ashcroft & Ashcroft, 1990). Reduced intracellular ATP/ADP ratio is the main signal to open the K_{ATP} channels that will shift the membrane potential toward the equilibrium potential for potassium ion. Conversely, high ATP/ADP ratio makes this channel closed, which will induce depolarization of the plasma membrane under physiological ionic concentration (Ashcroft & Ashcroft, 1990; Yokoshiki et al, 1998). In pancreatic β-cells, the K_{ATP} channels act as a glucose sensor with respect to the release of insulin. When blood glucose levels rise, the b-cells respond to the increasing intracellular ATP concentration and close this channel which depolarizes the cell, causing the activation of voltage-sensitive Ca^{2+} channels, the influx of Ca^{2+}, and insulin release (Ashcroft et al, 1984; Cook & Hales, 1984). In addition to the insulin secretion, the K_{ATP} channels are implicated in various cellular functions, such as modulations of the action potential duration in cardiac myocytes (Noma, 1983), the excitability in skeletal muscle cells (Spruce et al, 1985), and the contractility in vascular smooth muscle cells (Standen et al, 1989; Zhang & Bolton, 1996).

The recent molecular characterization of K_{ATP} channels has revealed their precise structure and localization in various tissues. The K_{ATP} channel is a heterologous octameric structure composed of four Kir6.x subunits and four sulfonylurea receptor (SUR) subunits: Kir6.x subunits form the inwardly rectifying K^+ permeating pore and SUR subunits have a regulatory function enabling sensitivity to the ATP/ADP ratio and channel blocker sulfonylurea (Aguilar-Bryan et al, 1995; Inagaki et al, 1996; Tucker et al, 1997). Several combinations of these two subunits have been identified in native cells, such as Kir6.2-SUR1 in pancreatic β-cells, Kir6.2-SUR2A in cardiac and skeletal muscle, and Kir6.1 (or 6.2)-SUR2B in smooth muscle (Seino, 1999). Each combination shows distinct single channel conductance, blocking potency of ATP, and varying sensitiveness to K_{ATP} channel openers or blockers (Yokoshiki et al, 1998).

The K_{ATP} channels are also widely distributed in the neurons of central nervous system, having been identified in striatal cholinergic interneurons (Lee et al, 1998), CA1 hippocampal pyramidal neurons (Zawar et al, 1999), mid-

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ABBREVIATIONS: MPG, major pelvic ganglia; K_{ATP}, ATP-sensitive K^+; SUR, sulfonylurea receptor; LVA, low-voltage activated

brain dopaminergic neurons (Guatteo et al, 1998), and pituitary gland (Bernardi et al, 1993). The compositions of Kir6.x and SUR subunits are different depending on neuronal types (Zawar et al, 1999). Recently, K_{ATP} conductance has been identified in dissociated neurons from intracardiac ganglia in adult rats, which may contribute to changes in neural regulation of cardiac function during ischemia-reperfusion (Hoggs & Adams, 2001). To the best of our knowledge, it is the only report of K_{ATP} channels in peripheral neurons from mammalian autonomic ganglia.

The major pelvic ganglia (MPG) provide autonomic innervation to the lower bowel, urinary bladder, prostate, and penis (Keast, 1999). Physiologically, these ganglia play important roles in various autonomic reflexes, including micturition and penile erection (de Groat & Booth, 1993). A peculiar feature of MPG that differentiates them from other autonomic ganglia is the colocalization of both sympathetic and parasympathetic postganglionic neurons within the same ganglion capsule (Keast, 1999). In the present study, we observed the K_{ATP} conductance in a subpopulation of neurons dissociated from MPG in male rats. In the present study, we recorded K_{ATP} currents on relatively small and phasic type cells, which were suggested to be parasympathetic postganglionic neurons. Since the major constituents of K_{ATP} channel complex are Kir6.2 with SUR1, which is known to be highly sensitive to metabolic inhibition (Liss et al, 1999), these findings suggest the possibility that K_{ATP} channels in parasympathetic MPG neurons are involved in electrophysiological changes during hypoxia and/or metabolic inhibition.

