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Activation of galanin receptor 2 stimulates large conductance Ca^{2+}-dependent K^+ (BK) channels through the IP3 pathway in human embryonic kidney (HEK293) cells

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ABSTRACT

The large conductance Ca^{2+}-activated K^+ (BK) channels are widely distributed in the brain, and act as intracellular calcium sensors in neurons. They play an important feedback role in controlling Ca^{2+} flux and Ca^{2+}-dependent processes, including neurotransmitter release and cellular excitability. In this study, the effects of the neuropeptide galanin on BK channels were examined by determining the whole-cell currents and single-channel activities in human embryonic kidney (HEK293) cells co-expressing GalR2 and the BK alpha subunit. Galanin enhanced the currents of BK channels, in a concentration-dependent and PTX-independent manner, with an ED50 value of 71.8 ± 16.9 nM. This activation was mediated by GalR2, since its agonist AR-M1896 mimicked the effect of galanin, and since galanin did not facilitate BK currents in cells co-expressing cDNAs of BK and GalR1 or GalR3. The galanin-induced BK current persisted after replacement with Ca^{2+}-free solution, suggesting that extracellular Ca^{2+} is not essential. Chelating intracellular Ca^{2+} by either the slow Ca^{2+} buffer EGTA or the fast Ca^{2+} buffer BAPTA abolished galanin-mediated activation of BK channels, indicating the important role of intracellular Ca^{2+}. The role of Ca^{2+} efflux from the sarcoplasmic reticulum/endoplasmic reticulum (SR/ER) was confirmed by application of thapsigargin, an irreversible inhibitor that depletes Ca^{2+} from SR/ER. Moreover, the inositol-1,4,5-triphosphate receptor (IP3R) was identified as the mediator responsible for increased intracellular Ca^{2+} activating BK channels. Taken together, activation of GalR2 leads to elevation of intracellular Ca^{2+} is due to Ca^{2+} efflux from ER through IP3R sequentially opening BK channels.

1. Introduction

The large conductance, Ca^{2+}-activated K^+ (BK or BK_{Ca}) channels are widely distributed in the central nervous system, with a particularly high expression in the cortex, amygdala, thalamus, hypothalamus, hippocampus and pineal gland [1,2]. When unitary conductance is determined from current–voltage plots with symmetrical $[K^+]$ (>100 mM) at both sides of the membrane, BK channels can be distinguished by a conductance at ~100–300 pS [3]. They can also be identified by the activation mechanism, that is they can be triggered not only by elevated intracellular Ca^{2+} levels $[Ca^{2+}]_i$ but also by membrane depolarization [4]. Therefore, BK channels are considered as intracellular calcium sensors in neurons, playing an important feedback role in controlling Ca^{2+} flux and subsequent Ca^{2+}-dependent processes, including neurotransmitter release and cellular excitability [3,5].

A functional coupling between BK channels and G-protein coupled receptors has been reported. For example, activation of dopamine receptor results in opening of BK channel through the protein kinase G pathway [6]. Moreover, activation of M2 muscarinic acetylcholine receptors may stimulate Giβγ-mediated excitation of adenyl cyclase/cAMP activities, which leads to phosphorylation of Ca^{2+} channels, resulting in influx of Ca^{2+} and opening of BK channels [7]. Several neuropeptide receptors, such as the μ-opioid receptor, the neuropeptide Y2-type receptor and the oxytocin receptor have been shown to mediate agonist activation of BK channels [8–10].

Galanin, a 29–30 amino acid neuropeptide [11], is widely expressed in the central nervous system and plays multiple roles in regulation of homeostatic and affective behavior through its three

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receptors, galanin receptor 1 (GalR1), -2 (GalR2) and -3 (GalR3) [12,13]. With regard to GalR2 an important transduction mechanism involves $G\alpha_{q/11}$ stimulating phospholipase C and phosphatidylinositol-phosphate production, resulting in transmitter release, but $G\alpha_{i/o}$ mediating inhibition of adenylyl cyclase activity has also been demonstrated [14–21]. Functionally galanin via GalR2 has been shown to have, e.g., trophic effects [22] and to be involved in pain threshold regulation [23], cognitive functions [24], consummatory behavior [25], neurodegeneration [26], anxiety- and depression-like behaviors [27], seizure activity [28] as well as addiction [29]. In the present study, the effects of galanin on BK channels were examined by determining the whole-cell currents and single-channel activities in human embryonic kidney (HEK293) cells co-expressing GalR2 and the BK alpha subunit.

