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Neuroscience Research 34 (1999) 13–19

NEUROSCIENCE RESEARCH

Expression of a non-inactivating K^+ channel driven by a rat heat shock promoter increased the resting potential in *Aplysia* silent neurons

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Received 13 October 1998; accepted 8 February 1999

Abstract

We assessed the role of a non-inactivating K^+ channel (aKv5.1) in the resting potential by overexpressing this channel by heat shock in the neurons. A reporter gene *lac*Z linked to a promoter region spanning from the -285 to the $+88$ base of the rat *HSP*70ib gene was induced 62.5-fold when this DNA construct was microinjected into the neurons of the marine mollusk *Aplysia* and treated with heat shock at 30°C for 3 h. Using this efficient induction system, we induced the expression of aKv5.1 by heat shock in cultured, electrically silent neurons of *Aplysia* and examined its effect on the resting potential. The channel expression increased the resting potential by approximately 10 mV. This increase was specific to heat shock induction and abolished by treatment with TEA, a specific K^+ channel blocker. These results provide the direct evidence that a low voltage-activated, non-inactivating K^+ channel can contribute to the resting potential.

Keywords: Non-inactivating K^+ channel; *Aplysia* neuron; Heat shock promoter; Transgenic expression; Resting potential; DNA microinjection

1. Introduction

K^+ channels play a critical role in the control of the resting membrane potential and membrane excitability (Hille, 1992). The electrical activity of neurons is determined by the combination of ion channel proteins residing in the plasma membrane. K^+ channels are thought to be important in determining the resting potential of neurons and in modulating the time course of repolarization of action potential.

Although there have been many reports about the molecular cloning of diverse K^+ channels, their physiological roles in the resting potential and membrane excitability have not been fully challenged so far. Therefore their cDNAs or genes need to be transferred into the excitable cells, positively for overexpression, or negatively for knockout of the specific channels. This genetic approach, in addition to the pharmacological approach, seems to be very powerful in understanding the functions of the specific channels in the nervous system in vivo.

The nervous system of marine mollusk *Aplysia* has been useful for the gene transfer study (Kaang et al., 1992, 1993; Kaang, 1996a). Using direct microinjection as a tool of gene transfer, we have been able to examine the function of gene products such as K^+ ion channels by overexpressing them under a strong constitutive promoter (Kaang et al., 1992; Zhao et al., 1994). By a

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0168-0102/99/$ - see front matter
PII: S0168-0102(99)00027-9

similar way, we could study the function of transcription factors (Kaang et al., 1993), synaptotagmin (Martin et al., 1995), and cell adhesion molecules (Bailey et al., 1997) in identified *Aplysia* neurons.

However, induced expression of foreign genes under a specific condition, rather than constitutive expression, may provide a more controlled way in assessing the function of an expressed gene product such as ion channels. The effect of overexpressed gene product on the neuronal functions can be assessed by finding any significant physiological difference between gene-induced and -uninduced conditions applied to the neurons that were microinjected with the same cDNA.

In this study, we developed a way to control by heat shock promoters the expression of introduced genes in *Aplysia* neurons. We tested the various rat heat shock protein genes (Sorger and Pelham, 1987; Lisowska et al., 1994; Mestril et al., 1994) and also compared their inducible expression under heat shock with the constitutive expression driven by *Rous sarcoma* viral promoter (Kaang et al., 1992) in *Aplysia* neurons. We next examined the expression of a specific cloned *Aplysia* K^+ channel (aKv5.1) using the heat shock promoter and examined whether this channel can directly contribute to the resting potential in electrically silent neurons. A previous study suggested that this channel may contribute to the resting potential and firing patterns of neurons (Zhao et al., 1994). They carried out the constitutive expression of the channel genes, but could not examine whether overexpression of aKv5.1 influences the resting potential in electrically silent neurons. Now we provide a direct evidence of this channel's contribution to the resting potential by using a heat inducible expression system.