METHODS

Preparation of pelvic ganglion neurons

MPG neurons were enzymatically dissociated by the method described previously with some modifications (Park et al, 2001). The dissociated neurons were incubated in RPMI 1640 containing 10% fetal calf serum and 1% penicillin-streptomycin (all from Life Technologies, Grand island, NY, USA). Neurons were then plated onto cover slips coated with poly-L-lysine and maintained in a humidified 95% air-5% CO_2 incubator at 37°C. In all the following experiments, neurons were used within 36 hours after plating.

RT-PCR analysis

Total RNA from dissociated MPG neurons was prepared using a modified guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi 1987). Synthesis of the first strand of cDNA was performed in an RT-PCR buffer containing 2 μg total RNA, 25 nmoles dNTP, 0.5 μg random hexamer, 20 U RNase inhibitor and 200 U murine leukemia virus reverse transcriptase (all from Promega, WI, USA) in a final volume of 25 μl at 37°C for 60 min. Specific sense and antisense primer pairs were designed based on the known cloned sequences of rat K_{ATP} channel subunits deposited in GenBank (Table 1). Single stranded cDNA products were denatured at 94°C for 5 min, and then subjected to PCR amplification (35 cycles). Each PCR cycle consisted of denaturing at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 1 min in a GeneAmp thermocycler (Perkin-Elmer, CT, USA). PCR buffer (50 μl) contained the transcribed cDNA, 10 pmoles primers, 10 nmoles dNTP, and 1.25 U of Taq polymerase (Perkin-Elmer, CT, USA). As a positive control, cDNAs of K_{ATP} channel subunits were amplified in rat insulinoma cells under the same conditions. The resultant PCR products were separated and visualized on a 1.1% agarose gel containing ethidium bromide.

Electrophysiology

ATP-sensitive K^+ currents were recorded using the whole-cell variant of the patch clamp technique (Hamill et al, 1981). Patch electrodes were fabricated from a borosilicate glass capillary (Corning 7052; Garner Glass Co, Claremont, CA, USA) using a P-97 Flaming Brown micropipette puller (Sutter Instrument Co., San Rafael, CA, USA). The patch electrodes were fire polished on a microforge (Narishige, Tokyo, Japan), and had resistances of 1~3 MΩ when filled with the internal solution described below. An Ag/AgCl wire was used to ground the bath. The cell membrane capacitance and series resistance were compensated electronically using an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany). Voltage protocol generation and data acquisition were performed using Pulse/Pulsefit (v8.50) software (HEKA Elektronik) on an IBM

Table 1. Primers used for RT-PCR analysis

Primers		Sequence (5' to 3')	Position	Product size	GenBank accession No.
Kir6.1	S	TTG GGT TTG GAG GGA GAA TG	428~447	411 bp	AB043637
	AS	ACA GGG GGC TAC GCT TAT CA	819~838		
Kir6.2	S	CTG CCT TCC TTT TCT CCA TC	892~911	385 bp	D86039
	AS	TTA CCA CCC ACA CCG TTC TC	1257~1276		
SUR1	S	TGG GGA ACG GGG CAT CAA CT	2484~2503	388 bp	AB052294
	AS	TGG CTC TGG GGC TTT TCT C	2853~2871		
SUR2	S	GCA AGA GCG TGG AAG AGA C	1805~1823	501 bp	AF087838
	AS	TGC CCC ATG AGA AGT ATC C	2287~2305		