2. Methods and materials

2.1. cDNAs and other reagents

Construct encoding mouse alpha subunit of the BK channel (mSlo1) was generously provided by Dr. Jiu-Ping Ding. A human galanin receptor 2 (hGalR2) construct was generated by PCR. It was tagged at the C-terminus with enhanced GFP (EGFP) by insertion into pEGFP-N1 plasmid (Clonetech, Madison, WI).

The following reagents were from commercial sources: galanin from Bachem (Torrance, CA), AR-M1896 and ryanodine from Tocris Bioscience (Bristol, UK), paxilline, thapsigargin, pertussis toxin (PTX) and BAPTA (1,2-Bis(2-Aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) from Sigma (St. Louis, MO).

2.2. Cell cultures

HEK293A cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in 5% CO_2. One day before transfection the cells were transferred to 24-well plates. Co-transfection was carried out with cDNAs encoding mouse BK channel α1 subunit (mslo1) and hGalR2 tagged with EGFP (hGalR2-EGFP) at an mslo1: hGalR2-EGFP ratio of 0.4:0.4 (μg)/well. At 70% confluence cells were transiently transfected using Lipofectamine2000 (Invitrogen, Carlsbad, CA). Electrophysiological experiments were performed 2 days after transfection.

2.3. Patch clamp recording

All experiments were performed at room temperature. As described previously [30], the pipette solution contained 150 mM KCl, 2.5 mM $MgCl_2$, 10 mM HEPES, 0.2 mM EGTA, 3.5 mM Na_2ATP and 0.5 mM Na_2GTP, pH = 7.4, and the bath solution 150 mM NaCl,

