Functional Uncoupling between the Receptor and G-Protein as the Result of PKC Activation, Observed in Aplysia Neurons

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Abstract: Application of either acetylcholine (ACh), dopamine (DA), histamine (HA), or Phe-Met-Arg-Phe-NH₂ (FMRFamide) induces a K⁺-current response in the identified neurons of Aplysia under voltage clamp. This type of response is mediated by a pertussis toxin (PTX)-sensitive G-protein, G₁ or G₂. Extracellular application of 60 μM phorbol dibutyrate (PDBu), an activator of protein kinase C (PKC), to these cells markedly depressed all the K⁺-current responses to ACh, DA, HA, and FMRFamide. The depressing effect of PDBu lasted for at least 60 min despite continuous washing with the normal perfusing medium. Application of PKC inhibitors such as 100 μM H-7 or 10 μM staurosporine and PKC₁₉₋₃₁, prior to the application of PDBu significantly decreased the depressing effects of PDBu. In contrast, an intracellular injection of okadaic acid (OA), an inhibitor of protein phosphatase 1 and 2A, significantly augmented the blocking effect of PDBu. Intracellular injection of the PKC catalytic subunit induced a similar depressing effect as observed with PDBu. The dose-response curves obtained with different transmitters all shifted downward after the activation of PKC, but the ED₅₀ of each transmitter remained unchanged. Furthermore, the K⁺-current responses induced by the intracellular application of GTPγS were not depressed at all, even after the receptor-induced K⁺-current responses of the same cell were markedly depressed. These results strongly suggest that PKC phosphorylated a certain coupling site between the receptor and G-protein, and impaired the signal transduction necessary for triggering the K⁺-channel opening. [Japanese Journal of Physiology, 47, 241–249, 1997]

Key words: K⁺-channel, protein kinase C, ACh-receptor, dopamine-receptor, histamine-receptor.

According to Higashida et al., stimulation of the bradykinin receptor identified in the NG108-15 cell produces a diphasic response (i.e., initial hyperpolarizing response followed by gradual depolarization) [1, 2]. This receptor stimulation is known to activate phospholipase C (PLC), and produce inositoltrisphosphate (IP₃) and diacyl glycerol (DAG) inside the cell. They determined that the initial hyperpolarizing response is due to opening the Ca²⁺-dependent K⁺-channel, which is activated by IP₃-induced Ca²⁺-release from the endoplasmic reticulum, and that the second depolarizing phase is due to closing the specific K⁺-channel (so called M-channel) as the result of PKC-induced phosphorylation, which is facilitated by the presence of DAG. PKC activation in this cell seems to occur following intracellular Ca²⁺-release by IP₃. However, PKC can be directly activated by extracellular application of PKC activators, or the catalytic subunit of PKC can be injected intracellularly to observe the effect of PKC-induced phosphorylation. There are a number of papers describing that receptor-induced K⁺-channel opening is readily blocked by the direct activation of PKC, regardless of the nature of either receptor or K⁺-channel [3–7].

Previously, we proposed the following working hypothesis on the mechanism of receptor-induced K⁺-
channel opening: All receptors capable of $K^+$-channel opening must be functionally coupled to the PTX-sensitive G-protein (G<sub>i</sub> or G<sub>o</sub>), the activation of which is primarily responsible for opening the specific $K^+$-channel [8–10]. This $K^+$-channel is known to be specific, and is called the “G protein-operated $K^+$-channel,” which is different from the axonal $K^+$-channel or Ca<sup>2+</sup>-dependent $K^+$-channel and also different from aminopyridine-sensitive or M-channels.

As described above, $K^+$-channel opening is the final process of receptor stimulation, and there are many steps in signal transduction to reach the final process. Accordingly, the actual acting site of PKC to block the final process may not only be $K^+$-channels of different kinds but also any steps of the signal transduction process to reach the final process. Therefore, further investigations using neurons with different receptors, G-proteins, and $K^+$-channels are required to clarify the actual acting site of PKC responsible for the blocking action of the receptor-induced $K^+$-channel openings.

This paper is to report our recent results, which suggest that the possible acting site of PKC could be the coupling site between the receptor and PTX-sensitive G-protein.

A preliminary report of this study has been published elsewhere [11].