*S: sense, AS: antisense

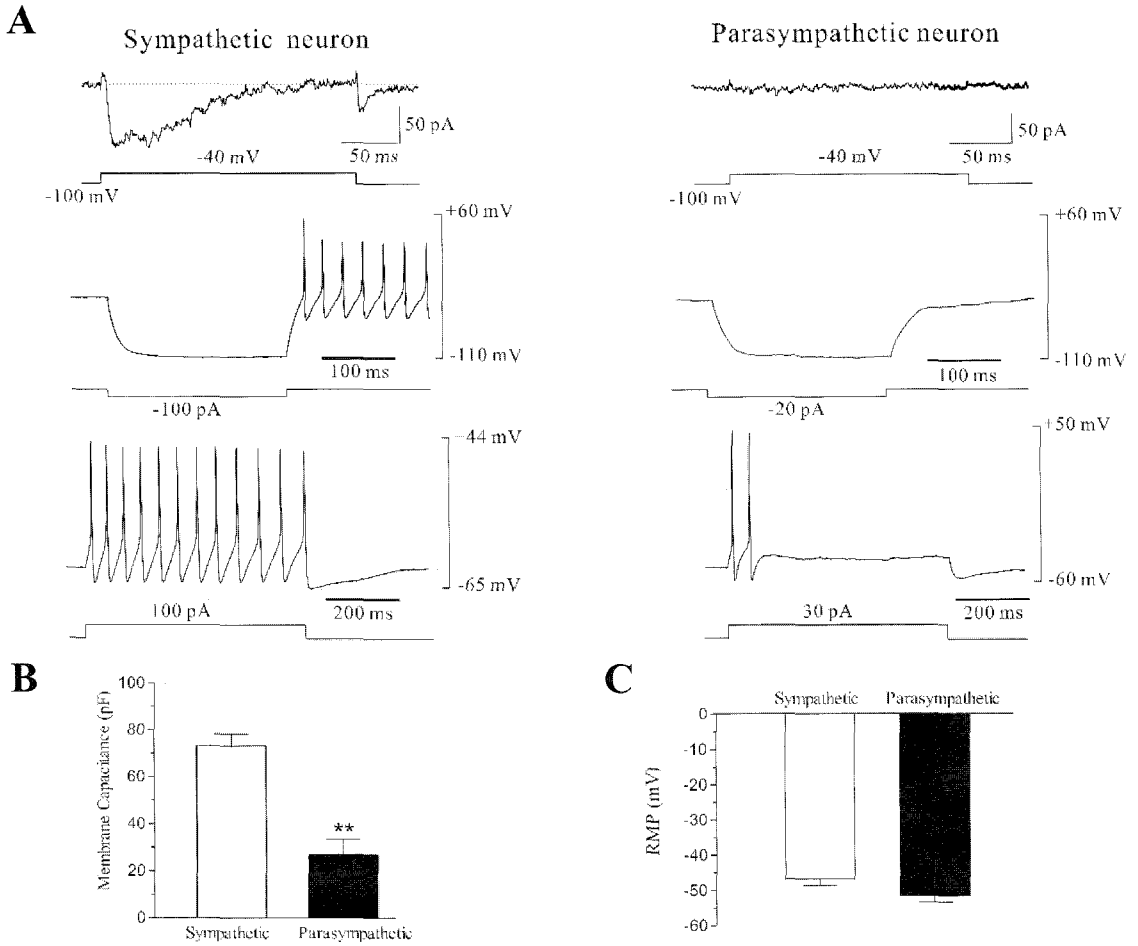

Fig. 1. The electrophysiological properties of sympathetic and parasympathetic neurons of MPG. A: The presence of T-type Ca^{2+} currents and rebound spikes after anodal break were observed in sympathetic but not in parasympathetic neurons. In response to long depolarizing current injection, sympathetic neuron showed tonic firing patterns that maintained a steady generation of action potential. In contrast, rapidly adapted phasic firings during depolarization were observed in parasympathetic neuron. B and C: Comparison of the mean capacitances and the resting membrane potentials between sympathetic and parasympathetic neurons of MPG. Data represent the mean± S.E.M. **$p<0.001$.

computer. Current traces were generally low-pass filtered at 3 kHz and digitized at 10 kHz using 8-pole Bessel filter in the amplifier. Current-clamp recordings were performed under the gramicidin-perforated whole cell configuration of the patch-clamp technique using an EPC-9 amplifier and Pulse/Pulsefit software. All experiments were performed at room temperature (20~24°C).