2. Materials and methods

2.1. Animal and cell culture

Aplysia kurodai was purchased from a local supplier in Pusan, South Korea and maintained in recirculating sea water tanks at 14°C before use. From *A. kurodai* weighing 100–130 g the ganglia were dissected and incubated at 18°C for 15–16 h in 1% protease (type IX, Sigma) dissolved in equal volume of isotonic L15 and artificial sea water (ASW: 460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, 10 mM HEPES, pH 7.6) and washed several times with ASW. Dissociated cell culture from the pedal ganglia was done basically following the protocol modified from Schacher and Proshansky (1983) and Lim et al. (1997). Cultures were maintained at 18°C by incubator. The cultured cells with extensive neurites were used for experiments 3 days after plating.

2.2. Construction of plasmids

We isolated by polymerase chain reaction (PCR) the genomic sequences of promoters from rat heat shock cognate 73 (r*HSC*73) (Sorger and Pelham, 1987), heat shock protein (r*HSP*70) (Lisowska et al., 1994), and inducible heat shock protein (r*HSP*70ib) gene (Mestril et al., 1994). The template DNA for PCR was prepared from the tails of Sprague–Dawley rats following the protocol described in Sambrook et al. (1989).

The primers used in PCR were as follows: 5′aagaagcttgccattggtccgttgcgggtgtaccc3′ and 5′aag-aagcttggcccagctgtgctgatgacgagaccac3′ for r*HSC*73; 5′aa-caagcttttgagaaaatttctgcgtccgccatcctg3′ and 5′aagaagcttc-cgcttctctgcttctctgcgagtggaacc3′ for *HSP*70; 5′aa-caagcttc-catggcaacactgtcacaaccggaacaag3′ and 5′aag-aagctttgccc-gaggttcggaacgcgccgcttgc3′ for r*HSP*70ib (*Hin*dIII site is underlined).

The PCR reaction amplified the promoter sequences spanning from −422 to +65 of r*HSC*73 (487 bp), from −432 to +62 of *HSP*70 (494 bp), and from −285 to +88 of *HSP*70ib (373 bp), respectively. Each PCR fragment was digested with *Hin*dIII and substituted for *Hin*dIII fragment of constitutive promoter in p*NEX*–*lac*Z (Kaang et al., 1992) to generate r*HSC*73–*lac*Z, r*HSP*70–*lac*Z, and r*HSP*70ib–*lac*Z, respectively. Right orientation of promoters in relation to the reporter *lac*Z was determined by DNA sequencing.

p*NEX*δ–aKv5.1 (Zhao et al., 1994) and r*HSP*70ib–*lac*Z were digested with *Bam*HI and *Kpn*I. The *Bam*HI/*Kpn*I fragment of p*NEX*δ–aKv5.1 containing aKv5.1 was substituted by ligation reaction for the *Bam*HI/*Kpn*I fragment of r*HSP*70ib–*lac*Z containing *lac*Z, thus generating r*HSP*70ib–aKv5.1.

2.3. Microinjection of plasmids into Aplysia neurons

All plasmid DNAs used in microinjection were prepared by a standard midi-prep procedure using Qiagen-tip 100 (Qiagen). Microinjection of DNA plasmids was done as previously described (Kaang et al., 1992, 1993). Microinjection solution composed of: 0.7 mg/ml each DNA construct (r*HSC*73–*lac*Z, r*HSP*70–*lac*Z, r*HSP*70ib–*lac*Z, or p*NEX*δ–*lac*Z), 0.3 mg/ml p*NEX*2-luciferase, 10 mM Tris–Cl (pH 7.3), 100 mM KCl, and 0.1% fast green. As an internal control for expression, firefly luciferase gene was allowed to express from a weak constitutive expression vector p*NEX*2 (Kaang, 1996a). A total of 10–20 neurons per ganglia were microinjected with DNA injection solution at room temperature (20°C). Injected ganglia were incubated at either 18°C (no heat shock) or 30°C (heat shock) for 3 h after 1 h of recovery at 18°C following microinjection. All ganglia were further incubated at 18°C for 12 h until the extracts were prepared for reporter gene analyses.

