CHAPTER - VIII

ROLE OF CALCIUM AND CALMODULIN IN MODULATION OF TAIL REGENERATION IN THE LIZARD, *HEMIDACTYLUS FLAVIVIRIDIS*.

Calcium, one of the universal ionic messengers conveys signals received at the cell surface to inside of a cell and regulates a large number of cellular functions (Rasmussen, 1989). The cellular concentration of calcium ions is maintained by controlling the movement of calcium across plasma membrane. Calcium ions pass through 'calcium channels' which are transmembrane proteins that open in conformation with passive flux of Ca^{2+} ion across the plasma membrane, down the electrochemical gradient (Hess, 1990). The intracellular Ca^{2+} ion concentration is thus maintained in a very low level by this elegant mechanism. Besides regulating several metabolic processes and hormone secretion, calcium ions are important modulators of cell growth (Whitefield *et al.*, 1979; Metcalf *et al.*, 1986). Direct evidences implicate Ca^{2+} as a major regulator of cell proliferation and DNA synthesis (Brennan and Lichtman, 1973; Rasmussen, 1975; Whitefield *et al.*, 1980; Engstrom *et al.*, 1982) and an increase in the intracellular Ca^{2+} may be the primary intracellular division signal (Berridege, 1975; Nagle and Egrie, 1981). Current concepts speculate that calcium is required in the late G_1 phase of the cell division cycle for progression into S phase (Hazelton *et al.*, 1979; Metcalf *et al.*, 1986).

Numerous studies have shown that calcium exerts its regulatory functions through interaction with its high affinity calcium binding protein, calmodulin. Calmodulin acts as an intracellular intermediary for Ca^{2+} ions by modulating the calcium regulation of cyclic nucleotide metabolism, microtubule assembly, calcium flux, cell motility and cell division (review, Cheung, 1980; Means and Dedman, 1980; Means and Rasmussen, 1988).

The cellular functions of calcium and calmodulin can be altered by pharmacological agents that limit the availability of calcium to the cells or prevent calcium-calmodulin binding. The most widely used organic calcium channel blockers are Verapamil and Diltiazem. Calmodulin functions can be specifically blocked by various antipsychotic drugs. Phenothiazines-Trifluoperazine and Chlorpromazine inhibit several calmodulin-dependent processes. The mitogenic properties of Ca^{2+} can be ascertained by increasing the intracellular calcium pool with Ionophore A23187, while intracellular calcium pool can be depleted by Papaverine.

A few reports are available on the influence of calcium ions on the epimorphic regeneration. The importance of Ca^{2+} in urodele limb regeneration is well recognised (Vethamany-Globus *et al.*, 1978). In the newt limb regenerate, calcium influx promotes and efflux depresses the mitotic index (Globus *et al.*, 1983, 1987). The magnitude at which calcium ions can regulate the regenerative events in the tail regeneration of lizards is so far not understood. The present study has been designed to ascertain the effect of ionic intracellular modifications brought about through calcium channel blockers, calmodulin antagonists, intracellular calcium depletor and calcium influx promoter in preblastemic and blastemic stages of tail regeneration in the lizard, *Hemidactylus flaviviridis*. The net effects of various treatments were monitored by measurement of tail growth and histological evaluation of the regenerate.

MATERIAL AND METHODS

As many as 275 lizards of both sexes were obtained from local dealer and acclimated in the laboratory, at $30 \pm 2°C$, for a week prior to experiments. Lizards were autotomized leaving 3 segments intact from the vent and allowed to regenerate. The animals were fed with cockroaches and water was given daily.

Series-A: Fifty six animals which attained the WE stage on the same day were selected and divided into 7 groups. The control group comprised 10 animals while 6-8 animals were in each treatment group. The animals in treatment groups received drugs (ip.) in two equally divided doses daily for 4 days, 24 hrs apart. Calcium ionophore was prepared in 0.5% DMSO. All other drugs were prepared in 0.6% saline.
The drugs and dosages were as follows:
Group - II : Diltiazem (DTM), 60 mg/kg b wt
Group - III : Verapamil (VRL), 5 mg/kg b wt
Group - IV : Trifluoperazine (TFP), 100mg/kg b wt
Group - V : Chlorpromazine (CPZ), 100 mg/kg b wt
Group - VI : Papaverine (PAP), 100 mg/kg b wt
Group - VII : Calcium ionophore A23187 at 5×10^{-7} M.

The animals in Group - I received 0.6% saline and served as control to the above treatment groups. A separate control group was also maintained to group - VII in which

animals received 0.5% DMSO.

Series-B: Fifty six animals which attained the BL stage on the same day were selected, grouped and treated in the similar way as in *Series - A*.

In both series (A & B) the growth rate of tail was measured at 48 hrs and 96 hrs after the treatment. For the histological studies, the regenerate was cut along with one original segment, fixed in Bouin's fluid for 48 hrs and processed as described in chapter - I.

Data Analysis: The data were analysed statistically using one way analysis of variance followed by Duncan's multiple range test at 0.050 level.

RESULTS

Series - A (Treatment at WE stage): The results are presented in table-1 and fig.1. Treatment with calcium channel and calmodulin inhibitors significantly depressed the growth rate of the regenerate, both at 48 hrs and 96 hrs. However, injection of ionophore had no effect on the tail growth rate. Maximum inhibition of tail growth was observed in animals treated with papaverine.

Histological Observations: *(Control 48 hrs)*: By 48 hrs after the formation of wound epithelium a well-formed blastema – a mass of undifferentiated cells – were observed in the regenerating tail (fig.3). Within the mesenchyme, highly eosinophilic myogenic cells were observed. The proximal areas showed early signs of differentiation with the aggregation of promuscle cells. The entire distal area were occupied by undifferentiated cells.