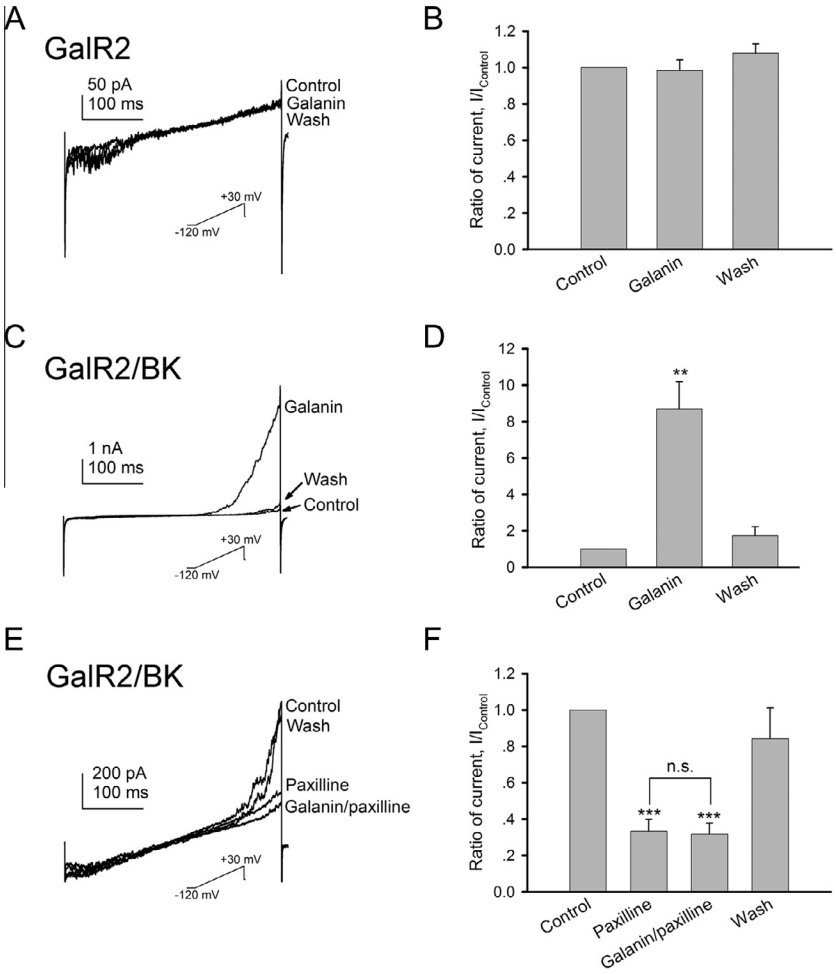

Fig. 1. Effect of galanin on whole-cell BK currents in HEK293A cells. Representative current–voltage plots obtained before (control) and after added galanin (400 nM) (A and C) or galanin with paxilline (10 μM) (E) during voltage ramps from −120 to 30 mV (300 ms duration). Summarized data of whole-cell currents obtained before any application of drugs, and normalized whole-cell currents obtained before and after added galanin (B, $n = 18$; D, $n = 8$) or galanin with paxilline (F, $n = 9$). The currents were normalized for each cell to $I_{control}$ recorded at 30 mV. Note that application of galanin did not significantly change the *I–V* curve in cells expressing GalR2 alone (A and B), but dramatically increased the current in cells co-expressing GalR2 and BK (C and D). The enhancement was completely inhibited by paxilline (E and F). Data are shown as mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ (Student's paired *t*-test).

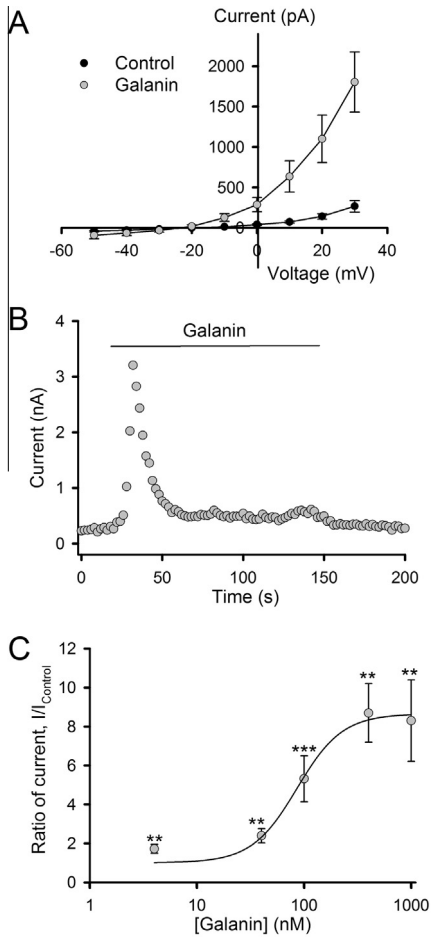

Fig. 2. Properties of galanin-induced BK current. (A) The mean *I–V* curves were plotted under control conditions (closed circles) and perfusion with 400 nM galanin (open circles). (B) Plots of current at +30 mV with a sampling interval of 2 s. The line indicated perfusion duration of galanin (400 nM). (C) Dependence of galanin-induced current on added galanin concentration. The fitted plots with the Hill Equation gave the ED50 = 85.9 ± 14.2 nM (the Hill coefficient $n = 2.1 \pm 0.7$). Data from 16 to 19 patches were pooled. Values are means ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with control.

5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM glucose, pH = 7.4. The recording was conducted with HEKA EPC-10 patch-clamp amplifier with associated software (PULSE and PatchMaster, HEKA Electronic Inc., Germany). The currents were typically digitized at 10 kHz. Macroscopic records were filtered at 2.9 kHz. Data were analyzed with Igor (WaveMetrics, Portland, OR) and Sigmaplot (Systat Software., Chicago, IL) software. The open probability of the channel was calculated using "single-channel search" function in the Clampfit software (Axon Instruments, Union City, CA). All data were presented as mean ± SEM. For data obtained from the same cell, paired *t*-test was used, whereas unpaired *t*-tested was adopted for analyzing data collected from a group of cells.

A microperfusion device (MPS-2; INBIO, Wuhan, China) with a fast exchange time (<100 ms) among eight channels was used to puff drugs locally to the cell in the study.

3. Results

3.1. Galanin facilitates Ca^{2+}-dependent K^+ channels

Whole-cell current was recorded from HEK293A cells. A single depolarization voltage step from −120 mV to +60 mV elicited a small background current, which was resistant to the selective BK channel blocker paxilline [31] in native HEK293A cells (data not shown) and HEK293A cells only expressing hGalR2-EGFP (GalR2 cells) (Supp. 1A). The properties of BK current in those HEK293A cells were similar to previously reported ones [32]. In cells expressing the BK channel alpha subunit (BK cells), the depolarization stimulation elicited a large outward current, which was inhibited by 10 μM of paxilline (Supp. 1B), indicating that this current is a BK current. Similar results were seen in the cells co-expressing the mouse alpha subunit and hGalR2-EGFP (GalR2/BK cells) (Supp. 1C), suggesting that co-expressing with hGalR2-EGFP does not influence BK channel activity.