r*HSP*70ib–aKv5.1 was mixed with p*NEX*δ-GFP (Kaang, 1996b) at 7:3 molar ratio in microinjection solution (total DNA concentration, 1 mg/ml) and microinjected into the acutely cultured, pedal neurons at room temperature. Each culture dish contained within a circle area (diameter, 14 mm) only 10–20 neurons dissociated from the pedal ganglia. Neurons were cultured at 18°C for 3 days before DNA microinjection. For channel expression, p*NEX*δ–GFP instead of p*NEX*2-luc was included in the microinjection solution as a marker of gene expression. Successful microinjection of DNA constructs into neurons was determined before electrophysiological recordings by detecting through fluorescence microscope the expression of GFP under a constitutive promoter of p*NEX*δ. In control experiment, p*NEX*δ–GFP alone was microinjected into cultured cells.

2.4. Luciferase and β-galactosidase assay

Ganglion extracts were made as previously described (Kaang et al., 1993) using 30 μl of 1 × Reporter Lysis buffer (Promega). Reporter gene assays and normalization of β-gal expression were done as previously described (Kaang, 1996a,b). Briefly, β-gal activity of the extract was determined by subtracting the total β-galactosidase activity of the extract with that of negative control extract (no DNA injection). Normalized β-galactosidase was represented by dividing the subtracted β-galactosidase activity by luciferase activity. Results are expressed as mean ± S.E.M.

2.5. Electrophysiology

Membrane potential was recorded at room temperature (20°C) 1 h after microinjection into cultured neurons using GeneClamp 500 (Axon Instruments, Inc.) with microelectrodes filled with 2 M K-acetate, 0.5 M KCl, 10 mM K-HEPES. The impedance of microelectrodes ranged from 10 to 15 MΩ. The neuron was impaled with a microelectrode and the resting potential (RP_1 in Fig. 2) was taken 5 min after impalement. Most of cultured, pedal neurons (~ 95%) were electrically silent. The neurons showing beating or bursting patterns were discarded. Only the neurons were subjected to further electrophysiological experiments if their resting potentials are more negative than − 35 mV. Data was stored on VCR recorder tapes through a digital data recorder (Model VR-10B, Instrutech, Corp.). Some of the cultured cells were subjected to heat shock at 30°C for 3 h immediately after membrane potential recording. After further incubating at 18°C for 12–16 h, the resting potential (RP_2 in Fig. 2) was recorded again in a similar way for the neurons expressing GFP which was detected simultaneously through an inverted fluorescence microscope (model Optiphot-2, Nikon). Some of the GFP positive neurons were voltage-clamped using two electrodes with GeneClamp 500 and pCLAMP program (Axon Instruments, Inc.). The medium was exchanged to Na^+–Ca^{2+} free ASW (460 mM Tris–Cl, 10 mM KCl, 66 mM $MgCl_2$, 10 mM HEPES, pH 7.6). Voltage-clamp experiments for analyzing activation and inactivation kinetics and tail currents and the subsequent data analyses were carried out as described in Zhao et al. (1994). Results are expressed as mean ± S.E.M.

3. Results

3.1. Induction of β-galactosidase activity by heat shock in Aplysia neurons

Heat shock at 30°C for 3 h produced statistically significant 8.6, 13.6, and 62.5-fold induction of *lacZ* expression from the neurons that were microinjected with DNA constructs, r*HSP*70–*lacZ*, r*HSC*73–*lacZ*, and r*HSP*70ib–*lacZ*, respectively (Table 1). By contrast, heat shock did not increase significantly expression from constitutive vector p*NEX*δ–*lacZ*. The results show that upstream region of rat *HSP* genes are readily recognized by yet unidentified *Aplysia* heat shock fac-

Table 1
Normalized β-galactosidase expression driven by the various promoters in the presence and in the absence of heat shock treatment[a]