Solution and drugs

For dialyzed whole cell recordings, the internal pipette solution contained (in mM) 10 NaCl, 102 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 EGTA, 10 HEPES, 0.1 ATP, and 1 GTP, adjusted to pH 7.2 with 38 mM KOH. The normal external solution contained (in mM) 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose, adjusted to pH 7.4 with Tris. The high-K^+ external solution contained 90 mM KCl and 50 mM NaCl. For gramicidin perforated patches, the composition of pipette solution (in mM) was 140 KCl, 5 NaCl, 0.5 $CaCl_2$, 5 EGTA and 10 HEPES, adjusted to pH 7.2 with Tris. A stock solution of gramicidin (Sigma) was prepared at 50 mg/ml in dimethylsulfoxide (Sigma) and diluted in the pipette solution to a final concentration of 50 μg/ml before use.

Data analysis

Data are presented as means±SEM. Statistical significance was determined using Student's t-test, and $P<0.05$ was considered significant. EC_{50} values were calculated by fitting the concentration-response curves to the logistic function of least-squares nonlinear regression using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Electrophysiological properties of MPG neurons

According to the previous reports on the characteristics of MPG neurons, sympathetic neurons can be differentiated

from parasympathetic neurons located within the same capsule, based on several differences in electrophysiological findings. The most prominent feature of sympathetic MPG neurons is the selective expression of the low voltage activated (LVA) T-type Ca^{2+} channels (Zhu et al, 1995; Park et al, 2001). The molecular identity of the T-type Ca^{2+} channels in sympathetic MPG neurons has already been clarified as α1H among the three cloned isoforms, α1G, α1H and α1I (Lee et al, 2002). As shown in Fig. 1B, we observed the presence of T-type Ca^{2+} currents in sympathetic neurons with membrane capacitance of 73.3±4.9 pF (n=6), which was significantly larger than parasympathetic neurons with 24.9±5.0 pF (n=11, $p<0.0001$). Resting membrane potential of sympathetic neurons (−44.7 ±1.7 mV, n=6) was slightly, but not significantly, more depolarized than that of parasympathetic neurons (−51.1 ±2.0 mV, n=12; Fig. 1C). At those membrane potentials, nearly 80% of T-type Ca^{2+} channels reside in the inactivated state. Therefore, injection of hyperpolarizing current into sympathetic neurons to recover T-type Ca^{2+} channel from inactivation produces rebound action potential firing after anodal break, according to our earlier findings (Lee et al, 2002). In consistent with the findings of absent T-type Ca^{2+} currents, parasympathetic MPG neurons were silent after anodal break (Fig. 1A).

Generally, autonomic neurons can be differentiated on the basis of action potential patterns to depolarizing current pulses, which is classified as being either tonic (slowly adapting) or phasic (rapidly adapting). The term 'adapting' refers to a decrease in frequency of action potentials observed during a maintained depolarization (Adams and Harper, 1995). In rat MPG, all of the tested sympathetic neurons showed tonic firing patterns that maintained a steady generation of action potential during long depolarizing pulses (n=6), whereas rapidly adapted phasic firing patterns were observed in parasympathetic neurons (n=13). These findings could be a useful hallmark to discriminate the sympathetic and parasympathetic neurons, which were isolated from rat MPG (Fig. 2).

