Prevention of Ginsenoside-induced Desensitization of Ca\(^{2+}\)-activated Cl\(^{-}\) Current by Microinjection of Inositol Hexakisphosphate in Xenopus laevis Oocytes

IN Volvement of GRK2 and \(\beta\)-Arrestin I*

We demonstrated that ginsenosides, the active ingredient of Panax ginseng, enhance endogenous Ca\(^{2+}\)-activated Cl\(^{-}\) currents via Ga\(_{12/13}\)-phospholipase C-\(\beta\) pathway in Xenopus laevis oocytes. Moreover, prolonged treatment of ginsenosides induced Cl\(^{-}\) channel desensitization. However, the molecular mechanisms involved in ginsenoside-induced Cl\(^{-}\) channel desensitization have not yet been determined precisely. To provide answers to these questions, we investigated the changes in ginsenoside-induced Cl\(^{-}\) channel desensitization after intraoocyte injection of inositol hexakisphosphate (InsP\(_6\)), which is known to bind \(\beta\)-arrestins and interfere with \(\beta\)-arrestin-occupied receptor-down-regulation, and cRNAs coding \(\beta\)-arrestin I/II and G-protein receptor kinase 2 (GRK2), which is known to phosphorylate G protein-coupled receptors and attenuate agonist stimulations. When control oocytes were stimulated with ginsenosides, the second, third, and fourth responses to ginsenosides were 69.6 ± 4.1, 9.2 ± 2.3, and 2.6 ± 2.2% of the first responses, respectively. Preintraoocyte injection of InsP\(_6\) before ginsenoside treatment restored ginsenoside effect to initial response levels in a concentration-, time-, and structurally specific manner, in that inositol hexaphosphate had no effect. The EC\(_{50}\) was 13.9 ± 8.7 \(\mu\)M. Injection of cRNA coding \(\beta\)-arrestin I but not \(\beta\)-arrestin II blocked InsP\(_6\) effect on prevention of ginsenoside-induced Cl\(^{-}\) channel desensitization. Injection of cRNA coding GRK2 abolished ginsenoside effect enhancing Cl\(^{-}\) current. However, the GRK2-caused loss of ginsenoside effect on Cl\(^{-}\) current was prevented by coinjection of GRK2 with GRK2-K220R, a dominant-negative mutant of GRK2. These results indicate that ginsenoside-induced Cl\(^{-}\) channel desensitization is mediated via activation of GRK2 and \(\beta\)-arrestin I.

Ginseng, the root of Panax ginseng C. A. Meyer, has been used as a representative tonic for several hundreds years in such countries as Korea, China, and Japan. Currently, ginseng is one of the most famous and precious herbal medicines consumed in around the world (1, 2). Recent accumulating evidence shows that ginsenosides are the pharmacologically active ingredient of ginseng. Ginsenoside is one of the derivatives of triterpenoid dammarane, consisting of 30 carbon atoms.

Recent studies have suggested that G proteins mediate some ginsenoside effects. For example, Nah and McCleskey (3) and Nah et al. (4) showed in neuronal cells that ginsenosides inhibit voltage-dependent Ca\(^{2+}\) current in sensory neurons through the activation of pertussis toxin-sensitive G protein. Choi et al. (5, 6) demonstrated that ginsenoside treatment increased Ca\(^{2+}\)-activated Cl\(^{-}\) current through the signaling pathway that activates pertussis toxin-insensitive Ga\(_{12/13}\) proteins coupled to PLC-\(\beta\) in Xenopus laevis oocytes. Choi et al. (5) also showed that Ca\(^{2+}\)-activated Cl\(^{-}\) current produced by ginsenoside treatment diminished spontaneously after reaching peak amplitude, even in the continued presence of ginsenosides in X. laevis oocytes. However, very little is known about molecular mechanisms involved in Cl\(^{-}\) channel desensitization induced by ginsenoside treatment.

