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INCREASED PROTEIN KINASE C ACTIVITY IN THE CENTRAL NERVOUS SYSTEM OF THE NEWT DURING LIMB REGENERATION

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SUMMARY: Protein kinase C (PKC) activity was examined in the CNS of the newt *Pleurodeles waltlii* undergoing regeneration after limb amputation. In the spinal cord and brain of control newts, the level of PKC activity was virtually the same for the cytosolic and the particulate fractions. At days 7 and 14 after amputation of two limbs, a twofold increase in overall PKC activity occurred in the spinal cord and accounted for increased membrane-bound activity, while cytosolic activity was not significantly impaired. In contrast, overall PKC activity was not affected in brain. However, a twofold increase in the brain particulate fraction occurred at day 14 while cytosolic activity decreased proportionately. Similar alterations were observed in newts undergoing one or multiple limb amputations. Such changes in PKC activity neither occurred in the CNS of newt after limb denervation nor in the CNS of limb amputated frog *Rana temporaria*, an Amphibian which is unable to regenerate. Taken together, these results provide evidence that PKC of the CNS is involved in the regeneration process of newts. Changes in activation-associated PKC distribution proceeded through different mechanisms: long-lasting increase in membrane bound activity with a net increase of overall activity in the spinal cord, and long-term redistribution of enzyme activity to the particulate fraction in brain.

Protein kinase C (PKC) is a ubiquitous enzyme which plays a key role in signal transduction and thus in cell adjustment to the environment. Evidence has recently accumulated that phosphorylation of ion channels regulates the neuronal properties (1,2,3). PKC has been implicated in long-lasting changes underlying associative learning, enhancement of transmitter release and long term potentiation (4,5,6,7,8). A role for PKC in these functions has been supported by the mimicry of phorbol esters which

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specifically activate this protein kinase (3,5,6) as well as by enzyme redistribution from cytosol to membranes (7,8,9), which is thought to be associated with enzyme activation.

It is known that amphibians are unable to regenerate an amputated limb after its denervation, and that peripheral nerves release a growth factor which stimulates the proliferation of blastema cells (10). Conversely, limb blastema cells produce a neurotrophic factor during regeneration which leads to rapid restoration of peripheral nerves in the blastema (11).

We investigated the possibility that PKC of the CNS is involved in regeneration following limb amputation. We report here that long-term subcellular redistribution of PKC occurs in brain, while a net increase in the activity of spinal cord enzyme takes place in membranes.

EXPERIMENTAL PROCEDURES

Animals and chemicals : Forelimbs of 6-month to 1-year old *Pleurodeles waltlii* were amputated through the distal stylopod under anesthesia. PKC was assayed in spinal cord and brain of intact (day 0) or amputated newts at 7 days (early-bud stage), 14 days (mid-bud stage) and 24 days (2 digits stage) after surgery. Similar studies were done in parallel on newts to which anterior non amputated limbs were denervated by cutting spinal nerves 3, 4 and 5 at the brachial plexus level. Experiments were also performed on CNS of adult *Rana temporaria*, an Amphibian which is unable to regenerate after amputation. All chemicals were provided by Sigma (USA) except for DEAE cellulose and P81 paper, obtained from Whatman (USA), and phorbol ester 12-O tetradecanoyl phorbol 13- acetate (TPA) purchased from CCR Inc (USA).

Preparation of cell extracts: A large section of spinal cord and the whole brain of control or regenerating animals were rapidly cut off and homogenized in a glass-glass conical microgrinder in 1 ml of buffer A containing 20mM Tris-HCl (pH 7.4), 10mM EGTA, 2mM EDTA, and 250 mM sucrose, 2mM PMSF and 0.01% leupeptine. Homogenates were centrifuged for 1 h at 100,000 g and the supernatant was used as cysosolic fraction. The particulate pellet was suspended in 0.5 ml of buffer A containing 0.5% Triton-X100 (w/v), sonicated 30 s, kept in ice for 10 min and centrifuged at 100.000 g for 1 h. The detergent soluble fraction was referred to as the particulate fraction. To partially purify the enzyme and eliminate Triton-X100, the particulate and the cytosolic fractions were, in some experiments, passed through an anion exchange DEAE column equilibrated with 20mM Tris-HCl buffer (pH 7.4) containing 2mM EGTA, 2mM EDTA, 2mM PMSF and 10mM β-mercaptoethanol. The enzyme was eluted with a 0.0-0.3M NaCl gradient at 0.1M NaCl.