To study the effect of galanin on BK current, repeated 300-ms ramp command voltages from −120 mV to +30 mV were applied on the cells with an interval of 2 s. The current was measured at the voltage of +30 mV and normalized to the value before galanin application, i.e. the ratio of current ($I/I_{Control}$). Application of galanin at 400 nM did not significantly increase the outward current in GalR2 cells (Fig. 1A and B). However, in GalR2/BK cells the same application caused an 8-fold enhancement of the outward current as compared to controls (Fig. 1C and D; Fig. 2A). Pre-incubation with paxilline at 10 μM abolished the galanin-induced enhancement (Fig. 1E and F), suggesting that galanin facilitates the outward current by activation of BK channels. Application of galanin with the same concentration did not have any significant effect on the outward current in cells co-expressing cDNAs of BK and GalR1, or GalR3 (data not shown), indicating that the galanin-induced facilitation of BK current is solely mediated by GalR2 in the HEK293A cells. To further define the receptor subtype involved in galanin-induced activation of BK, the GalR2 agonist AR-M 1896 was applied and found to mimic the effect of galanin. Thus, the BK current was enhanced by application of AR-M 1896 at 500 nM (Supp. 2).

3.2. Properties of galanin-induced BK current

In time-lapse the galanin-induced BK current was shown to rapidly decline, even under conditions of continuous application of galanin (Fig. 2B). To determine the quantitative relationship between galanin concentration and ratio of induced BK current, a dose–response curve was plotted and fitted using Hill Equation, which yielded an ED50 = 85.9 ± 14.2 nM with the Hill coefficient $n = 2.1 \pm 0.7$ (Fig. 2C). Moreover, the cell-attached mode was used to record single channel activity. The channel open probability (*N*Po, *N* = number of channels, Po = open probability of channel) and the amplitude of single channel current were calculated using Clampfit. Single BK channel current could be detected under depolarization voltage, with a conductance of ~130 pS under physiological conditions, as reported previously [33]. As a result, the single channel recording showed that galanin increased the channel activity (*N*Po) of BK channels but did not affect the single channel current (Fig. 3A and B), suggesting that galanin facilitated BK channel opening without changing its channel pore structure.

3.3. Mechanism of galanin-induced BK current

It has been demonstrated that activation of BK channels depends on elevation of intracellular Ca^{2+} level [34], and that activation of GalR2 can lead to a surge of intracellular Ca^{2+} concentrations in HEK cells [35]. Chelating intracellular Ca^{2+} by either the slow Ca^{2+} buffer EGTA (10 mM) or the fast Ca^{2+} buffer BAPTA (10 mM) in the pipette solution [36] abolished galanin-mediated activation of BK channels (data not shown), indicating an important role of intracellular Ca^{2+} in the galanin-induced BK current. To identify the source of the Ca^{2+} in response to

galanin application, the bath solution containing 2 mM Ca^{2+} was replaced by Ca^{2+}-free solution. The galanin-induced increase of BK current was not affected (Fig. 3C), excluding that influx of extracellular Ca^{2+} underlies the BK channel activation. To determine whether increased intracellular Ca^{2+} originates from sarcoplasmic reticulum/endoplasmic reticulum (SR/ER), the cells were pretreated with a potent inhibitor of the SR Ca^{2+} ATPase, thapsigargin, at 10 μM [37,38]. The enhancement of BK current induced by 100 nM galanin perfusion was abolished after this pretreatment (Fig. 3D), suggesting that galanin-induced BK channel activation is mediated by Ca^{2+} efflux from the SR/ER. Both ryanodine receptor (RyR) and inositol-1,4,5-triphosphate receptor (IP3R) have been shown to be responsible for Ca^{2+} efflux from SR/ER [39]. Thus, the RyR antagonist ryanodine (20 μM) [37] and the IP3R antagonist 2-APB (100 μM) [40–42] were applied, respectively, to verify which receptor is involved in the GalR2 signaling pathway. The galanin-induced BK channel activation was abolished by 2-APB (Fig. 3D), suggesting that the intracellular elevation of Ca^{2+} via activation of GalR2 is due to Ca^{2+} efflux through IP3R from ER.

Several signaling pathways have been suggested to be involved in activation of GalR2 [12,13]. For example, GalR2 has been reported to initiate signaling pathways in antiepileptogenesis by coupling to G(q) and G(i) proteins [43]. To examine the involvement of G(i) proteins in galanin-induced BK channel activation, cells were pretreated with 250 ng/mL pertussis toxin (PTX) for over 4 h. The effect of galanin on BK channel persisted under this condition (Fig. 3D), suggesting that GalR2 mediates galanin-induced activation of BK channel in a PTX-insensitive mode.