Desensitization of a receptor is defined as the diminished or abolished response to an agonist after repeated stimulation. The desensitizing process of G protein-coupled receptors (GPCRs), which are mainly coupled to Ga\(_{12/13}\), Ga\(_{12/13}\)-adenylate cyclase, or Ga\(_{12/13}\)-phospholipase C, is well characterized (7–9). The main specialized regulatory proteins for homologous GPCR desensitization process are GRKs and \(\beta\)-arrestins (10, 11). GRKs mediate the phosphorylation of the receptors that are occupied by agonists. The phosphorylated receptors create a binding site for regulatory proteins, \(\beta\)-arrestins, which are involved in the endocytosis of desensitized receptors and bindings of \(\beta\)-arrestins to the phosphorylated receptor facilitate to uncouple it from its target G proteins for termination of effector stimulation (10, 12–14). On the other hand, recent reports showed that InsP\(_6\) blocks visual arrestin interaction with phosphorylated rhodopsin by a direct binding to visual arrestin, resulting in elimination of light-induced inactivation of rhodopsin (15, 16).

In the present study, to further characterize the molecular mechanisms underlying ginsenoside-induced Ca\(^{2+}\)-activated
Results
Rapid Desensitization of Ginsenoside- or ACh-induced Cl- Current Responses after Repeated Application of Ginsenosides or ACh—In the present study, we examined the changes in ginsenoside effect on the Cl- current after repeated application of ginsenosides. As shown in Fig. 1A, treatment of ginsenosides induced Cl- current enhancement in a voltage-dependent manner. The reversal potential was near -20 mV, indicating that ginsenosides activate endogenous Ca2+-activated Cl- currents in X. laevis oocytes (5, 6). Oocytes first stimulated with ginsenosides were washed with ND96 for 3–5 min until the basal current was recovered again and then restimulated with ginsenosides. The second, third, or fourth Cl- current responses for ginsenosides were dramatically diminished, and the magnitudes of Cl- current were 69.6 ± 4.1, 9.2 ± 2.3, and 2.6 ± 2.2%, respectively, of the first responses of ginsenosides (n = 15 oocytes each from three different batches of donors) (Fig. 1B, inset). As a positive control, cRNA coding m1 muscarinic acetylcholine receptors (mACHRs) was injected into oocytes; receptor activation also enhances Cl- currents via Gq/11-PLC-insitol triphosphate pathway (18). Repeated treatment of oocytes expressing m1 AChR with ACh resulted in a gradual attenuation of the magnitude of Cl- current. The second, third, or fourth Cl- current responses for ginsenosides were diminished, and the magnitudes of Cl- current were 14.2 ± 7.1, 8.3 ± 6.3, and 4.1 ± 3.2%, respectively, of the first responses of ACh (n = 13 oocytes each from three different batches of donors) (Figs. 3C and 5B).

Effect of InsP6 on the Rapid Desensitization of Ginsenoside- or ACh-induced Cl- Current Responses—Because preintraoocyte injection of InsP6 prevents the desensitization on Ca2+-activated Cl- currents induced by repeated treatment of substance P or lysophosphatidic acid in X. laevis oocytes expressing substance P receptors or lysophosphatidic acid receptors (12), the above results prompted us to test the possibility that InsP6 might also prevent ginsenoside-induced Cl- channel desensitization. As the first step toward testing this hypothesis, we investigated the effect of InsP6 on the rapid loss of Cl- current responses after short-term treatment with ginsenosides or ACh. We injected InsP6 (100 μM intracellular concentration) into oocytes 20 min before treatment with ginsenosides or ACh. Preintraoocyte injection of InsP6 neither changes basal membrane current compared with H2O-injected oocytes (H2O/InsP6 = 0.24 ± 0.05/0.22 ± 0.07 μA, n = 15 each) nor affects ginsenoside- or ACh-induced Cl- current responses (Figs. 1D, inset, and 5B). However, as shown in Fig. 1, C and D, preintraoocyte injection of InsP6 not only prevented the loss of ginsenoside- or ACh-induced Cl- current response but also slightly enhanced ginsenoside- or ACh-induced Cl- current response (Fig. 1D, inset and Fig. 5B). The second, third, or fourth Cl- current response for ginsenosides was maintained, and the magnitudes of the current were 106.6 ± 3.1, 134.8 ± 5.3, and 138.7 ± 7.2%, respectively, of the first responses of ginsenosides (n = 12 oocytes each; from three different batches of donors) (Fig. 1D, inset). For m1 AChR, the second, third, or fourth Cl- current response for ACh was also maintained, and the magnitudes of the current were 102.4 ± 10.1, 94.8 ± 12.3, and 114.7 ± 13.2%, respectively, of the first responses of ACh (n = 12 oocytes each; from three different batches of donors) (Fig. 5B).

