Transmitter regulation of voltage-dependent K⁺ channels expressed in *Xenopus* oocytes

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Voltage-dependent K⁺ channels (RBK1, RBK2 and RGK5) were co-expressed in *Xenopus* oocytes with 5-hydroxytryptamine (5-HT₂) receptors. K⁺ currents measured 2–4 days later were inhibited by 5-HT (100 nM–10 μM, 20–30 s application) by up to 90%. The effect of 5-HT was mimicked by intracellular injection of Ins(1,4,5)P₃. Increasing the Ca²⁺ concentration at the inner surface of excised membrane patches did not decrease the K⁺ current.

INTRODUCTION

Many hormones and transmitters open or close membrane ion channels by acting through intermediate second-messenger molecules. Both voltage-dependent and voltage-independent channels can be modulated in this way, and the mechanism may or may not involve channel phosphorylation [1]. In *Aplysia*, the amplitude of a voltage-dependent current in bag cells is decreased by 5-hydroxytryptamine (5-HT) and by several neuroactive peptides that increase intracellular cyclic AMP [2–5], whereas in mammalian cardiac cells transmitters that increase cyclic AMP markedly increase the current through delayed rectifier K⁺ channels [6–9]. Although cyclic AMP is implicated as the second messenger in those examples, there are also cases where activation of protein kinase C is believed to be involved in the modulation of voltage-dependent K⁺ channels such as *Hermisenda* photoreceptors [10] and rat hippocampal pyramidal cells [11].

One approach to a further understanding of the mechanisms involved in transmitter modulation of ion channels is to reconstitute the coupling by expressing components of known molecular structure in heterologous cells. Many G-protein-coupled receptors can now be expressed from cDNA clones, and four main classes of voltage-dependent K⁺ channels have been cloned and expressed [12]. The mammalian members of the *Drosophila* Shaker class include rat brain clones RBK1 (ref. [13]; same as RCK1) and RBK2 (ref. [14]; same as BK2 and almost identical with RCK5) and rat genomic clone RGK5 (ref. [15]; almost identical with RCK3). The aim of the present studies was to determine whether the function of these voltage-dependent K⁺ channels, when expressed in *Xenopus* oocytes, can be modified by agonists acting through co-expressed receptors.

EXPERIMENTAL

Capped RNA was synthesized *in vitro* as previously described [13]. Methods for harvesting, injection and incubation of *Xenopus laevis* oocytes (stage V–VI) have also been reported [13–15]. Substance K receptor [16] was cloned by enzymic amplification after oligo(dT)-primed reverse transcription of bovine total stomach RNA as described previously [17]. Site-directed mutagenesis was performed on RBK1 subcloned in the phagemid pS after single-strand rescue with phage m13K07 as previously described [18]. In experiments involving co-expression of nicotinic acetylcholine-receptor subunits, 5 ng of each mRNA encoding α4 and β2 RNA was used (kindly provided by Dr. J. Patrick).

Two electrode voltage-clamp recordings were made from oocytes 24–96 h after injection with RNA, as described previously [13]. The oocytes were continuously superfused with ND-96 solution, of composition (mM): NaCl 96, KCl 2, CaCl₂ 1.8, MgCl₂ 1, Hepes 5 (pH 7.5). Drugs were applied by changing this solution to one that contained the drug. Currents were also measured in excised inside-out membrane patches [19]. In this case, defolliculated oocytes were stripped of vitelline membrane after pretreatment with hyperosmotic solution [19], recording electrodes (0.5–2 MΩ) contained ND-96, and the intracellular (bath) solution was (mM): KCl 98, MgCl₂ 2, Hepes 10, with varying CaCl₂ concentrations.

