

## Research report

PKC-dependent reduction of the acetylcholine-evoked inward Na current in *Aplysia* D-neurons: effect of injected PKC and PKC activatorsAndrás Papp<sup>\*</sup>, Jörg Hoyer<sup>1</sup>

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Abstract

The effect of elevated PKC activity on the membrane depolarization (D-response) evoked by extracellular ACh, applied on the soma of *Aplysia* neurons, was studied. Intracellularly injected PKC and certain PKC activators were used to elevate PKC activity. ACh-induced current was measured in voltage clamp. The neurons were treated extracellularly with the PKC activators: PDAc, SC-10, R-59949, (–)-ILV; or with purified PKC injected into the neuron through the recording electrode. PKC injection and treatment with any of the PKC activators caused a similar reduction of the ACh-induced inward Na current response (corresponding to D-response), while the non-activating  $\alpha$ -PDD had no effect. The results provide evidence that a PKC-dependent reduction of receptor responses also exists in this kind of *Aplysia* neurons. Furthermore, they show that the reduction of ACh response is indeed due to PKC activation (and not to a direct action of the phorbol ester).

**Keywords:** Protein kinase C; *Aplysia californica*; Acetylcholine; Acetylcholine receptor; Phorbol ester; Protein kinase C activator

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## 1. Introduction

Phosphorylation of receptors is an important means of regulation, where, among several others, the PKC-dependent phosphorylating system can be involved. Moran et al. [22] described, from *Xenopus* oocytes in which different receptors of mammalian neurons had been expressed, a reduction in ionic currents evoked by ligand activation of the metabotropic ACh and 5-HT as well as of the ionotropic GABA receptors. This effect was attributed to PKC-mediated phosphorylation; at the receptors or at a later link (e.g. the G-protein or its activation). The oscillatory response, produced by the endogenous muscarinic ACh receptors of *Xenopus* oocytes, was also desensitized by a PKC activator and, what is the more, sensitized by a PKC blocker [28]. PKC-dependent phosphorylation of the nicotinic ACh receptors in the *Torpedo* electric organ, and the role of this process in desensitization, was directly demonstrated by

Safran et al. [27]. Other reports, however, claim no [13], or an opposite [9,26] role of PKC in desensitization of cholinergic receptors.

Reaction of *Aplysia* visceral ganglion [7] neurons on ACh has been described by Tauc and Gerschenfeld [31]. These neurons show either a depolarization (the so called D-cells) or hyperpolarization (H-cells) on ACh application. A similar distribution of responses on ACh in neurons of the pleural ganglia of *Aplysia* was described by Kehoe [16]. Several types of ACh receptors: nicotinic D-type and H-type, and muscarinic H-type, have been found on the soma of different *Aplysia* neurons [10,16,30]. The presence and activity of PKC in *Aplysia* have also been demonstrated [5]. Recently, Sasaki et al. [29] described, in *Aplysia* abdominal ganglion neurons, a PKC- (and PKA-) dependent reduction of the K<sup>+</sup> current (i.e. an H-response) evoked through activation of G-protein-coupled ACh (and other) receptors.

The present study was based on some of our previous findings [17] in which PDAc, a PKC-activating phorbol ester, reduced the gross ACh response in various *Aplysia* neurons. Using PKC activators, a non-activating phorbol ester, and purified PKC; our aim was to prove that this effect, in *Aplysia* D-cells, was due to elevated PKC activity.

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## 2. Materials and methods

Isolated ganglia of *Aplysia californica* were obtained for the experiments. Identified neurons in the visceral ganglion [7] – first of all the R15 and RB group, having a nicotinic D-response – were preferentially used, but additional observations were also made of identified neuron groups in the pleural ganglia and unidentified neurons in the pedal ganglia (the latter also had a fast D-response). The ganglia were removed from the animal and the one to be used was fixed to the Sylgard bottom of the recording chamber by needles and continuously superfused with artificial sea water (ASW, composition (in mM): NaCl, 480; KCl, 10; CaCl<sub>2</sub>, 10; MgCl<sub>2</sub>, 20; MgSO<sub>4</sub>, 15; HEPES, 5. pH = 7.8; *T* = 16°C.)