Concentration- and Time-dependent Effects of InsP6 on Ginsenoside-induced Cl- Channel Desensitization—Fig. 2A shows the concentration-dependent response curve for prevention by InsP6 on the loss of ginsenoside-induced Cl- current responses. InsP6 prevented the loss of ginsenoside-induced Cl- current responses in a dose-dependent manner. The EC50 was 13.9 ± 8.7 μM. These inhibitory effects on desensitization were not simply a result of anionic charge, because the InsP6 analog-like inactive InsS6 had no effect, indicating that the attenuating effect on ginsenoside-induced desensitization of Ca2+-activated Cl- channel desensitization on Ca2+-activated Cl- current after intraoocyte injection of InsP6, which is known to bind ß-arrestins and block ß-arrestin-dependent receptor trafficking. We further examined the effects of GRK2 after intraoocyte injections of cRNAs coding GRK2. Herein, we present results suggesting that ginsenoside-induced Cl- channel desensitization involves the activation of GRK2/ß-arrestin I in X. laevis oocytes.
Cl\textsuperscript{−} current is specific to InsP\textsubscript{6} (Fig. 2A, inset). Fig. 2B shows the time course of onset of the inhibitory effect of InsP\textsubscript{6} on ginsenoside-induced Cl\textsuperscript{−} channel desensitization. The inhibitory effect reached a maximum level 15 min after intraoocyte injection, and the effect was persistent for at least 6 h.

**Effect of InsP\textsubscript{6} on Restimulation of Ginsenosides or ACh In Oocytes Undergoing Short- or Long-Term Treatment with Ginsenosides or ACh**—As a next step, we examined whether intraoocyte injection of InsP\textsubscript{6} to oocytes that have been desensitized by several repeated ginsenoside treatments or oocytes expressing m1 mAChRs that have also been desensitized by several repeated ACh treatments could prevent ginsenoside- or ACh-induced Cl\textsuperscript{−} channel desensitization. As shown in Fig. 3, InsP\textsubscript{6}-injected oocytes that were desensitized previously by short-term treatment with ginsenosides or ACh failed to respond to ginsenoside or ACh treatment. We also tested whether...
FIG. 2. Concentration- and time-dependent effect of InsP₆ on prevention of GTS-induced Cl⁻ channel desensitization. A, oocytes were injected with different concentrations of InsP₆ and incubated for 20 min in ND96. Other experimental procedures were the same as described in Fig. 1 (mean ± S.E.; n = 13–15 oocytes each). Inset, peak outward currents (mean ± S.E.; n = 14–16 each) recorded in oocytes injected with H₂O, InsP₆, or inactive analog form, InsS₆ (final 100 μM). B, the oocytes were injected with H₂O or InsP₆ and incubated for 20 min in ND96. The peak amplitudes of the outward currents recorded after fourth treatment of GTS (mean ± S.E.; n = 13–15 oocytes each). *, p < 0.05; **, p < 0.001; significantly different from the others.
intraoocyte injection of InsP₆ in oocytes that were desensitized by short- or long-term treatment with ginsenosides failed to induce GTS response on Ca²⁺-activated Cl⁻ current. 

A, inset, the traces of the current recorded at the time points 1 and 2 are superimposed. Histograms show that peak outward currents (mean ± S.E.; n = 14–15 oocytes each) recorded in oocytes injected with H₂O (first treatment with GTS) and InsP₆ (fifth treatment of GTS). B, inset, histograms show that ginsenoside induced peak outward currents (mean ± S.E.; n = 12–14 each) recorded in oocytes before (0 h) and after (16 h) ginsenoside treatment. C and D, injection of InsP₆ into oocytes expressing m mAChRs that were desensitized by short- or long-term treatment with ACh failed to induce ACh response on Ca²⁺-activated Cl⁻ current. C, inset, the traces of the current recorded at the time points 1 and 2 are superimposed. Histograms show that peak outward currents (mean ± S.E.; n = 13–14 oocytes each) recorded in oocytes injected with H₂O (first treatment with ACh) and InsP₆ (fifth treatment of ACh). D, inset, histograms show that ACh-induced peak outward currents (mean ± S.E.; n = 10–12 each) recorded in oocytes before treatment of ACh (0 h) and after ACh treatment (16 h). Bars denote bath application of GTS (50 μg/ml) or ACh (100 μM) and arrow indicates intraoocyte injection of InsP₆.
sitzation. Moreover, in oocytes that have been undergone prolonged treatment with ginsenosides or ACh, the loss of Cl\(^{-}\) current response to ginsenosides or ACh was not restored by intraoocyte injection of the InsP\(_6\).