Compounds used were A23187, acetylcholine, adenosine 5'-[γ-thio]triphosphate, alkaline phosphatase (type XXX-L), catalytic subunit of protein kinase A (bovine), cyclic AMP, dibutyryl cyclic AMP, forskolin, 5-HT creatinine sulphate, Ins(1,4,5)P₃, mastoparan, phenylmethanesulphonyl fluoride, 4β-phorbol 12,13-dibutyrate, 4β-phorbol 12,13-didecanoate, staurosporine, theophylline, trifluoperazine (all from Sigma Chemical Co., St. Louis, MO, U.S.A.), substance K, leupeptin (Peninsula, Belmont, CA, U.S.A.), atropine, ketanserin (Research Biochemicals Inc., Natick, MA, U.S.A.), *N*-[*N*-(1-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine (E64; Boehringer, Indianapolis, IN, U.S.A.), 1,2-bis-(2-aminophenoxy)ethane-*NNN'*-tetra-acetic acid (BAPTA; Molecular Probes Inc., Eugene, OR, U.S.A.), 1-(5-isquinolinesulphonyl)-2-methylpiperazine (H7) and calmidazolium (Calbiochem, La Jolla, CA, U.S.A.) and okadaic acid (kindly given by Dr. Y. Tsukitani, Fujisawa Pharmaceutical Co., Tokyo, Japan). Numerical values are expressed as means ± S.E.M.; numbers refer to the numbers of oocytes tested.

RESULTS

Oocytes that had been injected with RBK1 RNA synthesized *in vitro* showed outward currents of several μA when the membrane was depolarized from –80 to 0 mV. These currents activated within about 10 ms and declined by less than 15% during depolarizing commands of up to 4 s, and were in all other respects similar to the current previously described to result from RBK1 RNA injection [13,14,20]. Oocytes that had been injected only with 5-HT₂-receptor RNA did not exhibit voltage-dependent K⁺ currents when depolarized; application of 5-HT (1 μM) to these oocytes resulted in an inward current that rapidly (within 10 s) reached its peak amplitude and then declined into a smaller

Abbreviations used: 5-HT, 5-hydroxytryptamine; [Ca²⁺]_i, intracellular Ca²⁺ concentration; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-*NNN'*-tetra-acetic acid.

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Fig. 1. Inhibition of RBK1 K^+ current by 5-HT and other agonists

Traces (a)–(d) are pen recordings of membrane K^+ currents (outward current is upward) evoked at intervals of 30 s by depolarizing the oocyte from -80 to 0 mV. The bars above the records indicate the period during which the perfusing solution was changed to one that contained 5-HT or another agonist. (a) Oocyte previously injected with RBK1 and 5-HT₂ receptor RNAs. 5-HT ($1 \mu\text{M}$) evokes a transient inward current, and a sustained depression of the K^+ current. (b) A similar application of 5-HT has no effect in an oocyte previously injected only with RBK1 RNA. (c) Substance K (SK, 30 nM) has a similar action in an oocyte previously injected with substance K receptor RNA. (d) Oocyte previously injected with RBK1 RNA. Acetylcholine (ACh, $1 \mu\text{M}$) causes an inward current and slightly inhibits the K^+ current by activation of native muscarinic receptors. 5-HT ($1 \mu\text{M}$) further suppresses the K^+ current.

oscillatory current that often outlasted the 5-HT application. This current reversed polarity at about 25 mV and is the Ca^{2+} -activated chloride current previously described [21,22].

Oocytes injected with both RBK1 and 5-HT₂-receptor RNA showed voltage-dependent K^+ currents that were not obviously different from those seen in oocytes injected only with RBK1 RNA. In this case, 5-HT caused a large, long-lasting, inhibition of the voltage-dependent K^+ current (Fig. 1a). After a brief application of 5-HT (typically 30 s), the inhibition of the current began within 1–2 min and reached maximal inhibition only after $> 20 \text{ min}$ (Fig. 1a). The decrease in RBK1 current caused by 5-HT ($1 \mu\text{M}$) was $77 \pm 3\%$ (38 oocytes from 8 frogs), and the time to reach half-maximal inhibition was $10 \pm 0.3 \text{ min}$. The K^+ current recovered only slowly: with $1 \mu\text{M}$ -5-HT the effect was almost irreversible (time for half recovery was $> 25 \text{ h}$), but with 10 nM -5-HT reversal of the inhibition was observed with a half-time of about 4 h. The peak amplitude of the K^+ current was decreased by 5-HT, with no marked effects on the kinetics or voltage-dependence (Fig. 2a) [time constants for activation (-30 mV) were $8 \pm 1 \text{ ms}$ before 5-HT and $11 \pm 1 \text{ ms}$ after 5-HT ($n = 4$), and for deactivation (-50 mV) were $10 \pm 1 \text{ ms}$ both in control and after 5-HT]. Similar effects after 5-HT₂-receptor activation were seen with oocytes co-expressing the K^+ -channel clones RBK2 ([14]; $66 \pm 11\%$ inhibition, $n = 4$) or RGK5 ([15]; $72 \pm 4\%$ inhibition, $n = 4$). The same concentrations of 5-HT were effective to inhibit the K^+ current and to induce the chloride current (see above) (Fig. 2b). Both responses were blocked by the 5-HT₂-receptor blocker ketanserin (100 nM). 5-HT had no effect on RBK1 currents in oocytes that had not been also injected with 5-HT₂-receptor RNA (Fig. 1b; $n = 18$).