Phenotype-specific expression of the K_{ATP} channels

Under gramicidin-perforated current-clamp conditions, we tested the existence of K_{ATP} conductances in MPG neurons, determined by the hyperpolarizing responses to diazoxide (200 μM), a K_{ATP} channel opener. Fig. 2 demonstrates that diazoxide hyperpolarized the resting membrane potential only in small-sized phasic-firing parasympathetic neurons (8 out of 13 neurons). Diazoxide-induced hyperpolarization was completely blocked by glibenclamide (10 μM), a K_{ATP} channel blocker. The mean shift of resting membrane potential by diazoxide was 8.5±

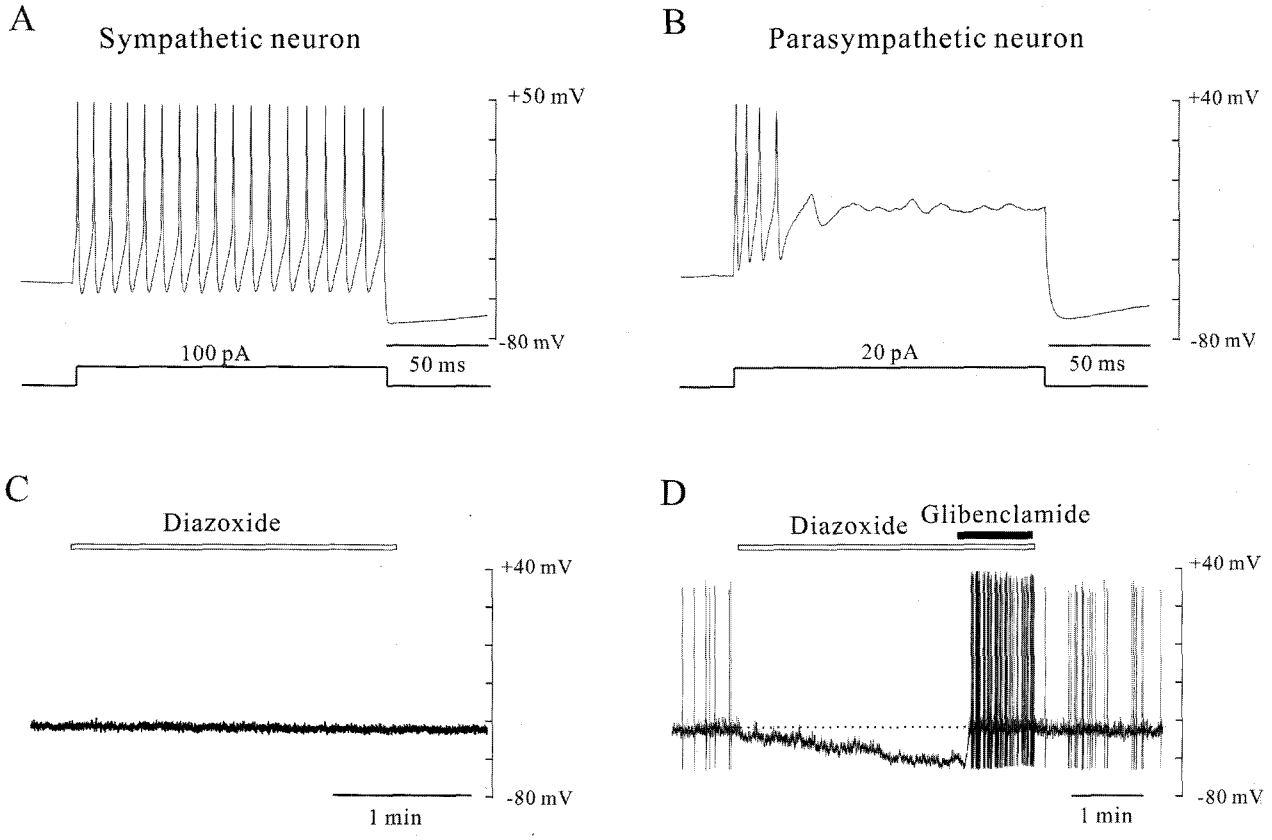

Fig. 2. The identification of the ATP-sensitive K^+ conductance in parasympathetic MPG neuron. Under current-clamp conditions with gramicidin perforated-patch technique, diazoxide (200 μM), an K_{ATP} channel opener, hyperpolarized the resting membrane potential of parasympathetic neuron (D), which showed phasic-firing in response to long depolarizing current injection (C). Diazoxide-induced hyperpolarization was completely blocked by the K_{ATP} channel blocker, glibenclamide (10 μM). In sympathetic neuron, tonic firing pattern were shown (A), however, diazoxide did not affect the resting membrane potential (B).