METHODS

Preparation and perfusing media. The abdominal or cerebral ganglia of Aplysia kurodai were dissected out and fixed in a perfusing chamber. The connective tissues covering the ganglion cells were carefully removed under a dissecting microscope to expose cells to the perfusing medium. Cells were normally perfused with artificial Aplysia medium using an ionic composition similar to that of Aplysia blood; Na<sup>+</sup> 587, K<sup>+</sup> 12, Cl<sup>-</sup> 671, Ca<sup>2+</sup> 14, Mg<sup>2+</sup> 52 mM [12]. The pH was adjusted to 7.4 with Tris and HCl, and the cells were continuously perfused at a constant flow rate of 5 ml min<sup>-1</sup>, keeping the temperature at 23 ± 2°C. The effective perfusing volume of the chamber was 0.2 ml.

Electrical recordings of transmitter-induced responses. Two microelectrodes filled with 1.8 M potassium citrate with a resistance of 2–3 MΩ were introduced into a single cell of the RC cluster [13], the LUQ cluster [14] of the abdominal ganglion, or the E cluster of the cerebral ganglia [15, 16] under binocular microscopic control. One electrode was connected to the input of a preamplifier installed in a Dagan 8500 clamp unit in order to record the change in membrane potential. The other electrode was connected to the output of an operational amplifier in the same unit and used to pass the current across the cell membrane. The membrane current was measured at the virtual ground of another operational amplifier of the clamp unit.

Both current and voltage clamp methods were used to evaluate the response of the cells to bath-applied or pressure-ejected transmitters or drugs, or to intracellularly applied reagents. In the current clamp method, current pulses of 3–10 nA with an 800 ms duration were introduced into the cell every 5 s in order to measure the change in membrane resistance during the voltage response to a transmitter. In the voltage clamp method, voltage pulses of 3–6 mV with an 800 ms duration were applied every 5 s to the command input of the operational amplifier in order to evaluate the change in slope conductance of the membrane during the current response.

All numerical data described in the results represent the mean of data from at least 5 different cells, each with a standard deviation.

Chemicals. Chemical used were dopamine (DA), acetylcholine (ACh), histamine (HA), Phe-Met-Arg-Phe-NH<sub>2</sub> (FMRFamide), okadaic acid (OA), phorbol 12,13-dibutyrate (PDBu), a catalytic subunit of protein kinase C (PKC), PKC inhibitor peptide (19–31) (PKCl<sub>19–31</sub>), guanosine 5′-O-(3-thiotriophosphate) (GTPγS), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), and staurosporine. DA, ACh, HA, PDBu, and the catalytic subunit of PKC were purchased from Sigma Chemical Co. Ltd. PKCl<sub>19–31</sub> and H-7 were from Kanto Chemical Co. Ltd. FMRFamide, GTPγS, OA, and staurosporine were purchased from Peptide Institute, Boehringer Mannheim Co. Ltd., Seikagaku Kogyo Co. Ltd., and Kyowa Medex Co. Ltd., respectively.

ACh, DA, HA, and FMRFamide were dissolved in normal artificial Aplysia medium and applied to the neuron by a constant flow as described above. Transmitter-induced response was evaluated by the maximal (plateau) current flow produced at each concentration of the transmitter. The duration of transmitter application was adjusted so as to obtain just the beginning of the maximal plateau response; for example, 10 s at 1 mM and 20 s at 0.1 mM in the case of DA. After each application of a given transmitter, the cells were washed with normal Aplysia medium for at least 10–20 min before any subsequent examination. In analyzing the effects of reagents on the transmitter-induced responses, the test medium was perfused 4 min before the application of a given transmitter unless otherwise specified.
Suppression of K⁺-Current Responses

PDBu, OA, and staurosporine were dissolved in dimethylsulfoxide (DMSO) as a stock solution and diluted with either normal perfusing medium for extracellular application (PDBu and staurosporine) or a 200 mM KCl solution for intracellular injection (OA). H-7 was dissolved in distilled water and diluted with normal perfusing medium. Either PKC, PKCl, or GTPγS was dissolved in the 200 mM KCl solution and intracellularly applied into the cells.

Intracellular application of chemicals.
Each solution was loaded into one side of a double-barrel microelectrode and intracellularly injected into the cell by a 1–2 kg cm⁻² pressure pulse with a duration of 200 ms. Intracellular concentrations of the ejected chemicals were estimated as follows. We measured the size of a droplet ejected from the micropipette tip in response to a single pressure pulse before penetrating the cell membrane and compared it to the size of the cell examined. The intracellular concentration of each chemical was thus calculated from the volume ratio between the cell and droplets ejected.