**Time Course of Recovery of Ginsenoside- or ACh-induced Cl\(^{-}\) Channel Desensitization**—To characterize recovery from Cl\(^{-}\) channel desensitization at longer times, we employed the following procedure: ginsenosides or ACh response was recorded in 13–14 oocytes (control group), and the mean control response was calculated. Test group oocytes were exposed to ginsenosides or ACh for 2–3 h, washed several times with ND96, and then the response to the second application of ginsenosides or ACh was measured in these oocytes during the indicated times. As shown in Fig. 4, initial recovery of ginsenoside-induced Cl\(^{-}\) current response during the first 5–60 min remained about 5–30% of control and was followed by 60% recovery after 2–4 h, whereas initial recovery of ACh-induced Cl\(^{-}\) current response during the first 5–120 min remained about 1–3% of control and was followed by 10–45% recovery after 4–8 h. Thus, recovery time on ACh-induced Cl\(^{-}\) response from desensitization was slower than that of ginsenoside-induced Cl\(^{-}\) response; the half-recovery time to control level from desensitization was about 145.8 ± 63 and 619.5 ± 26.9 min for ginsenosides and m1 mAChRs, respectively. However, ginsenoside- and ACh-induced Cl\(^{-}\) current responses had nearly recovered to initial control levels after 24 h.

**Overexpression of β-Arrestin I but Not β-arrestin II Blocks InsP\(_6\) Effect on Ginsenoside- and ACh-induced Cl\(^{-}\) Channel Desensitization**—As a next step, we tested whether or not overexpression of β-arrestins related with receptor desensitization could affect ginsenosides or ACh effect on Cl\(^{-}\) current. We also tested whether or not overexpression of these proteins could affect InsP\(_6\)-induced attenuation on ginsenoside- or ACh-induced Cl\(^{-}\) channel desensitization. As illustrated in Fig. 5A, intraoocyte injection of cRNA coding β-arrestin I or β-arrestin II did not affect either basal Cl\(^{-}\) current (H\(_2\)O/β-arrestin I = 0.22 ± 0.04:0.21 ± 0.06 μA; H\(_2\)O/β-arrestin II = 0.20 ± 0.04: 0.23 ± 0.03 μA; n = 15 each) or the Cl\(^{-}\) current evoked by initial ginsenoside treatment (H\(_2\)O/β-arrestin I = 3.5 ± 0.2: 3.2 ± 0.5 μA; H\(_2\)O/β-arrestin II = 3.5 ± 0.2:3.1 ± 0.1 μA; n = 15 each) (Fig. 5). However, intraoocyte injection of cRNA coding β-arrestin I significantly blocked the preventive effect of InsP\(_6\) on ginsenoside-induced Cl\(^{-}\) channel desensitization in the fourth treatment of ginsenosides (H\(_2\)O + InsP\(_6\)/β-arrestin I + InsP\(_6\) = 4.8 ± 0.8:1.3 ± 0.4 μA; \(p < 0.01\); measured at a \(V_c\) value of +60 mV, \(n = 15\) oocytes each, from three different batches of frogs), whereas intraoocyte injection of cRNA coding β-arrestin II failed to block the preventive effect of InsP\(_6\) (H\(_2\)O + InsP\(_6\)/β-arrestin II + InsP\(_6\) = 4.8 ± 0.8:3.9 ± 0.7 μA). Similarly, we could also observe that preintraoocyte injection of InsP\(_6\) prevented the loss of ACh-induced Cl\(^{-}\) current response in oocytes expressing m1 mAChRs and that injection of cRNA coding β-arrestin I (ACh + InsP\(_6\)/β-arrestin I + InsP\(_6\) = 4.6 ± 0.8:1.7 ± 0.4 μA; \(p < 0.01\); measured at a \(V_c\) value of +60 mV, \(n = 14\) oocytes each, from three different batches of frogs) but not β-arrestin II (ACh + InsP\(_6\)/β-arrestin II + InsP\(_6\) = 4.8 ± 0.8:4.0 ± 0.6 μA) blocked the effect of InsP\(_6\) on prevention of ACh-induced Cl\(^{-}\) channel desensitization (Fig. 5B).
Overexpression of GRK2 Inhibits the Effect of Ginsenoside or ACh on Cl\(^{-}\) Current Enhancement—Because the results above showed the possibility that \(\beta\)-arrestin I might participate in the desensitization signaling pathway on a loss of ginsenoside- or ACh-induced Cl\(^{-}\) current responses, we investigated the roles of GRK in the effect of ginsenoside or ACh on Cl\(^{-}\) current enhancement. We first injected cRNA coding GRK2 alone or co-injected cRNAs coding m1 mAChR and GRK2 into oocytes and observed the effect of ginsenoside or ACh on Cl\(^{-}\) current. As summarized in Fig. 6, A and B, ginsenosides or ACh applied to \(H_2O\)-injected vehicle oocytes enhanced the Cl\(^{-}\) current. In contrast, ginsenosides or ACh failed to enhance the Cl\(^{-}\) current in cells injected with cRNA coding GRK2 or cRNAs coding m1 mAChR and GRK2 (Fig. 6, A and B). However, intraoocyte injection of cRNA coding GRK2-K220R, which is a dominant-negative mutant of GRK2 and lacks kinase activity, had no effect on ginsenoside- or ACh-induced Cl\(^{-}\) current enhancement. Moreover, coexpression of GRK2 and GRK2-K220R abolished the inhibitory effect of GRK2 on ginsenoside- or ACh-induced Cl\(^{-}\) current enhancement. The extent of the blockade of the ginsenoside effect on Cl\(^{-}\) current by GRK2 cRNA injection was proportional to the amount of cRNA injected (Fig. 6C), and the time required for the injected cRNAs to work was \(>16\ h\) (Fig. 6D). Repeated treatment of ginsenosides or ACh in oocytes expressing m1 mAChR induced a loss of Cl\(^{-}\) current response in oocytes injected with cRNAs coding GRK2-K220R alone and cRNAs coding GRK2 and GRK2-K220R, respectively (Fig. 6, A and B).