The cells were impaled with a single microelectrode of 2–6 M $\Omega$  resistance, filled with 3 M KCl or with a special injecting solution (see Table 1B). An AXOCLAMP 2 device was used in all experiments. Responses to extracellular ACh injections (see below) were recorded in current or voltage-clamp mode, with cells kept mostly at –90 mV. (At this potential any ACh response appeared as an inward current but neurons were stable with no spike artifacts.) The response was sometimes recorded at different potentials to see its character and components. Beside ACh responses, action potentials (at unclamped membrane potential) were also recorded and visualized together with their first derivatives (dV/dt) by a Philips digital storage

scope. This record was used to monitor the state of the neuron. ACh responses were directly written out by a chart recorder and stored in a Nicolet digital storage scope with disk memory.

Activators of PKC were dissolved in the ASW and were applied through the perfusion. For full chemical names, concentrations and eventual dissolution procedure see Table 1A. ACh was extracellularly applied by pulse ejection of 1 M ACh-Cl solution from a pipette placed near (ca. 20  $\mu$ m) to the soma. The pulse length was individually adjusted for each neuron to get a clear response. The pulses were continually repeated at an interval of 3–4 min to keep desensitization moderate and to obtain uniform responses after 5–6 repetitions. PKC (purified from rat brain, dissolved as given in Table 1B) was intracellularly injected through the recording microelectrode. The solution was kept on ice and filled electrodes were used within 30 min. Intracellular injection and extracellular ACh application were done with ca. 200 kPa (~30 p.s.i.) pressure provided by a pulse-controlled injection device (Neuro-Phore, Medical Systems Corp., USA). Moderate injections (dyeing of the cytoplasm by Fast Green only around the electrode tip) yielded good effects, whereas stronger ones caused a general impairment of the neuron.

## 3. Results

In the first step of the experiments, we were going to confirm the effect of PDAc treatment [17] on the ACh response. Treatment of the neurons with extracellularly applied PDAc caused a consistent reduction of the ACh response in all neuron (and hence, at all receptor) types studied. The response always reacted rapidly to the presence of PDAc. In the case of the nicotinic D-type response of an R15 neuron (Fig. 1A) the peak response was, in the 4th min of PDAc application, already less than the control. The maximal reduction was reached in about 30 min (Fig. 1B). Maximal reductions of the ACh response (average values from various neurons having a D-response) induced by PDAc and other agents, are given in Table 2. In several types of neurons, the overall time course of the ACh response became slower. This could well be due to participation of more than one receptors, and hence, currents, in the response (see Discussion).

As it was known that phorbol esters can have effects which are independent of PKC activation, we compared the effect of PDAc with that of  $\alpha$ -PDD, a non-PKC-activating phorbol ester. As shown in Fig. 2A,  $\alpha$ -PDD had no effect on the ACh response. The result of this comparison supported the concept that the PKC-activating ability of PDAc was essential for the reduction of the ACh response.

To fully elucidate this question, we tried elevating the PKC activity level by injection of PKC preparation into the cell and examined the effect of some PKC activators

Table 1  
Solutions and substances

| Substance  | Amount          |
|--|-----------------|
| (A) PKC activators in extracellular application <sup>a</sup>   |                 |
| Phorbol-12,13-diacetate (PDAc, Sigma)  | 5 $\mu$ M       |
| $\alpha$ -Phorbol-12,13-dibutyrate ( $\alpha$ -PDD, Sigma)   | 5 $\mu$ M       |
| (–)-Indolactam-V, ((–)-ILV, Bachem)  | 3 $\mu$ M       |
| <i>N</i> - <i>n</i> -heptyl-5-chloro-1-naphthalenesulfonamide (SC-10, Calbiochem)  | 100–200 $\mu$ M |
| 3-[2-[4[bis(4-fluorophenyl)methylene]-1-piperidin-yl]ethyl]-2,3-dihydro-2-thioxo-4( <i>H</i> )-quinazolinone (R59-949, Calbiochem) | 10 $\mu$ M      |
| (B) Electrode filling for protein kinase C injection   |                 |
| Carrier solution: <sup>b</sup>   |                 |
| KCl  | 0.5 M           |
| Tris-Cl  | 20 mM           |
| Dithiothreitol   | 1 mM            |
| EDTA   | 0.5 mM          |
| EGTA   | 0.5 mM          |
| NaCl   | 10 mM           |
| Glycerol   | 20 w/v%         |
| Triton X-100   | 0.05 w/v%       |
| Fast green   | 0.2 w/v%        |

<sup>a</sup> PDAc and (–)-ILV could be dissolved in ASW. For SC-10 and R59-949, 0.5% dimethyl sulfoxide (DMSO) in the ASW and ultrasonic treatment was necessary.