The selectivity of this action of 5-HT for voltage-dependent K^+ channels was tested. Oocytes that had been injected with RNAs encoding nicotinic acetylcholine-receptor subunits ($\alpha 4$ and $\beta 2$), as well as RBK1 and 5-HT₂-receptor RNAs, gave inward currents in response to acetylcholine ($1 \mu\text{M}$) of $47 \pm 7 \text{ nA}$. A second application of acetylcholine, applied at a time when the K^+ current was decreased by $82 \pm 3\%$ as a result of a prior application of 5-HT ($1 \mu\text{M}$) 30 min previously, evoked an inward

current that was $107 \pm 9\%$ of the control value. These studies were carried out in a Ca^{2+} -free solution containing scopolamine ($1 \mu\text{M}$) and Mg^{2+} (10 mM) so as not to activate endogenous muscarinic receptors and to prevent any Ca^{2+} entry through nicotinic channels.

There was no long-lasting effect of 5-HT on the transient chloride current evoked by 5-HT application. In four oocytes, 5-HT ($1 \mu\text{M}$) evoked an average chloride current of $5.4 \mu\text{A}$ (at -80 mV); 30 min later, the RBK1 current was depressed by $89 \pm 3\%$. At this time, a second application of 5-HT evoked a chloride current with amplitude $117 \pm 14\%$ of that caused by the first application. 5-HT also did not change the leakage conductance (measured with hyperpolarizing steps in the range -70 to -120 mV).

The tachykinin substance K mimicked the effect of 5-HT in oocytes that had been injected with substance K receptor RNA (5 ng) in addition to RBK1 RNA. The oocytes gave typical chloride currents [16] in response to substance K, and substance K (30 nM) inhibited the voltage-dependent K^+ current by $46 \pm 16\%$ ($n = 5$) (Fig. 1c). Acetylcholine ($1 \mu\text{M}$) caused a small but long-lasting decrease in RBK1 currents ($13 \pm 4\%$, $n = 4$) even in oocytes previously injected only with RBK1 RNA, presumably through activation of native muscarinic receptors (Fig. 1d).

Coupling between some receptors and Ca^{2+} -dependent chloride channels in oocytes can be blocked by pertussis toxin [23]. K^+ currents in oocytes incubated in pertussis toxin ($3 \mu\text{g/ml}$) for 12–24 h were significantly less inhibited by 5-HT than those in control cells (Fig. 2d). In one group of control cells treated with heat-inactivated toxin, 5-HT inhibited the current by $78 \pm 1\%$ ($n = 5$; measured at 20 min), whereas in pertussis-toxin-treated cells the inhibition was $21 \pm 3\%$ ($n = 5$).

Two effects expected from G-protein activation of phospholipase C are increased levels of diacylglycerol and inositol phosphates. The involvement of these messengers in mediating inhibition of K^+ currents was therefore examined. 4-Phorbol 12,13-dibutyrate or didecanoate (30 nM) had no effect on the current or the inhibition by 5-HT. Injection of $\text{Ins}(1,4,5)\text{P}_3$

Fig. 2. Inhibition of K⁺ current by 5-HT and increased [Ca²⁺]_i.

