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PROTEIN KINASE C ACTIVATION IN *APLYSIA* NEURONS BY PHORBOL DIACETATE: COMPARISON OF EFFECTS FOLLOWING EXTRACELLULAR OR INTRACELLULAR APPLICATION

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The effects of protein kinase C activation on electrophysiological phenomena of neurons in *Aplysia californica* ganglions were studied. The enzyme was activated by phorbol-12,13-diacetate applied either extracellularly by perfusion, or by intracellular pressure injection. In both forms of application, an increase in the potential upstroke speed and amplitude as well as a reduction of the depolarization evoked by extracellular acetylcholine application was found. A protein kinase C blocker, H-7, had opposite effects on the action potential. All observed actions of the phorbol ester were consistently faster in intracellular application than extracellular.

Keywords: *Aplysia* neuron, protein kinase C, phorbol diacetate, H-7, action potential, acetylcholine response

Phosphorylating processes are of great importance in regulating nerve cell functions. One of the key enzymes, protein kinase C (PKC), has been detected in a wide variety of nerve cells and tissues including *Aplysia* neurons [8]. The activity level of PKC influences several electrophysiological phenomena of the neurons depending either on voltage-gated or agonist-gated ion channels.

Alterations of the action potential were observed in central and peripheral neurons of vertebrates and neurons of invertebrates. In *Aplysia*, DeRiemer et al. [9] found – working on the so-called bag cells – that the spike, being purely Ca-dependent in these cells, was increased in its amplitude by phorbol ester treatment or

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by intracellular PKC injection. This effect turned out to be a recruitment of a normally inactive Ca channel population [29]. In the visceral ganglion neurons of *Aplysia*, the spike is Na- and Ca-dependent to various proportions [17]. Previous studies on these neurons showed that the increase of spike amplitude and upstroke speed after treatment with a phorbol ester resulted mostly from increase of the fast Na^+ current, while Ca^{2+} currents showed no change or a slight decrease [18], with the exception of an increased L-type current in a group of burster neurons (a "dual action" similar to that seen in cultured mouse neurons [32]). Outward currents in visceral ganglion cells, including the Ca^{2+}-activated K^+ current, were reduced after PKC activation [27].

In vertebrate neurons, the activation of PKC caused either increase or decrease in various membrane currents. On one hand, an increase of both the fast Na^+ current and the Ca-activated K^+ current was found in cat spinal motoneurons [33]. Similarly, in neurons of the cat motor cortex, the spike overshoot and the fast afterhyperpolarization were increased while the slow afterhyperpolarization was decreased by intracellular phorbol ester injections [2]. On the other hand, an inhibition or reduction of currents was found in several mammalian preparations. Extracellularly applied phorbol esters abolished the spike afterhyperpolarization in rat hippocampal cells [24], and reduced the calcium currents both in chicken dorsal root ganglion cells [14, 25] and guinea pig hippocampal neurons [10]. In mouse neuroblastoma cells, a reduction of Na^+ and Ca^{2+} currents [20] was found and in cultured mouse neurons a reduction of Ca^{2+} and K^+ currents [32] was observed. The Na^+ current and the fast K^+ current through mammalian ion channels, which had been expressed in *Xenopus* oocytes, were inhibited by PKC activation or direct injection of the enzyme [7, 21].

PKC activity has important influence on the synaptic transmission, a process where agonist-gated channels have a central role. In smooth muscles from different mammalian organs, the transmitter action was either decreased or increased by PKC activation [1]. An increase of EPSPs and decrease of IPSPs by presumably postsynaptic action was found by Baranyi et al. [3] after injecting phorbol ester into pyramidal neurons. In the case of the nicotinic acetylcholine (ACh) receptor, phosphorylation by PKC and the resulting desensitization were directly demonstrated [26]. Receptors of this type have been described from *Aplysia* neurons by Kehoe [16]. In other preparations, the enhancement of EPSPs was presumably of presynaptic origin, for example at the neuromuscular junction [28] or on the RC-EPSP in neuron R-15 of *Aplysia* [19].