Protein kinase C assay : PKC was assayed as already described (12,13). The reaction mixture (125 µl) contained 2.5 µmol Tris-HCl buffer at pH 7.4, 5 µg phosphatidylserine, 62.5 nmol EGTA, 0.5 nmol magnesium acetate, 2.5 µmol (γ^{32}P)-ATP (approximately 3.10^5 cpm), 0.05 nmol TPA, 15 µg histone type H III-S. To start the reaction, an aliquot of crude preparations equivalent to 5 µg protein or 0.5 µg of partially purified enzyme were added. Incubation was carried out for 5 min at 30°C. The reaction was stopped by transferring a 50- µl aliquot onto squares of Whatman P81 paper which were soaked in 1% phosphoric acid and washed five times. Triton-X 100 did not inhibit particulate enzyme activity, as long as it was below 0.15 0/00 in the assay (data not shown). Enzyme activity, as the difference between ^{32}P incorporation into histone in the presence and in the absence of phosphatidylserine plus TPA, was expressed in µmol γ^{32}P-ATP transferred per

min. Specific activity was in µunits, with one unit of enzyme transferring 1 mol of phosphate per min per mg of protein in our assay conditions.

RESULTS

Spinal cord PKC activity was assayed in newts at days 0, 7, 14 and 24 after amputation of the two anterior limbs. As in mammals, the enzyme in *Pleurodeles waltlii* belonged to both particulate and cytosolic fractions, with an activity ratio (particulate over cytosolic) almost equal to 1 in intact animals. During the regenerative process, the overall (particulate + cytosolic) PKC activity increased up to twofold at day 14 before returning to control values at day 24 (Fig. 1). It should be pointed out that membrane-bound specific activity, which was about half the cytosolic activity in controls, increased twofold after amputation. In contrast, the level of cytosolic enzyme activity was not significantly impaired during regeneration (Table I). The effect of amputation of one or several limbs was examined at day 14 after surgery. Overall enzyme activity and membrane bound specific activity were increased in all the amputated animals, with major changes in PKC occurring in animals which regenerated two limbs (Fig.2). These results were obtained using crude enzyme. A similar rise in membrane-bound enzyme was also shown in experiments in which PKC was purified by DEAE-cellulose chromatography before being assayed.

PKC was concomitantly assayed in brain of regenerating newts. The levels of brain specific activity in both particulate and cytosolic fractions were much higher than those of spinal cord. In control brain,

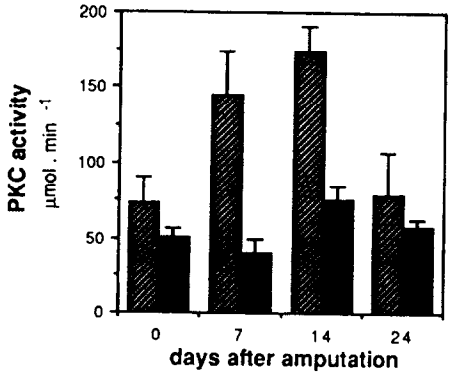

Fig. 1

Changes in PKC activity in spinal cord of newts during the process of limb regeneration. Animals were amputated of the two anterior limbs. Enzyme activity of particulate (▨) and cytosolic (■) fraction was expressed as µmol phosphate transferred per min. Data are mean value ± SE of 4 independent experiments performed on pooled spinal cords from 2 newts.