4. Discussion

In the present study, for the first time, a facilitatory effect of galanin on Ca^{2+}-dependent K^+ current was demonstrated. The galanin-induced BK channel activation is mediated by GalR2, and IP3R on ER is responsible for increased intracellular Ca^{2+} that activated BK channels.

A number of studies have suggested that galanin and its three receptors are involved in many physiological functions as well as in the pathophysiology of neurological and mental disorders [13]. However, information of possible galanin effects on BK channels via GalR2 is limited. Recently, BK channels have been suggested to be involved in GalR2-mediated galanin-induced microglial migration [44]. In our study, both whole-cell and single channel recording showed that galanin enhances BK currents in a concentration-dependent manner. The galanin-induced activation of BK was only seen in the cells coexpressing cDNAs of BK with GalR2, but not with either GalR1 or GalR3. Thus, even if AR-M 1896 is not only a GalR2 agonist [45] but also acts at GalR3 [46], our data strongly suggest that the facilitatory effect of galanin on Ca^{2+}-dependent K^+ current is mediated by GalR2.

Several neurotransmitter receptors have been reported to have a functional linkage with BK channels involving calcium. Thus, activation of adenosine A1 receptors [47,48] or NMDA receptors [37,49] on the postsynaptic membrane results in opening of BK channels by influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels. In smooth muscle the activation of BK channels is due to intracellular Ca^{2+} efflux from the ryanodine receptor in ER [50]. Ca^{2+} imaging studies have shown that activation of GalR2 can lead to a surge of intracellular Ca^{2+} concentrations in various