In this study, the effects of a PKC activating phorbol ester, phorbol-12,13-diacetate (PDAc), on *Aplysia* central neurons were investigated. Effects on the action potential and the response evoked by extracellular ACh injection were observed and compared in case of extracellular versus intracellular application of PDAc. A PKC

inhibitor, 1-[5-isoquinolinylsulfonyl]-methylpiperazine (H-7, [13]) was used to prove that the actions of PDAc are PKC-mediated.

Materials and Methods

Preparation

The experiments were carried out on neurons of isolated central ganglia of *Aplysia californica*. Identified neurons in the visceral ganglion [12] as well as unidentified ones in the pleural and pedal ganglia were used. Prepared single ganglions were fixed to the Sylgard bottom of the recording chamber by needles and were continuously perfused with artificial sea water (ASW). The composition of ASW was (in mM): NaCl, 480; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 20; $MgSO_4$, 15; HEPES, 5. The pH was set to 7.8; the chamber and inflowing ASW were kept at a constant temperature of 16 °C.

Recording

The cells were impaled with a single microelectrode of 2–6 MOhm resistance, which was filled with 3 M KCl or a special injecting solution (see below). An AXOCLAMP 2 device was used in all experiments. Action potentials were recorded in bridge mode, and responses to extracellular ACh injections in current or voltage clamp mode. Action potentials and their first derivatives (dV/dt) were visualized and plotted by a Philips digital storage scope. ACh responses were directly written out by a chart recorder and stored in a Nicolet digital scope with disk memory.

Substances and pressure injections

Organic substances were obtained from Sigma, inorganic salts from Merck. Phorbol diacetate (PDAc) was made up to a 5.6 mM stock solution in 10% dimethyl sulfoxide (DMSO) – 90% distilled water. This was added for perfusion to the ASW to give 2.5–10 μM final concentration (the final DMSO concentration was thus maximally 0.02%, i.e. only 1/15 of that necessary to induce direct DMSO effects, [10]). For intracellular injection normal recording electrodes were filled with a solution containing PDAc, 0.5 mM; KCl, 2 M; Fast Green dye, 2 mg/ml. H-7 was dissolved in distilled water and was applied solely by the perfusion in 5–50 μM concentration. Extracellular ACh application was performed by ejecting 1 M ACh-Cl solution from broad-tipped pipettes (5–10 μm). Injections were done with ca. 200 kPa (~30 psi) pressure yielded by a pulse-controlled injection device (Neuro-Phore, Medical Systems Corp., USA). Before penetrating a neuron, the recording electrode was set under steady pressure and was pushed against the connective tissue around the ganglion until a fine trace of filling was forced out through the tip. The electrode now had about 2 MOhm resistance and was suitable both for recording and injection. The dyeing of the cytoplasm by Fast Green was used to determine the appropriate amount of injection. Moderate injections with only a light green staining around the electrode tip gave good effects, stronger ones caused a general impairment of the neuron.

Results

Effects of PDAc on the action potential

Treatment with PDAc caused a change in several features of the action potential, and the effects were qualitatively the same in either form of application. When applying the drug by perfusion, a consistent increase of the spike upstroke speed was found on a total of about 100 neurons including all neuron types in the ganglions. Figure 1 demonstrates this increase, indicated by the first time derivative (dV/dt) as an increase of the positive peak (Fig. 1C and D vs. A). An increase of the spike amplitude and the negative dV/dt peaks, although usually present, was less characteristic. Intracellular application of PDAc by pressure injection exerted its effect also mainly on the positive dV/dt peak as shown in Fig. 2 (parts C and D vs. A). In a fully developed state, i.e. over 20 min for extracellular and at 10 min for intracellular application (see below), the positive dV/dt peak increased to 122.00 ± 14.57% of the control in extracellular and 121.88 ± 15.91% in intracellular application of PDAc (mean ± S.D.; calculated from 14 and 8 neurons, respectively). To prove the PKC-dependence of the above changes, we treated the neurons with the known and potent PKC inhibitor, H-7, in several experiments. The alterations seen