Table I . Subcellular distribution of spinal cord protein kinase C activity in regenerating newts

Days after amputation	Specific activity			
	Particulate fraction		Cytosolic fraction	
	µunits	% controls	µunits	% controls
0	100.4 ± 11.5	100	190.8 ± 21.2	100
7	197.4* ± 42.1	197	156.1 ± 36.1	82
14	227.0* ± 26.7	226	213.4 ± 21.8	112
24	122.2 ± 28.0	122	216.0 ± 39.3	114

Enzyme activity was expressed in µunits as defined in "Experimental procedures". Results are mean values ± SE of 4 independent experiments from pooled brains of two animals. * Statistically significant difference compared to controls using student's t test (* $p<0.05$).

overall PKC activity reached approximately 340 µunits, whereas in control spinal cord it was less than half that of brain (Table II). In contrast to what was observed in the spinal cord, overall brain activity was not significantly changed during the regenerative process following amputation of two limbs. However, limb regeneration impaired enzyme distribution in brain. Increased PKC activity occurred in the particulate fraction, which was associated with a proportionate decrease in the cytosolic fraction at day 14.

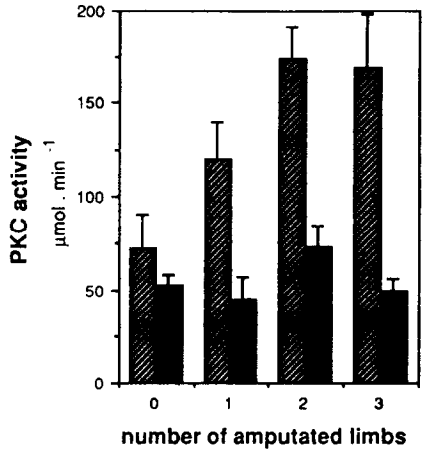

Fig. 2

Changes in PKC activity in spinal cord of newts following amputation of 1, 2 or 3 limbs. Results are mean values ± SE of 4 independent experiments performed 14 days after amputation on pooled spinal cords from 2 newts.
▨: particulate fraction, ■: cytosolic fraction.

Table II . Subcellular distribution of brain protein kinase C in regenerating newts

Days after amputation	Particulate fraction			Cytosolic fraction		
	PKC activity	Specific activity		PKC activity	Specific activity	
	µmol . min^{-1}	µunits	% controls	µmol . min^{-1}	µunits	% controls
0	297.7 ± 24.4	274.4 ± 14.4	100	257.4 ± 20.1	459.7 ± 35.3	100
7	201.5 ± 21.1	216.0 ± 21.1	79	328.3 ± 40.7	451.6 ± 66.5	98
14	305.8 ± 26.2	* 390.1 ± 16.2	142	* 98.1 ± 8.6	* 275.6 ± 19.2	60
24	259.7 ± 27.2	245.2 ± 18.2	90	215.2 ± 20.4	413.5 ± 35.4	90

Enzyme activity was expressed as defined in "Experimental procedures". Results are mean values ± SE of 4 independent experiments from pooled brains of two animals.
* Statistically significant difference compared to controls using student's t test (* $p<0.05$).

Changes in enzyme distribution were undetectable before day 14, indicating that PKC activity following amputation was altered later in brain than in spinal cord. Enzyme redistribution from cytosolic to particulate fraction was further confirmed as a function of the number of sectioned limbs. As shown in Fig.3, particulate activity increased, whereas cytosolic enzyme activity markedly decreased with the number of regenerating limbs.

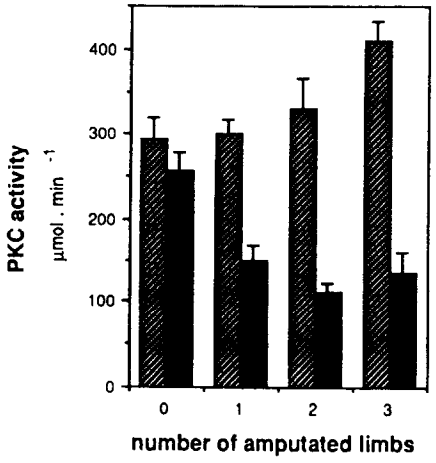

Fig. 3

Redistribution of brain PKC activity of newts following amputation of 1, 2 or 3 limbs. Results are mean values ± SE of 4 independent experiments performed 14 days after amputation on pooled brains from 2 newts.
▨: particulate fraction, ■: cytosolic fraction.