(a) 5-HT inhibits K⁺ current without change in time course. Records are outward current evoked by depolarizing the oocyte from -80 to 0 mV. Four superimposed traces are (from top to bottom) control current, current 10 min after adding 5-HT (1 μM), current 20 min after adding 5-HT, and leakage current. (b) Similar concentrations of 5-HT are required to inhibit the K⁺ current (●) and to induce the transient chloride current (○). (c) Intracellular injection of Ins(1,4,5)P₃ (10 pmol) inhibits the K⁺ current. (d) Inhibitory action of 5-HT (1 μM) is blocked by pertussis-toxin pretreatment (3 ng/ml; 24 h). (e) Inhibitory action of 5-HT (1 μM) is blocked by previous injection with calcium chelator BAPTA (2.5 nmol), which itself did not affect the current.

(10 pmol) into the oocyte decreased the K⁺ current with a time course similar to that observed after 5-HT application (Fig. 2c); the current was inhibited by $88 \pm 4\%$ at 20 min ($n = 5$). This effect was mimicked by direct intracellular injection of CaCl₂ (90 pmol; $32 \pm 9\%$ inhibition; $n = 5$), or by a brief (10–20 s) application of normal ND-96 Ca²⁺-containing solution to oocytes pretreated for 10 min with the Ca²⁺ ionophore A23187 in a Ca²⁺-free solution ($79 \pm 5\%$ inhibition; $n = 5$). The inhibitory effect of 5-HT was blocked by prior injection of the oocyte with 2.5 nmol of BAPTA, which itself did not change the K⁺ current (Fig. 2e).

The K⁺ currents in membrane patches removed from oocytes that had been injected with RBK1 RNA were not affected by changes in the Ca²⁺ concentration at the internal surface of the membrane. The Ca²⁺ concentration was changed in the range 2 μM–1 mM with no effect on the amplitude of the voltage-dependent K⁺ current recorded in the membrane patch ($n = 3$; currents of 100–200 pA were evoked by depolarizing the membrane from -70 to 30 mV for 300 ms, at 20 s intervals).

Several experiments designed to examine the role of possible intracellular transduction steps provided negative results. In an effort to test the role of channel phosphorylation by protein kinase A, a K⁺-channel subunit was made in which the Ser residues of a highly conserved consensus sequence [12–15,20] had been mutated to Ala. K⁺ currents in oocytes expressing this mutant channel (RBK1: Ser⁴⁴⁵ → Ala, Ser⁴⁴⁶ → Ala, Ser⁴⁴⁷ → Ala) were apparently normal, and 5-HT (1 μM) inhibited the current by $64 \pm 3\%$ ($n = 5$). Further evidence against involvement of protein kinase A was obtained: the K⁺ current and the action of 5-HT were unaffected by intracellular injection of the catalytic subunit of protein kinase A (2.1 Sigma units), cyclic AMP (500 pmol), adenosine 5'-[γ-thio]triphosphate (2.5 nmol) or staurosporine (2.5 pmol), and nor were they affected by extracellular application of staurosporine (10 μM) or theophylline (1 mM; an inhibitor of cyclic AMP phosphodiesterase). Further experiments to test the involvement of protein kinase C and calmodulin-dependent kinase were also negative. H7 (superfusion

with 20 μM), and the calmodulin antagonists calmidazolium (10 μM, by superfusion, or 15 pmol, by injection), trifluoperazine (3 μM, by superfusion) and mastoparan (50 nmol, by injection) had no effect. Indeed, evidence against the involvement of either kinase was the lack of any effect of injecting the oocytes with alkaline phosphatase (0.5 unit). The negative results with calmidazolium and alkaline phosphatase are particularly informative because they both caused at least 50% inhibition of the slow K⁺ current called I_{sk} by Takumi *et al.* [24] [calmidazolium (1 μM) by superfusion for 10 min: alkaline phosphatase (0.5 unit by injection) (A. E. Busch, M. P. Kavanaugh, J. P. Adelman & R. A. North, unpublished work)].