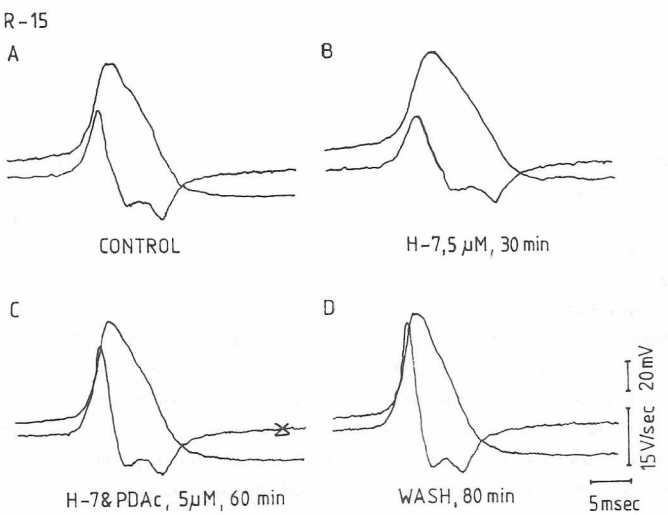

Fig. 1. Effects of PKC blocking and activation on parameters of the spike if PDAc is added to the ASW. Neuron R-15 in the visceral ganglion. Upper trace: action potential, lower trace: first time derivative (dV/dt). A: Control state. B: In presence of the PKC blocker H-7 both the first, positive peak of dV/dt and the spike amplitude are reduced. C: PDAc in the perfusion medium completely abolishes the effects of H-7. An increase of dV/dt and spike amplitude typical to PDAc action is seen. D: The action of PDAc is further increased by washing with ASW. The effects of H-7 could be washed out while those of PDAc were irreversible

under H-7 influence were opposite to those caused by PDAc both on the spike amplitude and speed (part B in Figs 1 and 2). They could, however, be reversed by subsequent PDAc administration, either extra- or intracellularly. The effects seen in the presence of both drugs were as described for PDAc above, but attenuated by the H-7 (part C in Figs 1 and 2). Washing out H-7 relieved the block and intensified the PDAc actions (part D in Figs 1 and 2). The changes of the spike had a characteristic difference in their time course for extracellular and intracellular application, as can be seen in column A of Figs 3 and 4. While the increase of dV/dt took always at least 25 min to develop when PDAc was added extracellularly, the effect of an intracellular PDAc injection was immediate and reached its peak in 10 minutes.

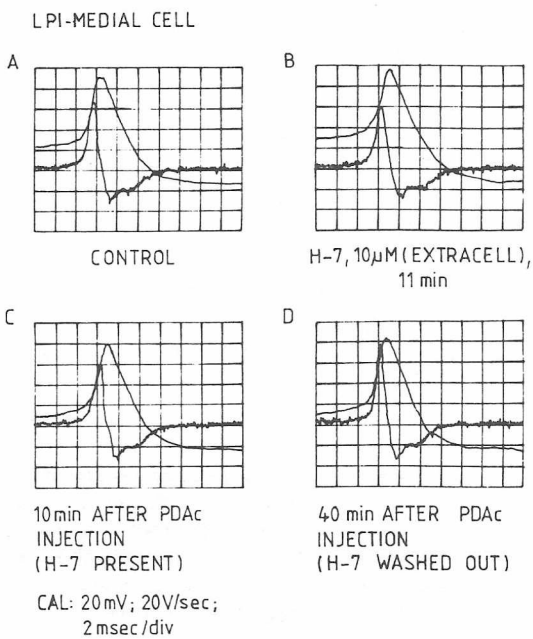

Fig. 2. Effects of PDAc blocking and activation in case of intracellular PDAc injection. Medial neuron in the left pleural ganglion. Same display as in Fig. 1. A: Control state. B: Treatment with H-7 decreases dV/dt and spike amplitude. C: After a moderate PDAc injection, the effects of PDAc were seen on the dV/dt peak and spike amplitude despite the presence of H-7. D: Washing out H-7 made the PDAc effects more pronounced