<sup>b</sup> In 50  $\mu$ l of this solution, an amount of PKC was dissolved representing 0.4 U activity. PKC was obtained from Calbiochem or kindly provided by Dr. W. Schieble (Hoechst AG, Frankfurt/M.).

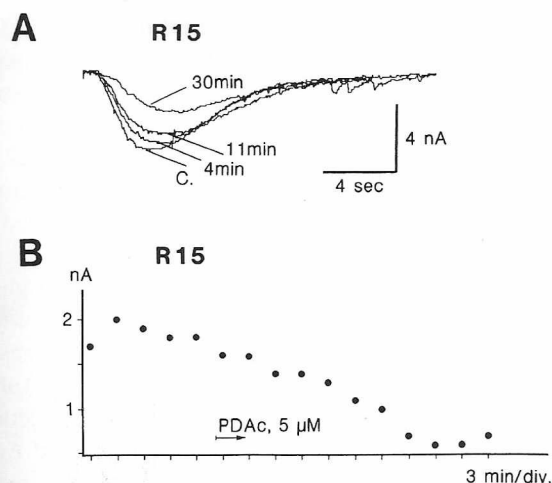


Fig. 1. Inward current responses evoked by extracellular acetylcholine pulses in identified *Aplysia* neurons. Effect of PDAc on the responses. Voltage-clamp records. A: treatment with the PKC activator PDAc (5  $\mu$ M, extracellular) caused a marked decrease of the nicotinic D-type ACh response in an R15 neuron.  $V_h = -90$  mV. Markings at the curves show control or the time elapsed under drug application. B: time course of the PDAc-induced reduction of the ACh response in another R15. Dots represent response peak amplitudes taken in 3-min intervals, and show the rapid onset and gradual development of the reduction on application of PDAc (marked).

chemically not related to phorbol esters. The PKC we used (see Materials and methods and Table 1B) was injected into the neuron by pressure through the recording electrode. The effect of the injection is shown in Fig. 2B. Similar to the effect of PDAc, the ACh-induced inward current was diminished. The clear, although moderate reduction developed, compared to that induced by PDAc, relatively fast; within 10 min. The intensity of the reduction was very variable which led to a greater standard deviation (see Table 2). This, however, was mainly due to the side effects of the injection and the broad-tipped electrodes needed to that and did not impair the significance of the effect. To get a better view of the side effects of the injection procedure itself, we injected several neurons with 'empty' carrier solution containing no PKC. As

Table 2  
Reduction of the ACh-evoked inward Na current response by injected PKC and by extracellularly applied PKC activators

| Treatment                       | Mean $\pm$ S.D.   | n  | P <     |
|---------------------------------|-------------------|----|---------|
| PKC injection                   | 73.18 $\pm$ 25.12 | 24 | 0.00005 |
| Carrier injection               | 95.93 $\pm$ 16.06 | 15 | n.s.    |
| PDAc 5 $\mu$ M                  | 65.24 $\pm$ 22.55 | 26 | 0.00005 |
| PDAc injection                  | 66.14 $\pm$ 28.98 | 22 | 0.00005 |
| $\alpha$ -PDD 5 $\mu$ M or inj. | 98.48 $\pm$ 26.01 | 23 | n.s.    |
| SC-10 100–200 $\mu$ M           | 66.63 $\pm$ 37.83 | 8  | 0.025   |
| R-59949 5 $\mu$ M               | 66.60 $\pm$ 25.28 | 5  | 0.01    |
| (-)-ILV 3 $\mu$ M               | 85.00 $\pm$ 34.40 | 16 | 0.01    |

The peak of the ACh response in control and treated state was measured. The mean  $\pm$  S.D. values give the average response after a given treatment in % of the control. Significance was tested with the one-tailed *t*-test.