2.4 mV (n=8).

Under the voltage-clamp mode, we recorded K_{ATP} currents in parasympathetic MPG neurons at a holding potential of −46 mV, which was close to the theoretical equilibrium potential for Cl^-. At this holding potential, the possibility of contamination with Cl^- currents would be minimized. Changing the external bathing solution from 5 mM to 90 mM K^+ resulted in steady-state inward currents due to raising the driving force across the neuronal membrane for K^+ inward movement. Application of diazoxide (100 μM) activated additional inward K^+ currents in parasympathetic neurons. The average amplitude of diazoxide-induced inward currents was 210±82 pA, and the normalized value to membrane capacitance was 14.5± 8.7 pA/pF (n=6). Glibenclamide (10 μM) completely blocked the diazoxide-activated currents (Fig. 3).

As described earlier, decreased intracellular ATP/ADP ratio can open the K_{ATP} channels and induce hyperpolarization. Thus, we applied respiratory chain inhibitors to stop mitochondrial ATP synthesis and observed the changes in membrane potential caused by K_{ATP} conductances. Rotenone and antimycin used in this study are known to block the electron transport chain complex I and III, respectively. The metabolic inhibition by rotenone (10 μM) and antimycin (10 μM) hyperpolarized the resting membrane potential by 7.5±2.1 mV (n=6; out of 8 neurons) in parasympathetic MPG neurons, but not in sympathetic neurons (0.8±0.6 mV, n=3). This hyperpolarization was also blocked, but not completely, by glibenclamide (Fig. 4).

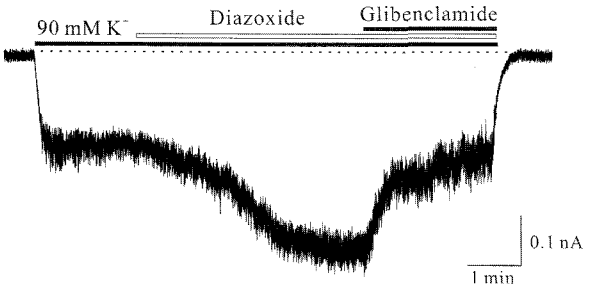

Fig. 3. Activation of the ATP-sensitive K^+ currents by diazoxide. Under voltage-clamp mode holding at −46 mV, changing the external bathing solution from 5 mM to 90 mM K^+ resulted in steady-state inward currents due to raising the driving force for K^+ movement. Application of diazoxide (100 μM) activated an additional inward current, which was blocked by glibenclamide (10 μM).

Molecular identification of KATP channel subunits expressed in MPG neurons

To identify the subunits of K_{ATP} channels expressed in MPG neurons, we performed RT-PCR analysis using primer pairs which were specific to Kir6.1, Kir6.2, SUR1, and SUR2 gene, respectively (Table 1). As shown in Fig. 5, the amplified products of Kir6.2 (estimated product size of 385 bp) and SUR1 (388 bp) from rat MPG neuron were detected abundantly, as were the results from rat insulinoma cells (pancreatic β-cell line). In MPG neurons, however, Kir6.1 and SUR2 were also expressed even though in small amounts, differing from the case of rat insulinoma cells.

DISCUSSION

The aim of the present study was to test whether K_{ATP} channels are located in MPG neurons, and, if so, to characterize the function and molecular identity of these channels using electrophysiological and molecular experimental techniques. Interestingly, we observed that the localization of K_{ATP} conductances in MPG was phenotype-specific, present only in parasympathetic neurons.