RESULTS

DA-, FMRFamide-, and ACh-induced hyperpolarizing responses

In the current clamp experiments, the application of DA or FMRFamide to the cells of the RC group, and ACh or FMRFamide to the cells of the LUQ group often induced a marked hyperpolarizing response of 15–25 mV, causing suppression of spontaneous repetitive firing as shown in Fig. 1A. Voltage clamp experiments of these responses demonstrated an outward current associated with an increase in membrane conductance (Fig. 1B). This current response is entirely due to opening the specific K⁺-channel. The details of

Fig. 1. A: DA-, FMRFamide-, and ACh-induced hyperpolarizing responses of the neurons in the RC and LUQ clusters of the abdominal ganglion of Aplysia. The cells were current clamped. Periodic downward deflections in the voltage record (V) show the electrotonic potentials induced by constant inward current pulses (I) of 800 ms, indicating the changes in effective membrane resistance. The cells showed spontaneous firing at a resting potential of −50 mV. B: DA-, FMRFamide-, and ACh-induced outward current (I) responses recorded from the same cells under voltage clamp at −55 mV. Periodic downward deflections in the current record indicate the changes in slope conductance (see command voltage (V) on the top).

Fig. 2. Depressing effect of PDBu on the DA (A), ACh (B), FMRFamide (C), and HA (D)-induced K⁺-current responses recorded from different cells. Control responses before application of PDBu are on the left, while test responses after PDBu are on the right. The 60 μM PDBu was applied for 4 min then washed out for 5 min before testing the effect. The depressing effects of PDBu on these transmitter-induced responses remained 60 min after washing with the normal perfusing medium. The resting membranes of these cells were clamped at −55 mV.
this K⁺-channel have often been published elsewhere [14, 16–20].

**Effect of PDBu on the K⁺ current responses**

PDBu is a phorbol ester known to be a potent activator of protein kinase C, relatively soluble to the perfusing media and penetrable through the cytoplasmic membrane. All the responses to DA, ACh, FMRFamide, and HA were markedly depressed after a 4 min application of 60 μM of PDBu as shown in Fig. 2. The rate of depression depended on the concentration and application time of PDBu, but remained almost constant for approximately 1 h after application. The relative values of the test responses after a 4 min application of 60 μM PDBu were 21.5±4.0% (n=10) of the control for DA-, 19.1±4.0% (n=7) for ACh-, 18.3±6.0% (n=15) for FMRFamide-, and 19.4±4.0% (n=5) for HA-induced responses, respectively.

The effect of this PDBu on the resting membranes of these cells was insignificant inward current (1–2 nA).

**Effect of PKC inhibitors on the depressing action of PDBu**

We examined the effects of protein kinase C inhibitors such as H-7, staurosporine, and PKCl19–31 on the depressing action of PDBu on the K⁺-current responses elicited by DA, ACh, HA, and FMRFamide. H-7 (100 μM) and staurosporine (10 μM) were applied extracellularly for 2 h, whereas PKCl19–31 was injected intracellularly to make intracellular concentration to be 1 μM. The top row (A) in Fig. 3 shows the typical depressing effect of 60 μM PDBu on the 100 μM DA-induced response (same as shown in Fig. 2A). The second row (B) in Fig. 3 shows little depressing effect of 100 μM PDBu on the DA-induced response when examined on the cell pretreated with H-7. Similar results were observed with the K⁺-current responses elicited by ACh, HA, and FMRFamide using H-7, staurosporine (data not shown), or PKCl19–31 (Fig. 3C).

**Effect of okadaic acid on the depressing action of PDBu**

Okadaic acid (OA) is known as an inhibitor for the 1 and 2A types of protein phosphatase [21]. A concentration of PDBu (10 μM) was chosen to be just the threshold for depression of both ACh- and DA-receptor-induced responses, so little depressing effect is seen in the control experiments, as shown on the left in Fig. 4. Intracellular injection of OA itself with the dose used in this experiment produced slight depression for both the ACh- and DA-induced responses, but not as marked as seen after PDBu treatment (see the right columns in Fig. 4). Statistical values for PDBu-induced depression before and after injection of OA are shown in Table 1.

Similar results were obtained with HA- and FMRFamide-induced K⁺-current responses in different neurons (data not shown). These effects of OA strengthened our assumption that the depressing action of PDBu was due to the activation of protein kinase C and subsequent phosphorylation of a certain protein necessary for generation of the transmitter-induced responses.