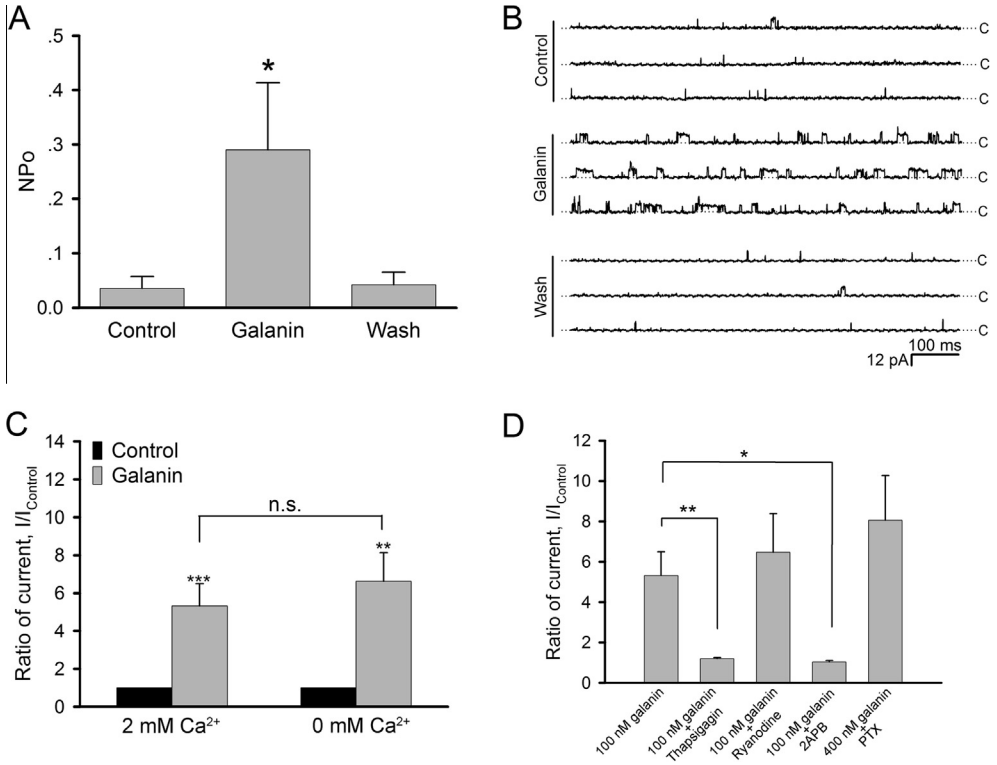

Fig. 3. (A and B) Effects of galanin on BK single-channel activities in cell-attached patches. Single channel activity of BK channel was recorded under cell-attached configuration. The channel open probability (*N*Po) under the command protocol of repeated 1-s depolarization voltage at +60 mV was analyzed before, during and after galanin (400 nM) treatment of the cell ($n = 6$). The traces represent 6 independent patches in HEK-GalR2/BK cells. The dotted line indicate close state. $^*p < 0.05$ as compared with Control. (C and D) Signaling pathways involved in GalR2 mediated galanin-induced BK activation. (C) Application of galanin at 100 nM facilitated the whole cell current of BK channels in both standard bath solution (2 mM Ca^{2+}, $n = 9$) and Ca^{2+}-free bath solution (0 mM Ca^{2+}, $n = 9$). (B) Summary of effects of thapsigargin (10 μM, $n = 13$), ryanodine (20 μM, $n = 18$), 2-APB (100 μM, $n = 8$), pertussis toxin (PTX) (250 ng/mL, $n = 11$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ n.s. is short for no significance.

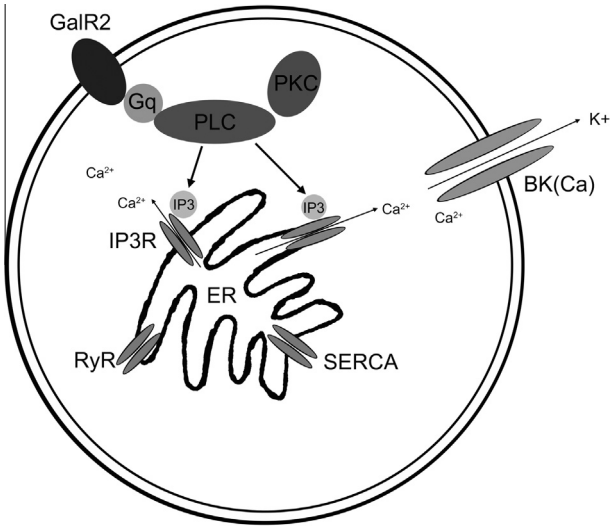

Fig. 4. Model of GalR2 signaling pathway in galanin-induced activation of BK channels. Galanin activates GalR2 coupling to Gq/11, resulting in activation of PLC and increase of IP3 leading to inositol-1,4,5-triphosphate receptor activation at ER. Thus, the intracellular Ca^{2+} mobilization leads to activation of BK channels.

cell types [43,51,52], including HEK cells [35]. In the present study, chelating intracellular Ca^{2+} with EGTA or BAPTA abolished the galanin-mediated activation of BK channels, pointing to a key role of Ca^{2+} in galanin regulation of BK currents. Since the effect was present in a Ca^{2+}-free solution, the galanin-mediated activation of BK channels is mainly due to increase of intracellular Ca^{2+}. In agreement, the galanin-induced BK current was abolished, when the Ca^{2+} pool was depleted from SR/ER by thapsigargin. Moreover, the IP3R antagonist 2-APB, but not the RyR antagonist ryanodine, blocked the galanin-induced BK current, suggesting that IP3R is responsible for Ca^{2+} efflux from SR/ER [53]. Taken together, galanin activation of GalR2 leads to elevation of intracellular Ca^{2+} levels due to Ca^{2+} efflux through IP3R from ER, which in turn activates BK channels.

The galanin-induced BK current rapidly declined even under continuous application of galanin. One possible explanation is fast internalization of GalR2 in HEK293 cells [35,54]. Another explanation is that the increased cytosolic Ca^{2+} level is quickly reduced by Ca^{2+} pumps located on both the ER and the plasma membrane [55].

GalR2 has, for example, been reported to initiate signaling pathways in antiepileptogenesis by coupling to G(q) and G(i) proteins [43]. There is also experimental evidence for a Gi-coupling of GalR2 in HEK293 cells [52], but it is unlikely that pertussis toxin-sensitive G(i) proteins play a pivotal role, as pertussis toxin failed to block the effect of galanin on BK. Moreover, the increase of intracellular Ca^{2+} release from ER via IP3R implies that coupling to G(q) protein is the main pathway downstream of GalR2, as also reported in other studies [19,56]. Thus, our hypothesis is that galanin activates GalR2 linked to G(q), resulting in increased phospholipase C activity [51] and subsequent hydrolysis of phospholipid PIP2. The hydrolytic product, IP3, then induces Ca^{2+} efflux from the ER through IP3R, and as a consequence, the BK channels are activated (Fig. 4).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.110.

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