DNA[b]	r*HSP*70ib–*lacZ*	r*HSP*70–*lacZ*	r*HSC*73–*lacZ*	p*NEX*δ–*lacZ*
Heat shock (+)[c]	4.123 ± 0.950 ($n = 7$)	1.232 ± 0.278 ($n = 6$)	5.304 ± 2.060 ($n = 6$)	12.66 ± 4.12 ($n = 6$)
Heat shock (−)[c]	0.066 ± 0.026 ($n = 8$)	0.144 ± 0.098 ($n = 6$)	0.391 ± 0.147 ($n = 6$)	8.06 ± 2.16 ($n = 6$)
Fold induction[d]	62.5	8.6	13.6	1.6

[a] Normalized β-gal expression is represented as β-galactosidase/luciferase activity. Results are expressed as mean ± S.E.M., n = number of trials.
[b] DNA solutions microinjected also contained p*NEX*2-luciferase as an internal control.
[c] One hour after DNA microinjection, injected cells were incubated at 30°C (heat shock +) or at 18°C (heat shock −) for 3 h. Cells were then further incubated at 18°C for 12–16 h.
[d] Normalized β-gal expression in the presence of heat shock divided by that in the absence of heat shock. Statistical significance between no heat shock and heat shock was tested by two-tailed unpaired t-test: r*HSP*70ib–*lacZ*, $P < 0.002$; r*HSP*70–*lacZ*, $P < 0.005$; r*HSC*73–*lacZ*, $P = 0.057$; p*NEX*δ–*lacZ*, $P > 0.3$.

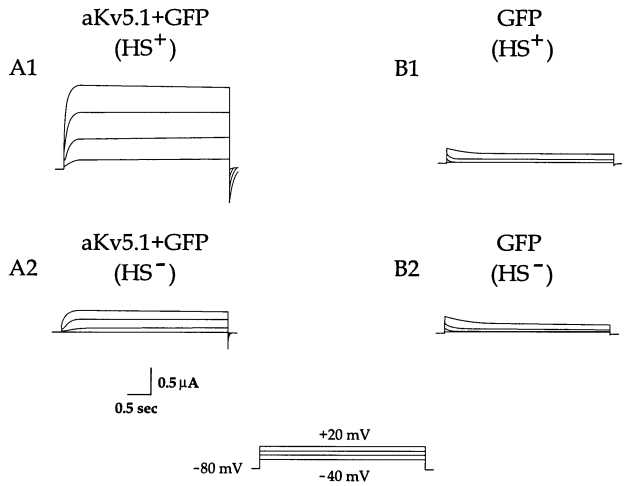

Fig. 1. Expression of transgenic aKv5.1 in cultured *Aplysia* neurons. The cultured neurons were microinjected with a mixture of r*HSP*70ib–aKv5.1 and p*NEX*δ–GFP (A1 and A2) or with p*NEX*δ–GFP alone as a control (B1 and B2). One hour after microinjection, some of the injected neurons were treated with heat shock at 30°C for 3 h (HS^+) (A1 and B1) and the others were not (HS^-) (A2 and B2). The neurons were further incubated at 18°C for 12–16 h before electrophysiological recording. GFP positive neurons were subjected to voltage-clamping experiments. Currents were evoked by 4-s depolarizing pulses from -40 to $+20$ mV in 20 mV clamping steps from a holding potential of −80 mV (bottom trace).

tors (*HSF*). Among these promoters regulated by heat shock, r*HSP*70ib seemed to be ideal for the study because it gave rise to the highest induction ratio with the lowest basal expression level in *Aplysia* neurons.

