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Modulation of transient type K channel cloned from rat heart

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Summary: We have cloned a transient type K channel from rat heart (RH10) and coexpressed a metabotropic glutamate receptor (mGluR5) to study the functional modulation of RH10 coupled to the phosphatidylinositol (PI) hydrolysis. Stimulation of mGluR5 suppressed peak amplitude of RH10 current and affected voltage dependence of activation and inactivation of the channel.

K channels play important physiological roles in excitable and non-excitable cells (1-3). For example, in excitable cells, delayed rectifier K channel regulates action potential reporalization and transient K channel affects action potential threshold and frequency.

Electrophysiological, pharmacological and molecular biological studies have extended our knowledge on structures and functions of K channels. Ion channels can be regulated through a variety of receptor-mediated mechanisms, in which most commonly observed mechanism is phosphorylation by protein kinase A (PKA) or protein kinase C (PKC). Physiologically, modulation of ion channels by phosphorylation is well studied (for review 4, 5). There are also some recent reports on receptor-mediated modulation of cloned K channels (6-9). The rat mGluR5 is coupled to G protein that activates phospholipase C (10, 11). In oocytes, as in most cells, activated phospholipase C produces inositol trisphosphate and diacylglycerol, resulting in the subsequent release of Ca^{2+} from intracellular stores and activation of PKC (12).

In this paper, we report modulation of cloned rat heart K channel by stimulation of coexpressed mGluR5 using *Xenopus* oocyte expression system.

Abbreviations: CHO,chinese hamster ovary; mGluR, metabotropic glutamate receptor; EGTA, ethylene glycol-bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid; PMA, 4-beta-phorbol 12-myristate 13-acetate; α-PMA, 4-alpha-phorbol 12-myristate 13-acetate; 8-Br-cAMP, 8-bromoadenosine 3', 5'-cyclic monophosphate; 8-Br-cGMP, 8-bromoguanosine 3', 5'-cyclic monophosphate.

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MATERIALS AND METHODS

cDNA cloning and sequencing

Cloning method has been described in detail elsewhere (8). In brief, degenerate oligonucleotides were synthesized according to conserved amino acid sequences of *Shaker* K channel family and primed a PCR reaction. PCR clones homologous to *Shaker* K channel family were used as hybridization probes to screen a rat heart cDNA library. One positive clone containing 3.2 kb insert, which we designated RH10, was selected for further characterization. Sequencing of RH10 was performed on both strands by the dideoxy chain termination method.

Expression of rat cardiac K channel and mGluR5

RH10 was subcloned into EcoRI site of the pBluescript vector to synthesize mRNA specific for RH10. The plasmid was cut with Hind III and Kpn I, and then 5' noncoding sequence of RH10 was deleted by exonuclease III up to -40bp from initiation codon. After religation, the resulting plasmid was digested with EcoRI and capped run-off cRNA was synthesized in vitro with T7 RNA polymerase. Transcribed RNA was dissolved in water at a final concentration of 0.2 μg/μl for oocyte injection. Messenger RNA specific for mGluR5 was synthesized from the cDNA clone pmGR5 (generous gift from Dr. S. Nakanishi) by using T7 RNA polymerase after linearization with Not I. Transcribed RNA was mixed with RH10 transcript in water at a final concentration of 0.2 μg/μl each.

Xenopus leavis oocytes were treated for two hours with collagenase (2 mg/ml) in modified Barth's medium and then were defolliculated manually with fine forceps. The oocytes were injected with RH10 transcript alone or with mixture of RH10 transcript and mGluR5 transcript (40-50 nl per oocyte). Injected oocytes were incubated for 2-5 days at 19°C in modified Barth's medium before electrophysiological assay. For electrophysiological assay, oocytes were bathed in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5). Standard two microelectrode voltage clamp method was used for recording whole cell current with 3 M KCl-filled electrodes. All experiments were carried out at 20-23°C.