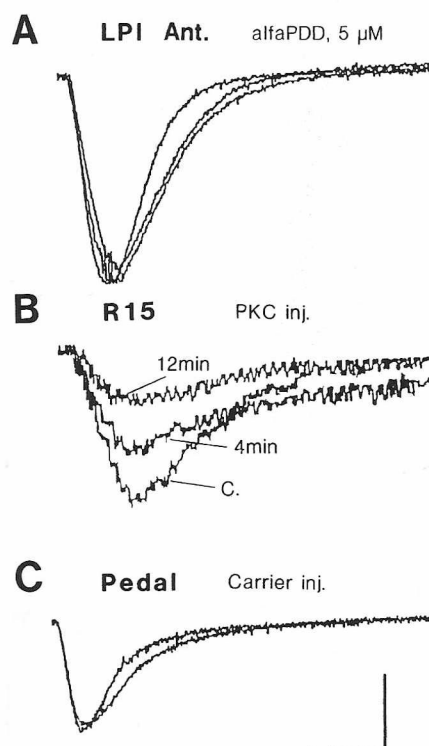


Fig. 2. Inward current responses evoked by ACh, recorded similarly as in Fig. 1. Reactions of the ACh response on treatments which do or do not increase PKC activity. Voltage-clamp records,  $V_h = -90$  mV. Markings at the curves show control or the time elapsed under agent application. A: treatment with the non-PKC-activating phorbol ester  $\alpha$ -PDD (5  $\mu$ M, extracellular; in an anterior neuron in the left pleural ganglion) caused no reduction in the ACh response. The response peaks do practically overlap in control and treated preparations. B: intracellular injection of PKC (into an R15 neuron) caused an ACh response reduction, the amount of which was comparable to the PDAc-evoked one (see Fig. 1A). C: a D-response neuron in the pedal ganglion, treated with 'empty' carrier solution (i.e. one with no PKC). No reduction of the ACh response was seen. Calibration: 4 nA, 10 s for A and C; 2 nA, 10 s for B.

seen in Fig. 2C, this treatment caused no reduction of the ACh response.

The non-phorbol PKC activators we used were: SC-10, (-)-ILV and R59-949 (see Table 1A). Compounds like SC-10 (chemically related to the well-known kinase blocker H-7 [8]) have been shown to activate PKC in vitro [25] and have been used in neurophysiological experiments [3,15]. As the substance is not water soluble, DMSO in ca. 0.5% concentration and ultrasonic treatment was necessary to dissolve it in ASW. As seen in Fig. 3A, the ACh response of an RB cell was reduced by SC-10. The response often had a time course change similar to that caused by PDAc, and the development of the SC-10-induced reduction was also slow, in some neurons it took even more time (up to 60 min) than the PDAc-induced reduction did. The other non-phorbol PKC activator, (-)-ILV, was first described by Basu [2]. It induced a reduction of the ACh response (Fig. 3B) which was, although less intense, significant (see Table 2). The third compound, codenamed R59-949 is, in fact, a diacylglycerol kinase inhibitor [4]. It is assumed that