3.2. Expression of a non-inactivating K^+ channel aKv5.1 mediated by rHSP70ib

We next examined the expression of a specific cloned *Aplysia* K^+ channel (aKv5.1) driven by a heat shock promoter r*HSP*70ib in cultured neurons dissociated from the pedal ganglia. Fig. 1 shows K^+ ionic currents activated by depolarizing voltage steps for 4 s from a holding potential (-80mV) in electrically silent, cultured *Aplysia* neurons. The neurons successfully injected with r*HSP*70ib–aKv5.1 yielded high levels of the steady-state current (Fig. 1A1). In these neurons the size of steady-state current at $+20$ mV was greater with heat shock than that without heat shock (0.832 ± 0.098 (S.E.M.) μA in 10 neurons vs. 0.343 ± 0.025 μA in 6 neurons; two-tailed unpaired *t*-test, $P < 0.005$) (Table 2, Fig. 1A). In our voltage-clamp condition, an endogenous steady-state current was also detected in the control neurons which was not injected or expressing only GFP. This endogenous current, however, was not affected by heat shock treatment (Table 2, Fig. 1B). Average amplitude of this background current was 0.162 ± 0.014 μA ($n = 18$ neurons) at $+20$ mV in the control neurons irrespective of heat shock treatment and much smaller than the steady-state current detected in the neurons expressing aKv5.1 channels. Therefore the steady-state current attributable to the expression of aKv5.1 may be calculated by subtracting total steady-state current (0.832 μA) by the mean value of endogenous background currents (0.162 μA) in control neurons. This approximate calculation yields the average value of 0.670 μA of aKv5.1 current with heat shock and average value of 0.181 μA without heat shock. Therefore induction of channel expression by heat shock is estimated to be 3.7-fold, based on the measurement of steady-state current at $+20$ mV.

Taken together, we conclude that the expression of aKv5.1 was induced by heat shock in *Aplysia* neurons microinjected with r*HSP*70ib–aKv5.1.

3.3. Properties of the expressed aKv5.1 channel in silent, cultured Aplysia neurons

The activation and inactivation properties of aKv5.1 heterologously expressed in silent *Aplysia* neurons are very similar to those reported in Zhao et al. (1994). aKv5.1 can be activated at least at -40 mV and does not inactivate. Activation curves of aKv5.1 expressed by heat shock were fit to Boltzmann distributions. The mean $V_{1/2}$, the midpoint of activation, was -28.0 ± 3.9 mV ($n = 5$) and the mean k, the slope factor, was 10.4 ± 1.3 ($n = 5$). The inactivation curve of aKv5.1 was also very similar to that described by Zhao et al. (1994) (data not shown).

We also determined by analyzing tail currents the reversal potential of the current through aKv5.1 channel expressed in neurons. In the bath solutions containing 2, 10, and 30 mM potassium ion, the reversal potential was -73.6 ± 2.1 mV ($n = 6$), -59.3 ± 2.6

Table 2
Steady-state current measured from cultured *Aplysia* neurons[a]

DNA microinjected[b]	aKv5.1 +GFP	GFP	None
Heat shock (+)[c]	0.832 ± 0.098 (10)[d]	0.206 ± 0.034 (5)	0.150 ± 0.028 (4)
Heat shock (−)[c]	0.343 ± 0.025 (6) $P < 0.005$[e]	0.140 ± 0.020 (5) $P > 0.1$	0.148 ± 0.021 (4) $P > 0.5$

[a] Steady-state current is the current amplitude at the end of a 4-s step pulse of +20 mV from holding potential of −80 mV. Mean ± S.E.M.; unit is μA.

[b] aKv5.1+GFP: r*HSP*70ib–aKv5.1 was mixed with p*NEX*δ–GFP at 7:3 molar ratio in microinjection solution (total DNA concentration, 1 mg/ml) and microinjected into the cultured pedal cells; GFP: p*NEX*δ–GFP alone was microinjected as a control. None: Nothing was injected into the neurons. When p*NEX*δ–GFP was injected, only GFP positive neurons were selected for voltage-clamp experiments.

[c] See footnote in Table 1.

[d] The figures in parentheses indicate the number of neurons tested.

[e] Two-tailed unpaired *t*-test.

mV ($n = 7$), and -44.3 ± 2.1 mV ($n = 6$), respectively. This indicates that the expressed aKv5.1 channels are highly selective to K^+ ions, as reported in Zhao et al. (1994).