Table III . Subcellular distribution of protein kinase C activity in spinal cord and brain of amputated frogs

Days after amputation	Spinal cord				Brain			
	Particulate fraction		Cytosolic fraction		Particulate fraction		Cytosolic fraction	
	µunits	% controls	µunits	% controls	µunits	% controls	µunits	% controls
0	194.3±13.7	100	337.8±12.9	100	219.4±21.9	100	404.2±20.9	100
7	121.4±17.5	90	321.1±18.0	95	197.0±10.2	90	300.8±38.2	74
14	152.2±14.7	113	326.5±26.5	97	293.4±30.1	134	480.6±25.6	119
24	151.7±24.0	113	306.2±9.6	91	182.2±22.8	83	350.9±11.2	87

Enzyme activity was expressed in µunits as defined in "Experimental procedures". Results are mean values ± SE of 4 independent experiments from pooled brains of two animals.* Statistically significant difference compared to controls using student's t test (* $p<0.05$).

To further implicate the observed alterations of PKC in the regeneration process, we examined the effects of denervation of the two anterior limbs, without limb amputation, upon this enzyme activity in CNS . We showed that the ratio of particulate over cytosolic PKC activity did not significantly change in either brain or spinal cord of animals at days 7, 14 and 24 after denervation. However, PKC activity slightly increased in both particulate and cytosolic fractions in spinal cord at day 14 after denervation (data not shown).

Additional experiments were performed on *Rana temporaria,* an amphibian which is unable to regenerate after amputation. We sectioned the two anterior limbs of frogs, and enzyme activity was assayed in experiments designed like those performed in the newts. Overall spinal cord PKC activity in frog was slightly higher than in newt, whereas overall brain enzyme activity was lower in frog brain than in newt. However, it should be stressed that neither overall activity nor enzyme distribution significantly changed in CNS of amputated frogs following amputation (Table III).

DISCUSSION

Present results show increased membrane-bound PKC activity in CNS of the newt undergoing limb regeneration after amputation. These changes in levels of PKC activity were part of the process of limb regeneration, since denervation of non-amputated limb did not evoke similar alterations in enzyme activity. Furthermore, in the amputated frog *Rana temporaria,* which is unable to regenerate, such an increase in PKC activity did not occur.

There is general agreement that, upon signal binding to a specific cell surface receptor, PKC interacts with membranes presumably at the site of diacylglycerol formation. Therefore, increased membrane-bound PKC is associated with increased cellular PKC activation. Enzyme activity was almost equally distributated between cytosolic and membrane fractions in brain and spinal cord of non-regenerating newt. Following limb amputation, marked changes in enzyme distribution took place in both brain and in spinal cord which proceeded through different mechanisms. Typical long-term redistribution of the PKC from cytosolic to membrane fraction occurred in brain where the enzyme became predominantly associated with membranes without a net increase in the overall PKC activity. In contrast, long-lasting high levels of membrane-bound PKC activity, were associated with a net increase in overall enzyme activity in spinal cord. These two modes of activation-associated enzyme redistribution suggest that PKC activity is differently controlled in brain and in spinal cord during limb regeneration. In addition, changes in PKC distribution occurred later in brain than in spinal cord favoring a different implication of their enzymes in the regeneration process. It should be pointed out that in spinal cord, a drop in the cyclic AMP content correlates in time with the maximal PKC activity, suggesting an antagonistic control of both PKC- and cyclic AMP-mediated signalling pathways in spinal cord of regenerating newts (14).

In mammalian brain, at least six cDNAs encoding for PKC isoforms have been isolated (15). Three protein fractions, referred to as type I, II and III, were resolved by chromatography on hydroxylapatite columns (16). The distribution of these isoforms in nervous tissues is extremely heterogenous. While the predominent form of PKC in brain is type I, the type III form predominates in the spinal cord, which lacks the type I enzyme (16). At present, no information is available concerning PKC isoforms and their regional distribution in amphibian CNS. However, it has recently been shown that different PKC isoforms may be involved in *Xenopus* embryogenesis (17). It is thus attractive to suggest that the involvement of different isoforms, fulfilling specific neuronal functions in response to limb amputation and subsequent regenerative process, may account for the different modes of PKC activation in brain and spinal cord.

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