The possibility was next considered that the increase in intracellular Ca²⁺ was activating a proteinase. However, there was no effect of injecting the oocytes with 250 pmol of either proteinase inhibitor E64 or phenylmethanesulphonyl fluoride, or of pretreating them with leupeptin (5 mM for 2 h).

DISCUSSION

Several lines of evidence indicate that the voltage-dependent K⁺ current is decreased as an indirect consequence of an increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i). These include the findings that the effect can be mimicked by treatments such as intracellular injection of Ins(1,4,5)P₃, permeabilization of the oocyte to Ca²⁺ with A23187, or direct intracellular injection of Ca²⁺. In addition, the effect can be mimicked by activation of other receptors known to couple to phospholipase C and to increase [Ca²⁺]_i in oocytes, including the substance K receptor [16] and the endogenous muscarinic receptor [25]. Further evidence for a role of Ca²⁺ comes from the finding that intracellular injection of the calcium chelator BAPTA blocks the action of 5-HT on K⁺ currents. Therefore, it is concluded that the first step in the action of 5-HT on K⁺ channels is activation of a pertussis-toxin-sensitive G-protein which results in Ins(1,4,5)P₃ generation and an increase in [Ca²⁺]_i.

The mechanism by which an increase in $[Ca^{2+}]_i$ decreased the current is less clear. Four possibilities might be distinguished. First, high $[Ca^{2+}]_i$ might directly gate the channel; this is not likely, because of the very slow time course and because the effect was not seen in excised patches. Second, the increase in $[Ca^{2+}]_i$ might stimulate endocytosis, with resulting loss of membrane channels; this also seems unlikely, in view of the magnitude of the effect, together with the lack of any effect on other membrane channels (leak conductance, nicotinic acetylcholine receptor, or 5-HT-activated chloride channels). Third, the channel might be affected by a change in phosphorylation state. However, pharmacological manipulation of phosphorylation pathways revealed no evidence of a role in the 5-HT₂-receptor-mediated decrease in K^+ currents; forskolin, staurosporine, H7, dibutyryl cyclic AMP, catalytic subunit of A kinase, theophylline, trifluoperazine and calmidazolium neither mimicked nor blocked the action of 5-HT. In addition, one mutant channel (RBK1: Ser⁴⁴⁵ → Ala, Ser⁴⁴⁶ → Ala, Ser⁴⁴⁷ → Ala) was still susceptible to the action of 5-HT. The result with intracellular alkaline phosphatase provided no evidence that dephosphorylation was involved. A fourth possibility is specific proteolysis of the channel, perhaps by a Ca^{2+} -activated proteinase such as calpain [26]. However, proteinase inhibitors (E64, leupeptin) at concentrations known to be effective to block calpain in oocytes [26] had no effect on the current inhibition, nor did intracellular injection of the serine proteinase inhibitor phenylmethanesulphonyl fluoride.

Inhibition of K^+ currents in *Xenopus* by activation of co-expressed 5-HT receptors has recently been reported by others [27,28]. Hoger *et al.* [28] found that pretreatment with the calmodulin inhibitors W7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide; 50 μ M] and trifluoperazine (100 μ M) partially prevented the inhibition by 5-HT; because the kinase inhibitor H7 did not alter the inhibition of the K^+ current, but prevented recovery from inhibition, they concluded that the channel is "normally in a phosphorylated state" and is dephosphorylated by a calmodulin-dependent phosphatase. Our finding of lack of effect of calmidazolium and alkaline phosphatase argue against this conclusion, and raise the possibility that the observed effects of W7 and trifluoperazine are due to actions other than inhibition of calmodulin (e.g. [29]).

Modulation of K^+ channels by neurotransmitters is a crucial mechanism underlying many changes in cell function [1]. Specifically, K^+ -channel inhibition can lead to functional changes ranging from transient increases in neuronal excitability to more prolonged phenomena such as long-term potentiation [30,31]. The observations in the present study suggest the possibility that voltage-dependent K^+ channels can be specifically regulated by activation of neurotransmitter receptors through a mechanism dependent on increases in cytoplasmic Ca^{2+} .

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