RESULTS

Sequence analysis revealed that a primary structure of RH10 was identical to that of RHK1 (rat cardiac K channel cloned by Tseng-Crank et al. (13)). RH10 corresponds to Kv1.4 according to the nomenclature proposed by Chandy et al. (14). RH10 had putative phosphorylation sites for PKA at amino acid residues 86-90 and 596-600 , and for PKC at amino acid residues 169-171, 471-473, 475-477 and 664-666 (15-17). Voltage-dependent transient outward current was observed in oocytes injected with RH10 cRNA, and its kinetics and voltage dependence were found to be similar to those described (13).

Cloned mGluR5 was coexpressed with RH10 in *Xenopus* oocytes. When voltage was clamped at a holding potential -80 mV, application of 0.1 mM L-glutamate to such oocytes caused a transient activation of endogenous Cl channels (Fig.1A). The effect of mGluR5 stimulation on RH10 current (I_{RH10}) was evaluated by measuring the I_{RH10} amplitude before and after application of L-glutamate. I_{RH10} was evoked by a voltage step from -80 mV to +20 mV. The time course of changes in I_{RH10} amplitude caused by mGluR5 stimulation is shown in Fig. 1B. The amplitude of I_{RH10} was decreased to 21.7 ± 0.1% of original value at 30 min after the application of 0.1 mM L-glutamate. Current-voltage (I-V) relationships before and after application of 0.1 mM L-glutamate are shown in Fig. 1C. The peak amplitude of I_{RH10} was suppressed more significantly than the sustained phase of I_{RH10} by mGluR5 stimulation (Fig. 1D). Steady state activation and inactivation curves were obtained by fitting experimental current-voltage relationship data to Bolzmann distributions (Fig. 2). Effects of mGluR5 stimulation on I_{RH10} were assessed by comparing steady state activation and inactivation