The expressed channels were inhibited by a K^+ channel blocker tetraethylammonium (TEA, 10 mM) (Eastman Kodak Co., Rochester, NY, USA). In an experiment, steady-state K^+ current at $+20$ mV was reduced by $46.7 \pm 2.9\%$ ($n = 6$) from 0.780 ± 0.114 to 0.420 ± 0.067 μA ($n = 6$). This inhibition rate for the expressed aKv5.1 in *Aplysia* neurons is very similar to that reported in Zhao et al. (1994).

These data illustrate that aKv5.1 expressed heterologously in cultured, pedal neurons share the similar properties of K^+ channel with that overexpressed in R15 (Zhao et al., 1994) such as TEA sensitivity, non-inactivation kinetics, and low threshold for channel activation.

3.4. Effect of overexpressed non-inactivating K^+ channel on the resting potential

Next we examined whether the induced expression of the low voltage-activated non-inactivating channel aKv5.1 can influence on the resting potential in electrically silent neurons. The induced expression of aKv5.1 by heat shock increased the resting potential (Fig. 2A). In electrically silent neurons that expressed aKv5.1 by heat induction, the resting membrane potential became 9.6 ± 1.7 mV more negative, coming from -45.6 ± 2.0 to -55.2 ± 1.1 mV ($n = 17$). This change in the resting potential is statistically significant ($P < 0.00005$, two-tailed paired t-test). After overexpression of aKv5.1, the mean of the resting potential (-55.2 mV) became close to the reversal potential (-59.3 mV) of this channel.

The change in the resting potential due to overexpression of aKv5.1 was blocked by treatment with 10 mM TEA. In an experiment, the resting potential was changed from -47.1 ± 1.8 to -53.4 ± 2.1 mV ($n = 9$) after induction of aKv5.1 by heat shock. This change in the resting potential is statistically significant ($P < 0.005$, two-tailed paired t-test). Treatment with 10 mM TEA to these neurons depolarized membrane potential to -46.7 ± 2.6 mV. This change in the resting potential with 10 mM TEA is statistically significant ($P < 0.001$, two-tailed paired t-test). After washout, the resting potential returned to -52.8 ± 2.3 mV. The change in the resting potential due to washout is statistically significant ($P < 0.001$, two-tailed paired t-test). In contrast, TEA treatment had only a little effect on the resting potential in the uninjected control neurons. Treatment of 10 mM TEA changed the resting potential from -45.2 ± 2.3 to -42.6 ± 2.2 mV ($n = 5$). The resting potential of these control neurons was -43.6 ± 2.2 mV after washout of TEA. This data suggest that the increase in the resting potential in the neurons microinjected with r*HSP*70ib–aKv5.1 and treated with heat shock is due to the expression of aKv5.1.