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**Fig. 3. Effect of H-7 and PKCl19–31 on the PDBu-induced suppression of K⁺-current response to DA.** A: Control experiment with an untreated cell indicating the normal suppressing effect of 60 μM PDBu on DA-induced K⁺-current response. B: Test experiment using a cell treated with 100 μM H-7 for 2 h. C: Similar to B but examined in the cell injected with PKCl19–31. Note that PDBu did not significantly suppress the DA-induced response of the cells pretreated with H-7 or PKCl19–31. The intracellular concentration of PKCl19–31 was estimated to be 1 μM. The traces in the middle and right columns were obtained at 3 and 30 min, respectively, after PDBu application. Neither treatment with H-7 nor PKCl19–31 affected the resting membrane or the DA-induced response.
Effect of a catalytic subunit of PKC

In order to obtain further supporting evidence for our assumption, we examined the effect of a catalytic subunit of PKC purified from bovine heart. Intracellular injection of the catalytic subunit significantly depressed all G-protein-mediated K⁺-current responses to DA, ACh, and FMRFamide (Fig. 5). The amplitudes of the responses depressed by PKC of the same dose were 45.5±3.9% (n=5) of the control value in the DA, 43.4±5.0% (n=4) in the ACh, and 44.7±4.5% (n=4) in the FMRFamide responses, respectively. The recovery time course was very slow, showing a considerable depression for at least 30 min after PKC injection (see the right column in Fig. 5). Similar
depressing effects of the catalytic subunit of PKC were observed in the HA-induced response (data not shown).

Depressing effect of PDBu examined with the change in dose-response curves of the transmitter-induced responses

As described above, most of the receptor-induced K⁺-current responses were depressed by PDBu. In order to examine if PKC-activation could depress the affinities of certain receptors to the agonist, we investigated the dose-dependent effect of PDBu for each transmitter-induced response. Application of PDBu caused a downward shift of all the dose-response curves without a change in ED₅₀, a reciprocal parameter of the affinity of each receptor for the transmitter, as shown in Fig. 6. Similar results were also confirmed with the HA-induced K⁺-current response (data not shown). These results suggest that the acting site of PKC is not the transmitter binding site of each receptor.

No depressing effect of PDBu on the GTPγS-induced K⁺-current

Guanosine triphosphatase (GTPγS) is a non-hydrolysable GTP analog and known to cause irreversible activation of G-proteins. Ganglia of *Aplosia* include many neurons that have various postsynaptic receptors in their cytoplasmic membranes; activation of which produces specific K⁺-channel openings [14, 16–20]. Intracellular injection of GTPγS to these neurons produces a slow, but irreversible opening of the same K⁺-channel as that activated by each receptor stimulation [8, 9]. If the acting site of PKC to block the receptor-induced K⁺-channel opening is not the receptor or G-protein but the K⁺-channel itself, GTPγS-induced K⁺-channel opening should also be blocked by the activation of PKC. Thus, we examined the effect of PKC activation on the GTPγS-induced K⁺-current response after the receptor-induced K⁺-current response was irreversibly and markedly depressed by PKC activation.

Figure 7 shows an example of such an experiment with a cell in the LUQ cluster having both ACh and FMRFamide receptors. ACh- and FMRFamide-induced K⁺-current responses of this cell were markedly depressed by the application of PDBu as shown in Fig. 7A. It was to our surprise that the injection of GTPγS to this neuron produced a marked K⁺-current response with a gradual increase in membrane conductance. The control for this response should have taken before the application of PDBu, but it was impossible because of the irreversible nature of this response. However, we confirmed later, in different identical cells, that this response (32±5 nA, n=7), shown in Fig. 7B, was a typical, normal response (34±6 nA, n=9) to the same GTPγS.

Similar results were obtained with the cells having D₂ or H₃ receptors (data not shown).

**DISCUSSION**

Depressing effect of PDBu on receptor-induced responses possibly as the result of phosphorylation by PKC. This study demonstrated that the extracellular application of PDBu, an activator of PKC, significantly depressed the G-protein-mediated K⁺-current responses to ACh, DA, HA,
and FMRFamide. Similar depressing effects of PKC activators have been reported with the K⁺-current responses of GABAergic and 5HT receptors in hippocampal neurons [3, 4, 7], and Glut, muscarinic, and DA receptors in Planorbarius neurons [5, 6].