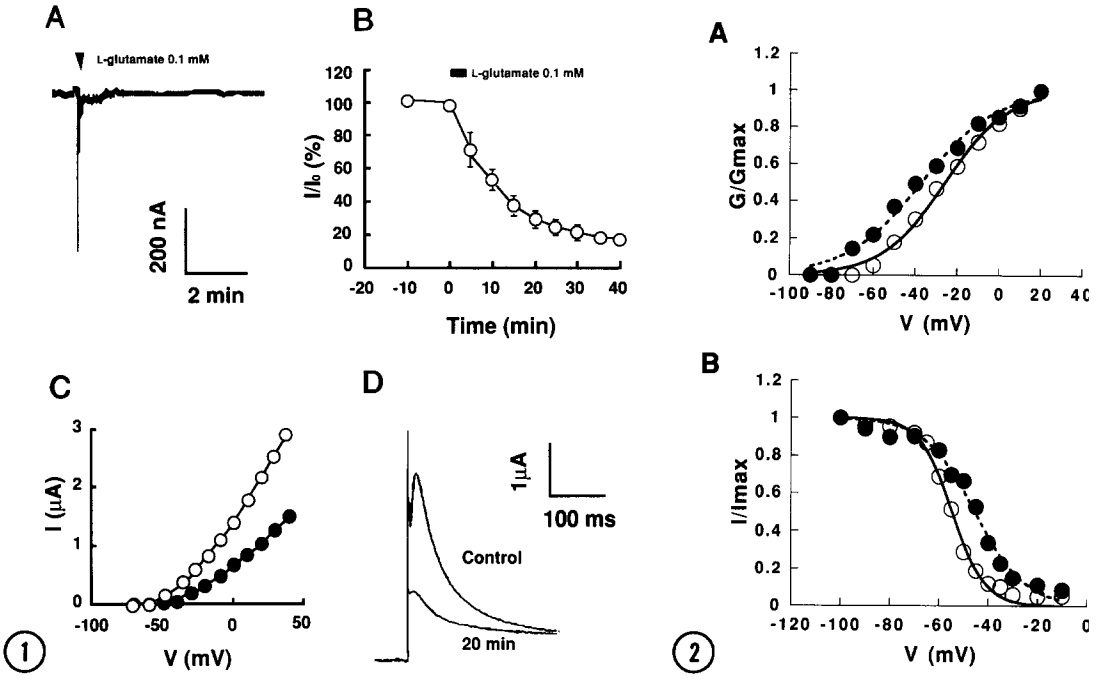

Fig. 1. Coexpression of RH10 with mGluR5 in *Xenopus* oocytes. (A) Transient inward current in response to the application of 0.1 mM L-glutamate (holding potential -80 mV). (B) Time course of the effect of mGluR5 stimulation on I_{RH10} amplitude (n=5, mean ± S.E.M.). I_{RH10} amplitude was plotted as a percentage of the I_{RH10} amplitude at time zero (I_0). The currents were evoked by a voltage step from -80 mV to +20 mV. (C) I-V relationships in control (open circle) and 20 min after application of 0.1 mM L-glutamate (closed circle) in an oocyte. (D) Actual current trace of control and 20 min after application of 0.1 mM L-glutamate.

Fig. 2. Steady state activation (A) and inactivation (B) curves of I_{RH10} before (open circle; n=5, mean ± S.E.M.) and after (closed circle; n=5, mean ± S.E.M.) application of 0.1 mM L-glutamate. Each curve was obtained by fitting the data from individual oocytes to the Bolzmann distributions (legend to Table 1).

parameters before and after application of L-glutamate. The results are summarized in Table 1. Stimulation of mGluR5 significantly reduced the activation parameter Gmax and shifted the half-maximum activation voltage (V_a) to the left, to more negative, without changing the slope factor (a_n), and the half-maximum inactivation voltage (V_h) to the right and increased its slope factor (a_h) significantly.

Several kinds of experiments were carried out to verify the involvement of intracellular second messengers in the suppression of I_{RH10}. To examine the effect of PKC on RH10 channel activity, oocytes were superfused with 1 μM staurosporine for one hour before and during experiments. Staurosporine did not affect I_{RH10} amplitude and kinetics, but slightly inhibited the effects of mGluR5 stimulation. In the presence of 1 μM staurosporine, I_{RH10} was reduced to 32.2 ± 0.1% of original value at 30 min after application of L-glutamate (Fig. 3A). The reduction was smaller than that occurred in oocytes which were not exposed to staurosporine. The PKC activator, PMA (0.1 μM), also reduced I_{RH10} amplitude but its effect was not so potent as that of mGluR5 stimulation. Effect of PMA was inhibited in the presence of 1 μM staurosporine. α-PMA (1 μM), an isomer of PMA which can not activate PKC, had no effect on

Table 1. Functional characteristics of channels expressed in *Xenopus* oocytes, alone or in association with mGluR5

	(A) Activation			(B) Inactivation	
	G_{max} (μS)	V_a (mV)	a_n (mV)	V_h (mV)	a_h (mV)
Control (n=6)	27.1 ± 1.0	−26.0 ± 2.3	14.6 ± 0.5	−54.8 ± 1.2	6.8 ± 1.6
mGluR5 (n=4)	16.2 ± 1.0**	−37.3 ± 2.9*	17.4 ± 1.3	−46.0 ± 2.3*	10.1 ± 2.1*
PMA (n=5)	23.4 ± 2.9*	−16.8 ± 5.2*	13.5 ± 0.4	−50.9 ± 1.4*	5.0 ± 0.4
mGluR5+Stauro. (n=4)	17.5 ± 1.1*	−24.5 ± 0.8	12.5 ± 0.6	−49.2 ± 4.0*	11.0 ± 1.9*