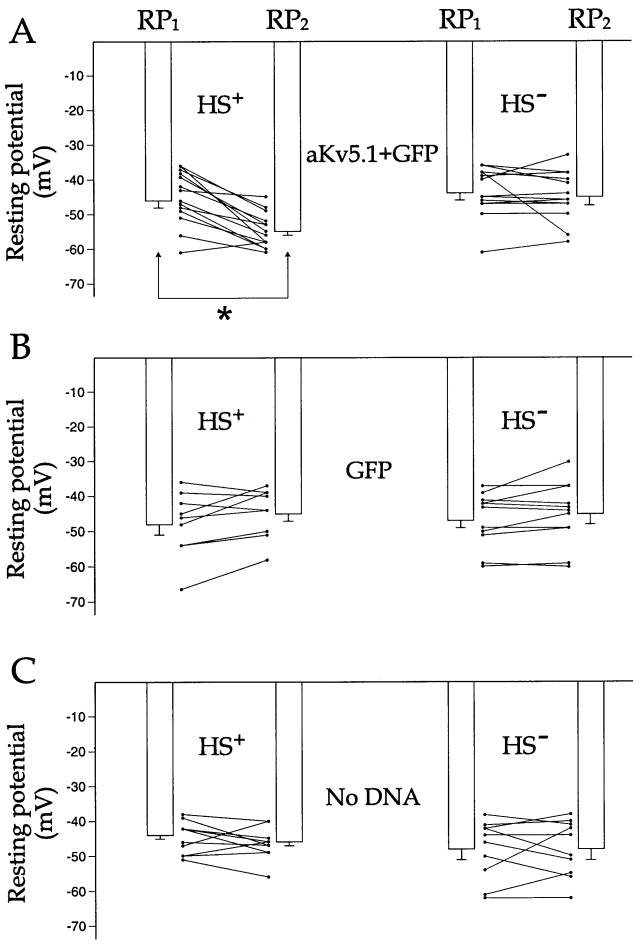

Fig. 2. The effect of overexpressed aKv5.1 on the resting potential. The neurons were microinjected with a mixture of r*HSP*70ib–aKv5.1 and p*NEX*δ–GFP (A) or with p*NEX*δ–GFP (B). (C) The control neurons were not injected with DNA solution. RP_1 is the resting potential recorded 1–2 h after microinjection of the DNA construct(s). Heat shock at 30°C (HS^+) or no heat shock at 18°C (HS^-) for 3 h were applied to the neurons immediately after recording of RP_1. RP_2 is the resting potential recorded 12–16 h after the heat shock period. Each pair of RP_1 and RP_2 shown between the bars represents a shift of the resting potential in a neuron. $^*P < 0.00005$, two-tailed paired t-test. Bars indicate mean + S.E.M.

However, when the microinjected neurons with r*HSP*70ib–aKv5.1 were not treated with heat shock, the resting potential became only 1.2 ± 1.6 mV ($n = 13$) more negative, which does not seem statistically significant ($P > 0.1$, two-tailed paired t-test) (Fig. 2A). In control experiments p*NEX*δ–GFP alone or no DNA was microinjected into *Aplysia* cultured neurons. In these cells there were no significant changes in the resting potential irrespective of heat shock treatment (Fig. 2B and C).

Taken together, these data suggest that the resting potential was increased specifically by overexpressed non-inactivating K^+ channel, aKv5.1.

4. Discussion

In this report, our first goal was to establish the transgenic gene induction system for *Aplysia* giant neurons based on the microinjection of plasmid DNA constructs and heat shock treatment thereafter. Secondly, we studied the role of a non-inactivating K^+ channel in the resting potential by inducing the transgenic channel gene using this gene induction system.

Heat shock factors that recognize the heat shock promoters such as *HSC*, r*HSP*70, and r*HSP*70ib appear to be highly conserved during evolution and therefore induce the transcription of downstream reporter gene *lac*Z in the nervous system of the marine mollusk *Aplysia*. Among the promoters tested, r*HSP*70ib gave the highest induction ratio by heat shock. A promoter region spanning from -285 to $+88$ base from r*HSP*70ib gene gave rise to 62.5-fold induction with heat shock of reporter gene driven by this promoter region. This promoter region also showed very little basal level of reporter gene expression without heat treatment. It contains two heat shock elements, two Sp1, and two CAAT box elements including a TATA box (Mestril et al., 1994). DNA sequences of r*HSP*70 and r*HSP*70ib genes are very similar to each other with 98% homology including the promoter and the coding region. The fragment of r*HSP*70 promoter used in our study contains an additional sequence spanning from -432 to -286 which does not exist in the fragment of r*HSP*70ib. It, however, is not certain whether the removal of this additional sequence or a few point mutations within the fragment of r*HSP*70ib promoter are responsible for difference in induction ratio between two promoters. On the other hand, r*HSC*73 promoter drove the highest basal level among heat shock promoters of *lac*Z expression as was known in mammalian systems (Sorger and Pelham, 1987).

r*HSP*70ib drove under heat shock the ectopic expression of a non-inactivating channel aKv5.1. The voltage-clamp data showed that this current was approximately ten times larger than the endogenous background currents in the neurons, but also readily detected even at -40 mV step. This implies that a significant fraction of overexpressed aKv5.1 channels are open near at the resting potential.