The depressing effects of PDBu on receptor-induced responses may be ascribed to the PKC-induced phosphorylation of a certain protein involved in the signal transduction pathway for K⁺-channel opening. The results described above seem to offer at least three supporting pieces of evidence for this assumption: First, OA, an inhibitor of type 1 or type 2A protein phosphatase, significantly facilitated the depressing effect of PDBu. Second, inhibitors of PKC such as H-7, staurosporine, and PKCl, markedly decreased the depressing effect of PDBu. Third, the intracellular injection of a PKC catalytic subunit significantly depressed the receptor-induced K⁺-current responses.

Possible site of phosphorylation by PKC. We reported previously that most of the receptors, activation of which produces the opening of K⁺-channel, are coupled to PTX-sensitive G-protein, Gₒ or Gα [8, 9]. Therefore, possible sites of phosphorylation by PKC are the receptor, G-protein, and/or K⁺-channel in order to explain the depressing effects.

It is unlikely that the agonist binding sites of entirely different receptor molecules such as M₁, D₂, H₂, or FMRFamide receptors are nonspecifically phosphorylated by PKC. In addition, the dose-response curves obtained from the D₂, M₂, and FMRFamide receptors showed no significant change in receptor affinities after the application of PDBu (see Fig. 6).

More possible sites of phosphorylation by PKC may be PTX-sensitive G-protein because this G-protein was commonly coupled to all receptors examined in this experiment. It has been known that the molecular dissociation of this G-protein into α and βγ subunits triggers the opening of K⁺-channel. If phosphorylation of this G-protein by PKC would impair the dissociation of these subunits, it should depress the subsequent K⁺-channel opening as well. Indeed, our results showed that K⁺-channel openings induced by the stimulation of different receptors were all depressed for at least 1 h after activation of PKC (see Figs. 3 and 7A).

In contrast, K⁺-channel opening induced by GTPγS, a direct activator of the G-protein, was not appreciably depressed even when the receptor-induced K⁺-channel opening of the same cell was markedly depressed, as shown in Fig. 7B. This finding seems to exclude the possibilities that the site of phosphorylation may be the K⁺-channel protein itself or the triggering site for K⁺-channel opening. These results strongly suggest that PKC might phosphorylate certain sites necessary for interaction between the receptors and G-protein and impairs the signal transduction from receptors to the G-protein.

Biochemical studies have suggested that Gᵢ or Gₒ can be phosphorylated by protein kinase C, resulting in the impairment of the functional coupling between receptors and G-protein. This has been shown in in vitro experiments using the purinergic receptor of the turkey erythrocyte membrane [22], somatostatin-receptor in lymphoma cells [23], and glucagon receptor in hepatocytes [24]. Impairment of the functional coupling between the receptor and G-protein by phospholipase C (PLC) in the vascular smooth muscle [25], α₂-receptor in human platelet [26–28], and chemotactic peptide receptor in human leukemia cells [29].
Physiological significance. The K⁺-current responses elicited by the stimulation of various receptors described in the results of this paper are all blocked by the application of PTX, as reported elsewhere previously [8, 9]. On the other hand, the K⁺-current response to GTPγS was not blocked at all by the same dose of PTX. Accordingly, we determined that PTX must have impaired the functional coupling between the receptor and G-protein but not the functional coupling between G-protein and K⁺-channel.

It should be noted that the blocking effects of PKC on the K⁺-current responses shown in the results of this paper were quite similar to those of PTX observed previously. The uncoupling effect of PTX is now known to be due to the ADP-ribosylation at the SH group of cystein residue in the α subunit of Gs or Go. Further biochemical studies are needed to determine the actual site of PKC phosphorylation in order to exert a similar uncoupling effect.

There are at least 16 different G-proteins (except small molecule G-proteins) identified so far in biological materials. Among them, the G-proteins of which activation can trigger the K⁺-channel opening are known to be Gs and Go in most cases. If the receptor of a neuron is coupled to Gs, receptor stimulation should activate PLC in the presence of cytoplasmic Ca²⁺, and produce IP₃ and DAG inside the cell. Since DAG is known to activate PKC, it might phosphorylate the coupling site between the receptor and Gs-protein, thus depressing the generation of K⁺-current response. This should be a typical model of negative feedback for K⁺-dependent postsynaptic responses. However, more physiological and biochemical studies in the mammalian CNS are needed in order to prove that this type of negative feedback system exists within a single neuron and physiologically regulates the synaptic transmission of the neuron in CNS.

We thank Mr. Housaku Ito and Mr. Kiyonori Ichikawa for constant supply of Aplysia. This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and Iwate Medical University Keiryoukai Foundation (No. 56).

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