Effects of InsP\(_6\) on PMA-evoked Loss of Ginsenoside-induced Cl\(^{-}\) Current Responses—The activation of PLC produces lipid-
soluble 1,2-diacylglycerol, an endogenous protein kinase C (PKC) activator. Previous reports showed that activation of PKC by treatment with PMA, an active PKC activator, causes receptor phosphorylation and receptor uncoupling from PLC-mediated inositol phospholipid metabolism, resulting in a loss of Cl\(^-\) current responses by agonist stimulations (19–23). In the present study, we first examined the effects of the PKC activator PMA on the ginsenoside-induced Cl\(^-\) current enhancements. As shown in Fig. 7, treatment with PMA, but not 4α-phorbol 12,13-diacecanoate, an inactive PKC activator, induced a loss of ginsenoside-induced Cl\(^-\) current enhancement but PKA activators had no effect on ginsenoside-induced Cl\(^-\) current responses (data not shown). The inhibitory effect of PMA was dose-dependent, and the IC\(_{50}\) was 35.6 ± 4.7 nM (Fig. 7B, inset). But cotreatment of the PKC inhibitor staurosporine with PMA prevented PMA-induced inhibition of the ginsenoside effect on Cl\(^-\) currents, indicating that PMA-evoked loss of ginsenoside-induced Cl\(^-\) current responses is specific to PKC activation. We also tested whether preintraoocyte injection of InsP\(_6\) prevent PMA-induced inhibition on ginsenoside-evoked Cl\(^-\) current. However, as shown in Fig. 7B, preintraoocyte injection of InsP\(_6\) had no effect on PMA-induced loss of ginsenoside-evoked Cl\(^-\) current responses.

**DISCUSSION**

Although ginsenosides, the active ingredient of *Panax* ginseng, have been widely used as pharmacological agents for a long time, few reports have related ginsenoside signal transduction until now. In previous studies, we demonstrated that activation of membrane components by ginsenosides at the extracellular side enhanced a Ca\(^{2+}\)-activated Cl\(^-\) current via a Ga\(_{q/11}\)-phospholipase C-β pathway. We showed also that prolonged treatment with ginsenosides induced a desensitization of Cl\(^-\) current response after reaching peak amplitude in *X. laevis* oocytes (5, 6).