(A) Conductance-voltage relationships were fitted to Bolzmann distributions, $G/G_{max}=1/(1+\exp(-(V_m-V_a)/a_n))$, to obtain the mid point (V_a) and the slope factor (a_n) of the curves. (B) Prepulse inactivation-voltage relationships were fitted to Bolzmann distributions, $I/I_{max}=1/(1+\exp((V_p-V_h)/a_h))$, to obtain half-inactivation voltage (V_h) and slope factor (a_h) of the curves. Membrane potential was stepped from -80 mV to a conditioning voltage (V_p) ranging from -100 to -10 mV for 400ms and then to a test pulse of +20 mV. mGluR5: 0.1 μM L-glutamate was applied, PMA: perfused with 0.1 μM PMA, mGluR5+stauro.: L-glutamate was applied in the presence of 1 μM staurosporine.
All values are given as mean ± S.E.M. p was calculated using a two-tailed unpaired Student's t test.
* $p < 0.05$, ** $p < 0.01$ vs control.

I_{RH10} (Fig. 3B). The effects of PMA on steady state activation and inactivation parameters are summarized in Table 1. PMA shifted the half-maximum voltage of steady state activation and inactivation curves to the right without significant changes in slope factors.

To examine the effect of intracellular Ca^{2+} on RH10 channel activity, EGTA was injected (estimated intracellular concentration 5 mM) 10-15 min before application of L-glutamate. The effect of mGluR5 stimulation on I_{RH10} was inhibited by EGTA injection. In EGTA injected oocytes, I_{RH10} was reduced to 56.3 ± 0.1% of original value at 30 min after application of L-glutamate (Fig. 4A). The reduction was significantly smaller than that occurred in oocytes which were not injected with EGTA. Intracellular application of Ca^{2+} (estimated intracellular

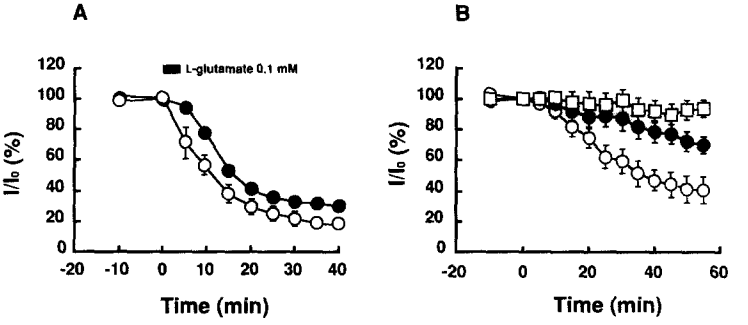

Fig. 3. (A) Time course of changes in I_{RH10} amplitude after application of 0.1 mM L-glutamate in the absence (open circle; n=5, mean ± S.E.M.) or presence (closed circle; n=5, mean ± S.E.M.) of 1 μM staurosporine. Oocytes were superfused with staurosporine before one hour and during experiments. (B) Time course of the effects of 0.1 μM PMA on I_{RH10} amplitude (open circle; n=5, mean ± S.E.M.), 0.1 μM PMA in the presence of 1 μM staurosporine (closed circle; n=5, mean ± S.E.M.) and 1 μM α-PMA (open square; n=3, mean ± S.E.M.). I_{RH10} amplitude was plotted as a percentage of the I_{RH10} amplitude at time zero (I_0). The currents were evoked by a voltage step from -80 mV to +20 mV.

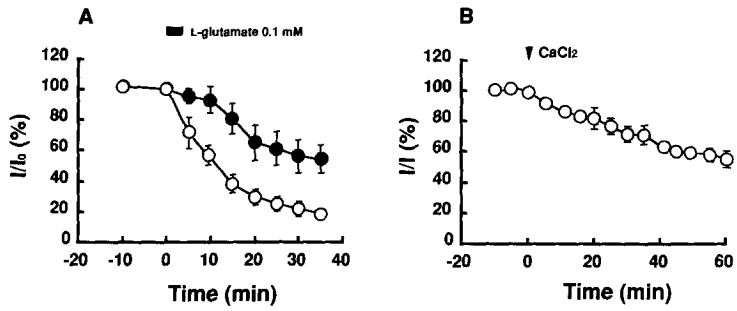

Fig. 4. (A) Time course of changes in I_{RH10} amplitude after application of 0.1 mM L-glutamate for control (open circle; n=5, mean ± S.E.M.) and EGTA injected oocytes (estimated intracellular concentration 5 mM; closed circle; n=4, mean ± S.E.M). (B) Time course of the effect of Ca^{2+} injection (estimated intracellular concentration 1 μM) on I_{RH10} (n=3, mean ± S.E.M). I_{RH10} amplitude was plotted as a percentage of the I_{RH10} amplitude at time zero (I_0). The currents were evoked by a voltage step from -80 mV to +20 mV.