MPG neurons consist of heterogeneous subpopulations, which receive different preganglionic inputs from sympathetic (hypogastric) and parasympathetic (pelvic) nerves (Purinton et al, 1973; Dail, 1993). These two subpopulations act as antagonistic regulators to each other, such as in contracting or relaxing the same urogenital muscle (De Groat & Booth, 1993), which takes an important part in functional autonomic reflexes. Therefore, the expressed levels of ion channels and neurotransmitter receptors could be quite different between the two subpopulations within the same capsule, and some differences have already been

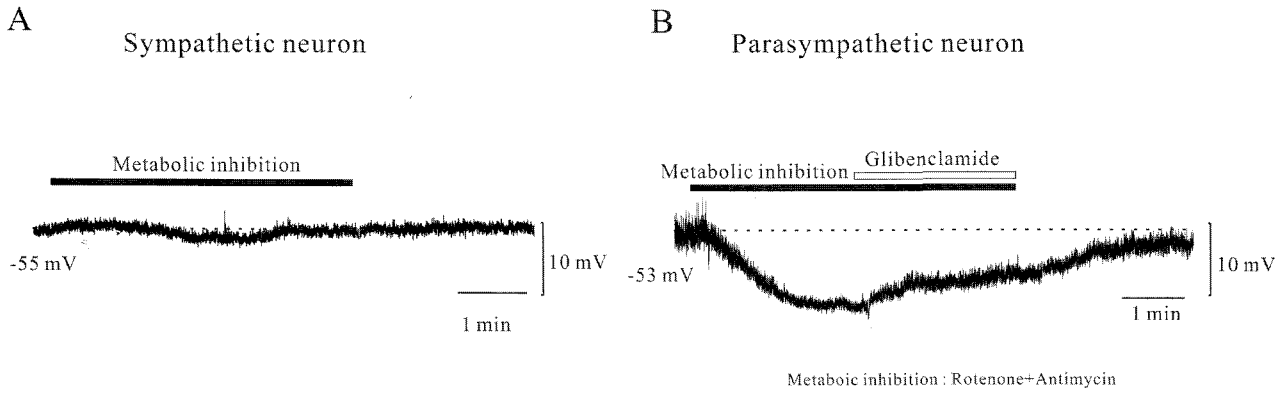

Fig. 4. The activation of the ATP-sensitive K^+ channels by metabolic inhibition. The metabolic inhibition was produced by applying inhibitors of the mitochondrial respiratory chain complex; complex I inhibitor rotenone (10 μM) and complex III inhibitor antimycin (10 μM). Under current-clamp conditions with gramicidin perforated-patch technique, metabolic inhibition caused membrane hyperpolarization only in parasympathetic neurons (B), which was blocked by 10 μM glibenclamide.

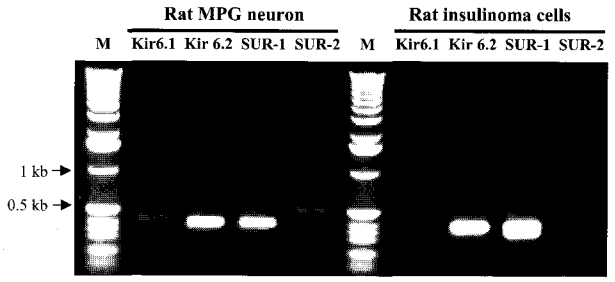

Fig. 5. RT-PCR analysis of mRNA encoding K_{ATP} channel subunits expressed in rat MPG neurons. Total RNA isolated from MPG neurons was reverse transcribed and amplified by PCR technique with primers specific to K_{ATP} channel subunits (Kir6.1, Kir6.2, SUR1 and SUR2) (Table 1). The resulting PCR products were separated and visualized on a 1.1% agarose gel containing ethidium bromide. As a positive control, the expressions of K_{ATP} channel subunits were observed in rat insulinoma cells under the same conditions.