The present study was performed to further characterize the desensitization signaling pathway of a loss of ginsenoside-induced Cl\(^-\) current response. In this study, we have provided three principal findings affirming that GRK2 and β-arrestin I are involved in the main molecular mechanisms mediating ginsenoside-induced Cl\(^-\) channel desensitization in *X. laevis* oocytes. First, we showed that short- or long-term treatment with ginsenosides induced a complete loss of ginsenoside effect on Cl\(^-\) current enhancement, whereas preintraoocyte injection of InsP\(_6\), but not InsS\(_6\) (an inactive analog of InsP\(_6\)), prevented the desensitization of Cl\(^-\) current induced by repeated treatment with ginsenosides. These results support the concept that the attenuating effect on ginsenoside-induced Cl\(^-\) channel desensitization is specific to InsP\(_6\). The EC\(_{50}\) (13.9 ± 8.7 μM) obtained from the present study was relatively high and suggests that InsP\(_6\) interacts with β-arrestin I with low affinity. However, there was structural specificity for InsP\(_6\) but not InsS\(_6\), and the potency of the Cl\(^-\) channel desensitization-blocking action of InsP\(_6\) was very similar to the concentration of InsP\(_6\) binding to arrestins (16). Moreover, because the concentration of InsP\(_6\) in variety of cells is in the range of 1–20 μM, the EC\(_{50}\) values for this pharmacological action might be physiologically significant (24–26). Interestingly, we observed that intraoocyte injection of InsP\(_6\) did not restore ginsenoside or ACh responses on Ca\(^{2+}\)-activated Cl\(^-\) channels after short- or long-term Cl\(^-\) channel desensitization caused by ginsenoside or ACh treatment that had already occurred (Fig. 3). The present study further suggested that membrane component(s) or m1 mAChRs that might be interacting with ginsenosides or ACh could be down-regulated after short- or long-term treatment with ginsenosides or ACh and that intraoocyte injected-InsP\(_6\) was no more helpful for the down-regulated membrane components or m1 mAChRs.

As for the second piece of evidence for the role of β-arrestins in ginsenoside-induced Cl\(^-\) channel desensitization, we demonstrated that overexpression of β-arrestin I cRNA, but not β-arrestin II cRNA, induced ginsenoside- or ACh-induced Cl\(^-\) channel desensitization in oocytes or in oocytes expressing m1 mAChRs that were preinjected with InsP\(_6\) before ginsenoside or ACh treatment. These results suggest that extra copies of β-arrestin I produced by injection of cRNAs coding β-arrestin I might bind with InsP\(_6\) and sequester free InsP\(_6\) by forming complexes with InsP\(_6\) and β-arrestin I. Therefore, the results of the experiments performed with β-arrestin I further suggest that β-arrestin I mediates ginsenoside- and m1 mAChR-induced Cl\(^-\) channel desensitization.

The third and final piece of evidence suggesting a role for GRK2 in ginsenoside- and m1 mAChR-induced Cl\(^-\) current desensitization comes from the experiments involving injections of cRNAs coding GRK2, which is known to phosphorylate...
GPCRs after agonist stimulation and facilitate β-arrestin binding to phosphorylated receptors for the receptor down-regulation process. The results of these experiments (i.e. blocking of the ginsenoside or ACh effect that enhances Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} current by GRK2 cRNA injection but not by dominant-negative GRK2-K220R, which lacks kinase activity, and blocking of GRK action by cRNA coinjection of GRK2 and dominant-negative GRK2-K220R) suggest that the kinase activity of GRK2 is a key role for the inhibitory effect on ginsenoside- and m1 mAChR-induced Cl\textsuperscript{−} current enhancement and that the kinase activity of GRK2 is also involved in signaling pathway of ginsenoside- or m1 mAChR-induced Cl\textsuperscript{−} channel desensitization. Moreover, these results further suggest that phosphorylation of membrane receptors by GRK2 that might interact with ginsenosides or phosphorylation of m1 mAChR by GRK2 could be enough for ginsenoside- or m1 mAChR-induced Cl\textsuperscript{−} channel desensitization (Fig. 6).

Recent studies have indicated that β-arrestin I as well as β-arrestin II are involved in GPCR desensitization after their respective agonist stimulations (10, 11). However, the present study does not support the idea that β-arrestin II functions as a direct mediator of ginsenoside- or m1 mAChR-induced Cl\textsuperscript{−} channel desensitization. Most of the GPCRs that couple to the activation of pertussis toxin-insensitive G protein and PLC and the release of Ca\textsuperscript{2+} from inositol triphosphate-sensitive intracellular stores showed indistinguishable affinity to both β-arrestin isoforms and formed a stable complexes with β-arrestin I or II even after 1 h (9, 12, 13). In present study, we also observed that once a loss on ginsenoside- or ACh-induced Cl\textsuperscript{−} current responses by short-term and repeated treatment with ginsenoside or ACh was initiated, the desensitization lasted for up to 8 h before complete recovery (Fig. 4). However, at present, we can offer no satisfactory explanation for the lack of effect of β-arrestin II cRNA injection on ginsenoside-induced Cl\textsuperscript{−} channel desensitization. To clarify the exact role of β-arrestins in ginsenoside-induced Cl\textsuperscript{−} channel desensitization, further studies will be needed.