concentration 1 μM) decreased the amplitude of I_{RH10} in slow time course (Fig. 4B). Effects of mGluR5 stimulation on steady state activation and inactivation parameters of I_{RH10} were examined in the presence of 1μM staurosporine (Table 1). Stimulation of mGluR5 reduced Gmax significantly without changing half-maximum activation voltage and slope factor (a_n). And it shifted half-maximum inactivation voltage to the right and reduced slope factor (a_h).

The application of 1 mM 8-Br-cAMP, 1 mM 8-Br-cGMP did not affect I_{RH10}. The application of 10 μM W7 did not inhibit the effect of mGluR5 stimulation (n=3, data not shown).

DISCUSSION

In this study, we demonstrated that the transient K channel cloned from rat heart was modulated by stimulation of coexpressed mGluR5.

Cloned mGluR5 was reported to couple to the stimulation of PI hydrolysis and transfection experiments using CHO cells indicated that mGluR5 did not increase cAMP level (18). Membrane permeable forms of cAMP did not affect RH10 channel activity despite the presence of potential PKA phosphorylation sites in RH10 sequence. So it was concluded that I_{RH10} reduction caused by mGluR5 stimulation was mediated through PI hydrolysis. I_{RH10} suppression caused by mGluR5 stimulation was inhibited by EGTA or staurosporine. In addition, direct activation of PKC alone or intracellular injection of Ca^{2+} alone did not elicit the same overall effects on the K current as mGluR5 stimulation. Therefore, both increase in intracellular Ca^{2+} and activation of PKC must contribute to this receptor-mediated modulation. Since RH10 has potential PKC phosphorylation sites, direct phosphorylation of the channel protein may possibly be responsible for the modulation by PKC activation. We observed the inhibitory effect of increased intracellular Ca^{2+} on RH10 channel activity. Similar inhibitory effects of Ca^{2+} on cloned K channel have been reported recently (6, 9). Increased intracellular Ca^{2+} has multiple actions. The activation of Ca^{2+} / calmodulin dependent enzymes is one of them. In the present experiments, however, calmodulin inhibitor W7 did not modify I_{RH10}

suppression by mGluR5 stimulation. The inhibition of the effect of mGluR5 stimulation by EGTA was greater than that by staurosporine. It might be partly due to the reduction of indirect stimulatory effect of Ca^{2+} on PKC: translocation of PKC and/or activation of phospholipase C.

mGluR5 stimulation reduced the maximum conductance, Gmax. It suggested a reduction of the number of functional channels and/or the average single channel conductance. For further investigation, single channel analysis is needed. Analysis of steady state activation and inactivation parameters revealed that mGluR5 stimulation could affect voltage dependence and channel gating charge. As reported by Moran et al., in cloned A channel, PMA reduced the maximum conductance and shifted voltage dependence of steady state activation and inactivation curves to the right without changes of slope factors (19). It was consistent with our results using PMA. They speculated that such a change could be mainly produced by the phosphorylation of the channel protein by PKC. In the present experiments, slope factors were significantly modified by mGluR5 stimulation but not by PMA. The difference may come from the effect of increased intracellular Ca^{2+}.

In isolated rat ventricular myocytes, a transient outward K current has been demonstrated (20). This current helps to shorten the action potential duration. It was reported that activation of α_1-adrenergic receptors led to action potential prolongation as a result of partial inhibition of transient outward K current and this effect was thought to be mediated by activation of PKC (21). Our results may support those observations.

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