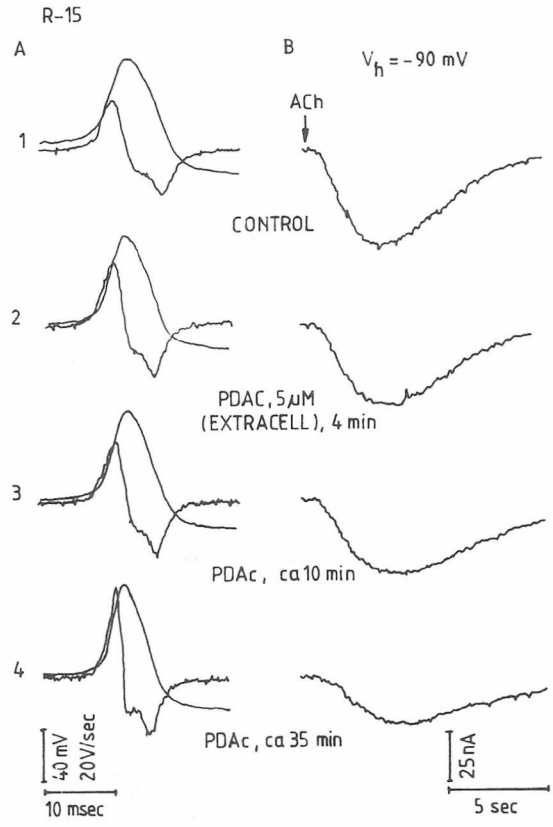

Fig. 3. Effect of extracellular PDAc on the ACh response and time course of PDAc effects. Neuron R-15. Column A: Spikes and dV/dt, meaning of traces like in Fig. 1. Column B: Inward currents corresponding to depolarizations evoked by 180 ms long ACh pulses recorded in voltage clamp (holding potential −90 mV). 1: Control state. In ASW containing 5 µM PDAc the spike shows alterations similar to those in Figs 1 and 2, and the ACh response is reduced. These changes could be seen already after 4 min (2) but reached full strength only after more than 30 min (3, 4)

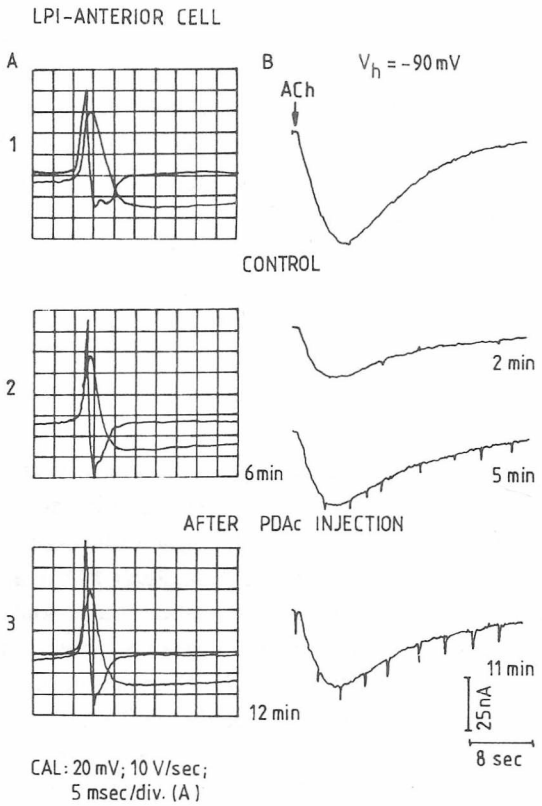

Fig. 4. Effect on the ACh response and time course of the effects in case of intracellular PDAc injection. Anterior cell in the left pleural ganglion. Column A: Spikes and dV/dt. Column B: Inward currents evoked by 2 s long ACh pulses recorded in voltage clamp (holding potential −90 mV). 1: Control state. Within 6 minutes after PDAc injection (2) the changes in the spike were just beginning while the reduction of the ACh response had already passed its maximum at 2 min. In the 12th min after injection (3), the increase of dV/dt was even stronger. At about the same time, the reduction of the ACh response was less strong but present and usually showed no further changes

Effects on responses evoked by ACh

PDAc had another characteristic influence on the depolarizations or hyperpolarizations elicited by extracellularly applied ACh (acting mostly on nicotinic receptors, [16]). In the present study, depolarizing responses recorded from certain neurons in the visceral ganglion, from the anterior neurons [16] in the pleural ganglion, or from neurons in the pedal ganglion were used. The ACh injections were repeated every 3 or 4 min and their size was set individually to achieve a clear and stable depolarizing wave. Treatment with PDAc resulted in a marked decrease in the ACh response [19]. On either extra- or intracellular application, a maximal decrease

to about 60% of the control (58.50 ± 22.87% in extracellular, 59.36 ± 32.76% in intracellular PDAc application – mean ± S.D. – on 14 neurons each) was found (column B in Figs 3 and 4). The time course of the change of ACh response was very different in the two modes of PDAc application, as seen on the spike. When giving PDAc extracellularly (Fig. 3), the decrease began quite early but needed at least ca. 30 min to reach its maximal value (Fig. 3 B2 to B4). However, when PDAc was injected intracellularly, the decrease was rapid and reached its maximum in 2–5 minutes (Fig. 4 B2). After that, the response showed a recovery but remained, with few exceptions, diminished (Fig. 4 B3, B4).