We also tested the role of second messenger-dependent protein kinase (i.e. PKC) activation on ginsenoside-induced Cl\textsuperscript{−} channel desensitization. As shown in Fig. 7, it is likely that the desensitization pathway via PKC activation on ginsenoside-induced Cl\textsuperscript{−} current enhancement might be mediated via a different pathway compared with β-arrestin I-mediated Cl\textsuperscript{−} current desensitization, because preintraoocyte injection of InsP\textsubscript{6} did not reverse PMA-evoked loss of ginsenoside-induced Cl\textsuperscript{−} current responses (27–29). What is the mechanism of desensitization by PKC if it does not involve β-arrestins? Previous reports demonstrated that treatment with a PKC activator (i.e. 12-O-tetradecanoylphorbol-13-acetate) not only attenuated ACh- or 5-HT-induced Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} current enhancement in X. laevis oocytes injected with rat brain mRNA but also increased phosphorylation of oocyte membrane proteins (27). But treatment with 12-O-tetradecanoylphorbol-13-acetate had no effect on Cl\textsuperscript{−} current enhancement evoked by intraoocyte injection of inositol trisphosphate or Ca\textsuperscript{2+} (28, 29). In the present study, PMA-induced desensitization was also not affected by intraoocyte-injected InsP\textsubscript{6}. These results suggest that the main role of PKC activation in terminating ginsenoside action on Cl\textsuperscript{−} current enhancement might be the phosphorylation of membrane proteins that interact with ginsenosides or other proteins involved in the ginsenoside signaling pathway, such as the Goq family, rather than β-arrestins (30). Similarly, intraoocyte injection of InsP\textsubscript{6} did not reverse 12-O-tetradecanoylphorbol-13-acetate-caused loss of substance P-induced Cl\textsuperscript{−} current responses (12). Because we could not explain exactly the mechanism of PMA-induced desensitization on ginsenoside-induced Cl\textsuperscript{−} current response, further studies will be needed to clarify the role of PKC activation in ginsenoside-induced Cl\textsuperscript{−} channel desensitization.

We also investigated recovery time kinetics from Cl\textsuperscript{−} channel desensitization in both ginsenosides and m1 mAChRs in X. laevis oocytes. The half-recovery time to control level from desensitization was about 145.8 ± 63 and 619.5 ± 26.9 min for ginsenoside and m1 mAChR, respectively. Interestingly, in myenteric neurons, substance P stimulation of neurokinin receptor-1, which is also coupled to the Gq/PLC-inositol triphosphate pathway, induced intracellular Ca\textsuperscript{2+} mobilization, and half-recovery time to control levels from desensitization induced by repeated treatment of substance P was about 20 min (13). Thus, it seems that the time course for resensitization on ginsenosides and m1 mAChR from Cl\textsuperscript{−} channel desensitization in X. laevis oocytes was much slower compared with neurokinin receptor-1 in neurons. However, recent reports also showed that various other GPCRs expressed in X. laevis oocytes did not recover as rapidly to control levels from desensitization as neurokinin receptor-1 in neurons did. For example, in oocytes expressing the endothelin\textsubscript{A} receptor, Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} current response to endothelin1 from desensitization was not completely recovered even after 90 min (31). Moreover, in oocytes injected with rat brain mRNAs, Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} current responses to ACh and serotonin from desensitization recovered partially after 60 min and recovered completely only after 240 min, respectively (23, 32). These different recovery times might be caused by different types of cells, and further studies will be needed to clarify the recovery time discrepancy between X. laevis oocytes and neurons.

In summary, using an X. laevis oocyte model system for explanation of ginsenoside signaling pathway that allows various foreign gene expressions, we obtained further results suggesting that GRK2 and β-arrestin I mediate ginsenoside-induced Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channel desensitization. This may be one of the desensitization signaling pathways that underlies ginseng action.
Prevention of Ginseng Desensitization by InsP₆

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