reported. For example, the expression of T-type Ca^{2+} channel has been identified only in sympathetic neurons, which provides a useful electrophysiologic criterion for classifying the phenotype of MPG neurons. Ca^{2+} channel modulations by α_2-adrenergic receptor agonist and NPY were also evident in sympathetic neurons, but not in parasympathetics (Zhu et al, 1995; Cha et al, 2001). In addition, we previously observed the expression of GABA-A receptors exclusively in sympathetic neurons (Kong et al, 2001), whereas that of serotonergic receptors in parasympathetic neurons (Unpublished observation). Based on the results described in the present study, the existence of K_{ATP} conductances could serve as an another marker for identifying parasympathetic neurons of MPG. Further characterization of functional differences between subpopulations of MPG will be helpful for the fundamental understanding of neuronal synaptic transmission and modulations through interneurons.

The discharge pattern to depolarizing pulses is variable depending on the functional type and location of autonomic ganglion neurons. In MPG, we observed that all sympathetic neurons had tonic firing patterns while parasympathetics had phasic firing patterns. In contrast, the superior cervical ganglion neurons showed phasic firing patterns even though they were entirely sympathetic. Mechanisms of different discharge patterns, which depend on the rate of adaptation, may be due to electrophysiological properties of the somatic membrane (Adams & Harper, 1995). Several ionic currents that are involved in the regulation of repetitive activity and adaptation have been characterized in autonomic ganglion neurons. The major determinant of adapting rate appears to be potassium channels, such as transient outward current (I_A), Ca^{2+} activated current ($I_{K,Ca}$), and muscarine-sensitive K^+ current (I_M). As shown in figure 2, glibenclamide not only blocked the hyperpolarizing effects of diazoxide but also increased the frequency of spontaneous action potential firings, which suggest that the resting conductance of K_{ATP} may influence the basal discharge pattern of this neuron. Likewise, we propose that the phenotype-specific expression of K_{ATP} channels might be partially involved in the rapidly adapting properties of parasympathetic MPG neurons.

Using RT-PCR analysis, we found that the dominant subunits of K_{ATP} channel in MPG neurons were Kir6.2 and SUR1. This combination of subunits is the most sensitive type to the intracellular ATP concentrations, and distributed mainly in glucose-responsive cells, not only pancreatic β-cells but also gastrointestinal enteric neurons (Liu et al, 1999). Kir6.1 and SUR2, however, were also expressed in MPG even though in small amount, but were absent in rat insulinoma cells. It is unclear from where these subunits originated. One of the possibilities could be the contamination of vascular tissue within the MPG capsule. SUR2B is known to be primarily located on vascular smooth muscle cells (Yokoshiki et al, 1998; Fujita & Kurachi, 2000). The other possibility is the heterogenous localization of these subunits with Kir6.2 and SUR1 in parasympathetic MPG neurons.

The decline of intracellular ATP concentration following ischemia or metabolic inhibition would activate the K_{ATP} channels in the nervous system, and this process induces hyperpolarization of neuronal membrane potential. Hyperpolarization provides an effective damping action on excessive action potential firing and a better preservation of cellular ATP levels, which act as a neuroprotective role against neuronal damage (Heurteaux et al, 1993). Liss et al (1999) reported that K_{ATP} channels in dopaminergic midbrain neurons expressed both SUR1 and SUR2 subunits with Kir6.2 subunits and importantly, the Kir6.2/SUR1 combination conferred the greatest protection against metabolic inhibition. In consistence with this finding, Kir6.2/SUR1 complexes in MPG neurons reacted to the metabolic inhibition and induced hyperpolarization in our present study. This hyperpolarization was not fully explained by the activation of K_{ATP} channel, which was proven by the fact that glibenclamide had only partial blocking action to the hyperpolarizing effect of metabolic inhibition. The remaining portion would be originated from another K^+ conductance, such as Ca^{2+} activated K^+ currents.

In conclusion, our data suggest that K_{ATP} channels in parasympathetic MPG neurons may contribute to the changes in excitability during hypoxia and metabolic inhibition. Further studies on the functional changes during pathological condition will be needed to understand the meaning of phenotype-specific distribution of K_{ATP} channels.

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