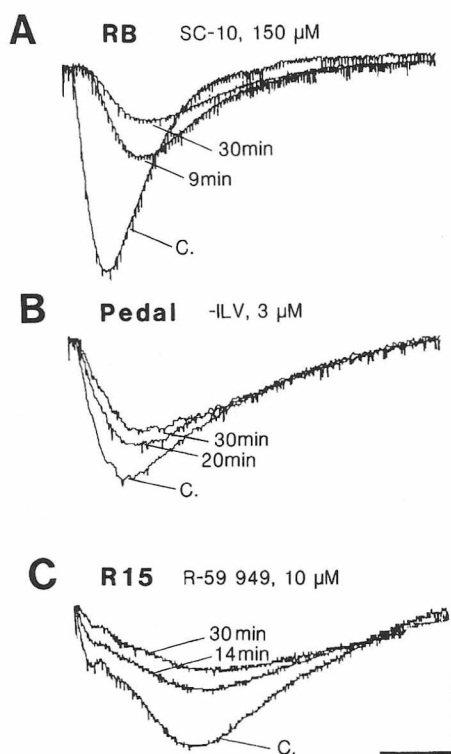


Fig. 3. Inward current responses evoked by ACh, taken similarly as in Fig. 1. Reaction of the ACh response on several PKC activators. Voltage-clamp records,  $V_h = -90$  mV. A: RB group neuron in the visceral ganglion. Treatment with the PKC activator SC-10 ( $150 \mu\text{M}$ , extracellular, with 0.5% DMSO) caused a slowly developing marked reduction of the ACh response. B: neuron in the pedal ganglion. Treatment with another activator  $(-)\text{-ILV}$  ( $3 \mu\text{M}$ , extracellular) also induced a reduction of the ACh response. C: neuron R-15. The substance R-59949, an indirect PKC activator (see text), was also able to reduce the ACh response. Calibration: 10 nA, 10 s for A; 4 nA, 4 s for B; 4 nA, 10 s for C.

the substance activates PKC indirectly, by raising the intracellular free (non-phosphorylated) diacylglycerol level [24]. This substance needed the same procedure to be dissolved as SC-10 did. Fig. 3C shows its effect on the ACh response of an R15 cell which was similar to those obtained with the other PKC activators.

#### 4. Discussion

The results above indicate a role of the PKC-mediated phosphorylation in the regulation of the nicotinic ACh receptors involved in the D-response of *Aplysia* neurons. As the effects of injected PKC on the one hand and of several, chemically different, PKC activators, on the other were identical, the role of PKC looks rather sure which is further supported by parallel findings on other preparations. In muscle, a connection between the influence of kinase activators – including activators of PKC – and enhanced desensitization of ACh receptor was described by Albuquerque et al. [1] and Eusebi et al. [6]. PKC-dependent phosphorylation of that receptor was demonstrated as well [21]. Nicotinic ACh receptors of the *Torpedo* electric

organ were also shown to be regulated by PKC- (and PKA-) dependent phosphorylation [27]. A similar mechanism was observed, for mammals, on  $\text{GABA}_A$  receptors [19,23] as well as on some G-protein coupled receptors like  $\alpha_2$ -adrenoceptors [11] and 5-HT receptors [14,22]. In *Aplysia*, the  $\text{K}^+$ -dependent H response, evoked through various G-protein-coupled receptors, was depressed by PKC (and PKA) activation [29].

A different reduction of early and late response phases after phorbol ester treatment, as we have seen, was also demonstrated on responses of ACh and 5-HT receptors expressed in *Xenopus* oocytes [14,22]. In various *Aplysia* neurons (e.g. the medial pleural ones [16], not detailed here) a multiple set of ACh receptors is activated by an ACh pulse [16,31]. It is therefore likely (also favored by some of our findings) that the time course alteration seen results from a different strong effect of the activated PKC on receptors responsible for the early and late phase of an ACh response. This, however, does not exclude a change in kinetics of the receptors themselves; firstly in neurons having a rather 'pure' response like the D-response of the R15 cell.

There is a similarity between the reduction of the ACh response of *Aplysia* neurons caused by PDAc (as described here and in [17]) and by 4-aminopyridine, described by Klee et al. [18]. The sensitivity of various ACh-evoked responses to the two agents are, however, not alike: in the pleural medial cell group, e.g. the slow (K-dependent) H-response, evoked via the muscarinic receptors, is the most sensitive to 4-AP, while it is less reduced on PKC activation than the faster (Cl-dependent) H-response of nicotinic receptors. Moreover, the effect of 4-AP is immediate. At this time, we have no data indicating how far the similarity reaches.

Phorbol esters are not free from side effects. Hence, a comparison with non-phorbol PKC activators was of interest. The correspondence of effects obtained using these activators and PDAc further supports the fact that these effects were indeed due to PKC activation. We were aware that the 0.5% DMSO needed to dissolve two of the agents was not necessarily without adverse effects. The similarity between effects of PKC activators used with and without DMSO indicate, however, that there were no negative side effects of DMSO in our experiments.

The facts that enhanced PKC activity leads to increased desensitization [12,28], and that activation of metabotropic receptors results in some later step in enhanced PKC activation [12,20] raise the possibility of a feedback, or cross-regulation. This underlines the importance of the phenomenon, the presence of which, in *Aplysia* neurons, we have here confirmed.

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