The current size in our experiments was 5–6 times smaller than aKv5.1 current overexpressed using p*NEX*δ vector in *Aplysia* neuron R15, described previously by Zhao et al. (1994). This result was well explained by the fact that the heat shock promoter r*HSP*70ib drives *lac*Z expression approximately two times less than RSV promoter of p*NEX*δ does (Table 1). Furthermore, the surface area of the pedal neuron is 2.5–3 times smaller than that of R15, which is calculated based on the size of cell body. As the result, the current size of aKv5.1 with activated heat shock promoter in our experiments are comparable with those reported in Zhao et al. (1994), in the case of the low-level expression with constitutive p*NEX*δ promoter.

Interestingly enough, the induction ratio of channel proteins in the presence of heat shock to in the absence of heat shock was estimated to be only 3.7-fold, which is far less than 62.5-fold induction calculated by using *lac*Z reporter. This implies that the neurons are less permissive to the heterologous expression of a membrane ion channel aKv5.1 than to that of a cytoplasmic, soluble protein β–galactosidase. The constraint of channel expression could be due to the limited sites or clustering factors (Kim et al., 1995; Zito et al., 1997) on the plasma membrane for the newly synthesized channel proteins. Alternatively, the neuron may have a protective mechanism at the genetic level in order not to allow any excessive expression of a channel, which can otherwise disturb the normal electrical physiology of the cells.

A role for aKv5.1 in determining the resting potential was previously suggested by Zhao et al. (1994). In their report, overexpression of aKv5.1 driven by strong promoter of p*NEX*δ eliminated completely the spontaneous bursting rhythm in an identified *Aplysia* neuron R15. They also reported that when R15 cells became silent and acquired new membrane potentials due to overexpression of aK5.1, the acquired membrane potential values of R15 correlated with the expression level of aKv5.1 current. This implied that aKv5.1 might play an important role in regulating the resting membrane potential.

In our report, however, we provide more direct evidence showing that aKv5.1 contributes to the resting potential. For transgenic expression of aKv5.1 we used cultured neurons dissociated from the pedal ganglia instead of R15 which shows a bursting electrical rhythm. Most of the pedal neurons are largely unidentified to our knowledge, but quite uniform in diameter of cell body (150–200 μm). By making dissociated neuronal culture, each neuron can be separated to one another and some of the neurons are more or less free of synaptic inputs. We chose in the cultures only the neurons that are electrically silent and that have stable resting potential. Therefore these culture conditions enabled us to record a stable resting potential from cultured neurons which were similar in cell size and input resistance. We then compared the changes in the resting potential of transgenically induced neurons by heat shock with those of neurons which were microinjected with the same DNA constructs but thereafter not induced with heat shock. All these careful considerations to minimize the experimental errors or factors seemed very critical in analyzing the subtle changes in the resting potential within the range of about 10 mV, which could be easily overwhelmed by experimental fluctuation factors.

In the present study, using a heat-inducible gene transfer system we directly demonstrated that a non-inactivating K^+ channel could contribute to the resting membrane potential, suggesting that aKv5.1 may play an important role in maintaining the resting potential. Now it will become interesting to see the real contribution of endogenous aKv5.1 to the resting potential in the nervous system. This would require identifying specific *Aplysia* neurons expressing aKv5.1 and quantifying its expression level in the identified cells as well.

The transgenic heat induction system may also provide a tool for accurately assessing in a more controlled way the effect of overexpressing transgenic proteins on the neuronal functions such as learning and memory (Kandel and Schwartz, 1982).

Acknowledgements

I thank Dr. Eric R. Kandel for critical reading of the manuscript. This research was supported by Korean Ministry of Science and Technology under the Brain Science Research program and KIST-2000 program.

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