Discussion

As a result of the experiments presented, one can conclude that the effects induced by PDAc are almost identical for extra- and intracellular application except for their different time courses. The only major difference is seen in the effect of PDAc on the ACh D-response. While in extracellular PDAc application the response decreases continually, in intracellular application the initial reduction is maximal followed by a partial recovery. The data concerning spike alterations presented here show a similarity to those of DeRiemer et al. [9] made on bag cell neurons. Increased amplitude and upstroke speed can enhance the excitability of the soma, as described for mammalian neurons [2, 33] and for *Aplysia* [18]. PDAc, however, did not change spike afterhyperpolarization, as it does in mammalian neurons [2, 33]. In the bag cells, the main inward current is carried by Ca^{2+}, and the activity level of PKC influences the neuropeptide secretion of these cells. In the central ganglion neurons of *Aplysia*, the action potentials are at least partly Na-dependent [17]. The observed increase of the EPSPs after PDAc treatment [19] is similar to that found on the neuromuscular junction by Shapira et al. [28]. This effect can, despite of the differences between the cells, be likened to the enhanced secretion and might be the base of synaptic modifications. A PKC-dependent enhancement of excitatory postsynaptic potentials resulting from enhanced transmitter release has, in fact, been described in *Aplysia* by Fossier et al. [11] and in mammalian hippocampus by Malenka et al. [23]. The decrease of ACh-induced membrane potential changes after phorbol ester treatment looks, in relation to the above-mentioned increase of the postsynaptic potential, controversial. It should be stressed, however, that the reduction of the response evoked by ACh pulses directed on the soma reflects the activity of (extrasynaptic) receptors and is in no way connected to possible presynaptic changes. A similar reaction was observed on rat $GABA_B$ receptors which showed a PKC-mediated inhibition of the agonist response [31]. Likewise, from cerebellar Purkinje cells, a reduction of glutamate sensitivity by phorbol esters has been reported [6]. Although H-7 binds to the catalytic domain of PKC, and not to the

regulatory one, which is the site of action of phorbol esters [15], it is a rather effective blocker of phorbol ester actions. In extracellular application, H-7 reduces the slope of the action potential upstroke (Figs 1 and 2) and the peak amplitude of EPSPs in *Aplysia* neurons [19]. It also reduces the spike amplitude in *Aplysia* bag cells (in high concentration, [4]), blocks the potentiation of field potentials in the dentate gyrus [22] and blocks the IPSPs in rat CA1 neurons [5]. When applied intracellularly, H-7 causes a reduction of the spike amplitude but has only a weak effect on postsynaptic potentials [30].

The general similarity between the effects achieved by extracellular or intracellular PDAc application, on one hand, and their sensitivity to H-7, on the other hand, indicates that these effects are in fact mediated by PKC. Comparing an activating and a non-activating phorbol ester, Doerner et al. [10] used the same argumentation supplemented by the fact that effects of the non-activating substance were not antagonized by H-7. Using diacylglycerol (DAG) analogues, Hockberger et al. [14] found a decrease in Ca^{2+} current in chick embryonal neurons only for extracellular application. As the effect was abrupt and not antagonized by different PKC blockers, they concluded that this action of DAG was not PKC-mediated. In our experiments, the antagonism between PDAc and H-7 was asymmetric, i.e. the effect of H-7 was reversed by PDAc (Figs 1 and 2) whereas that of PDAc was only minimally influenced by H-7, as demonstrated also on the monosynaptic RC-EPSP in neuron R-15 of *Aplysia* [19]. Despite these differences, one can conclude that the effects of PDAc on cellular electrophysiological phenomena in *Aplysia* neurons described here are PKC-mediated.

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