Control 96 hrs: The signs of early differentiation were well-marked by the aggregation of promuscle cells and procartilage cells (fig.4). The myoblast cells were found differentiating at the proximal areas and fuse to form myotubes while procartilage cells aggregate together and ependymal tube elongates towards the tip of the regenerate. The distal areas comprised proliferating blastemal cells (fig.5).

Diltiazem: Diltiazem injection and thus calcium channel blockage at preblastemic level inhibited the process of cell proliferation and differentiation (fig.6). The differentiation of myoblast cells and chondroblasts were largely affected. However, the proximal areas

Table - 1. Length of tail regenerated in lizards after treatment with calcium channel antagonists, calmodulin inhibitors and calcium flux promoter and inhibitor at preblastemic (WE) stage. The growth of the tail regenerate was measured at 48 hrs and 96 hrs after treatment. The tail length (in mm) is represented as mean ± SD.

Treatment	Length of tail regenerate	
	48 hrs	96 hrs
Control	2.57 ± 0.57	5.65 ± 0.66
	*	*
Diltiazem	1.68 ± 0.37	3.56 ± 0.49
	NS	*
Verapamil	2.44 ± 0.42	4.50 ± 0.46
	*	*
Chloropromazine	1.63 ± 0.44	3.31 ± 0.65
	*	*
Trifluoperazine	1.94 ± 0.42	3.27 ± 0.70
	*	*
Papaverine	1.38 ± 0.58	3.43 ± 0.73
	NS	NS
Ionophore A23187	2.93 ± 0.17	5.50 ± 0.53

* $P < 0.050$; NS - Nonsignificant

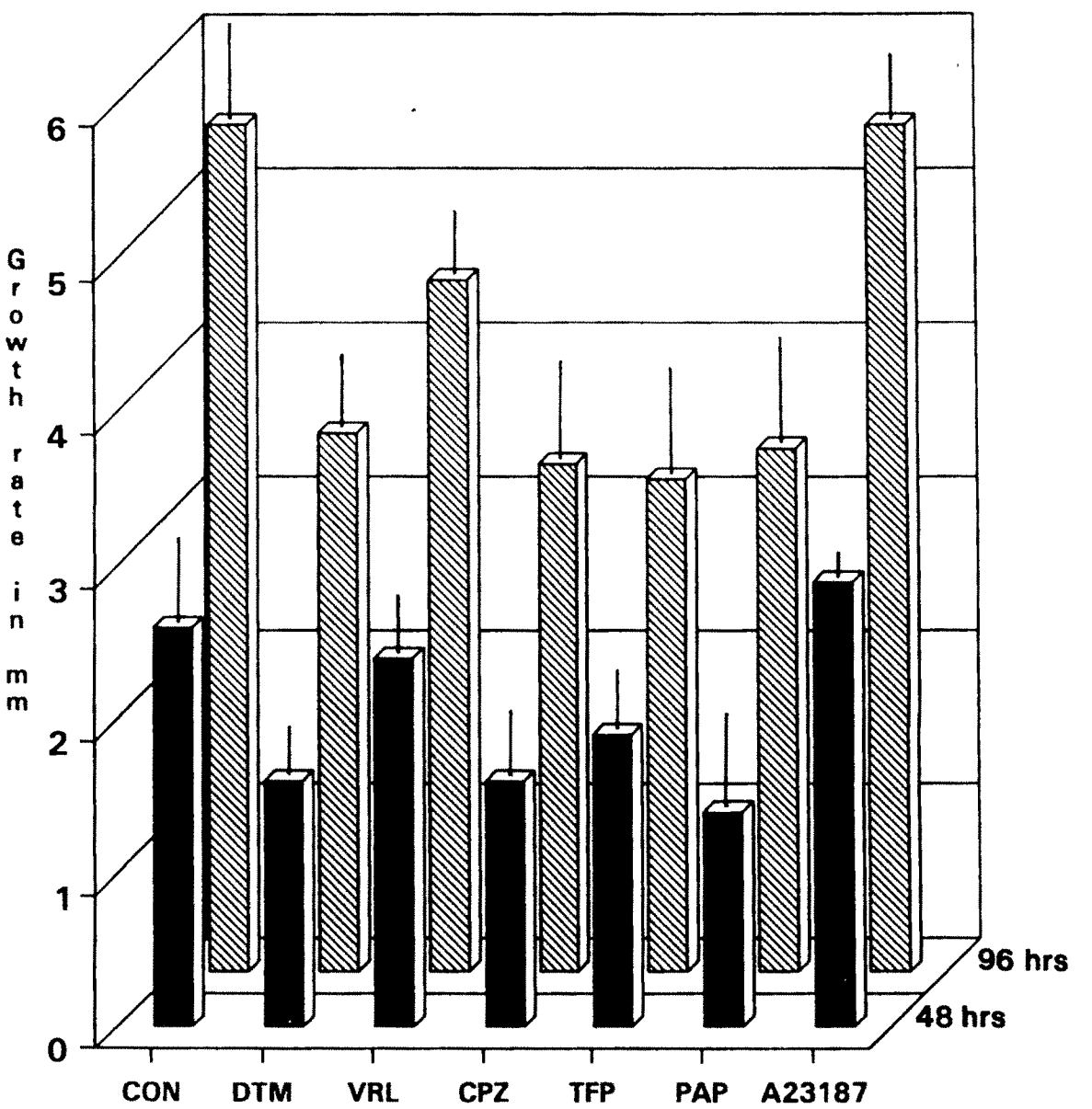


Fig.1. Length of tail regenerated in lizards treated with Ca^{++} channel antagonists, CaM inhibitors and Ca^{++} flux modifiers at WE stage. N-6-8 animals per group.

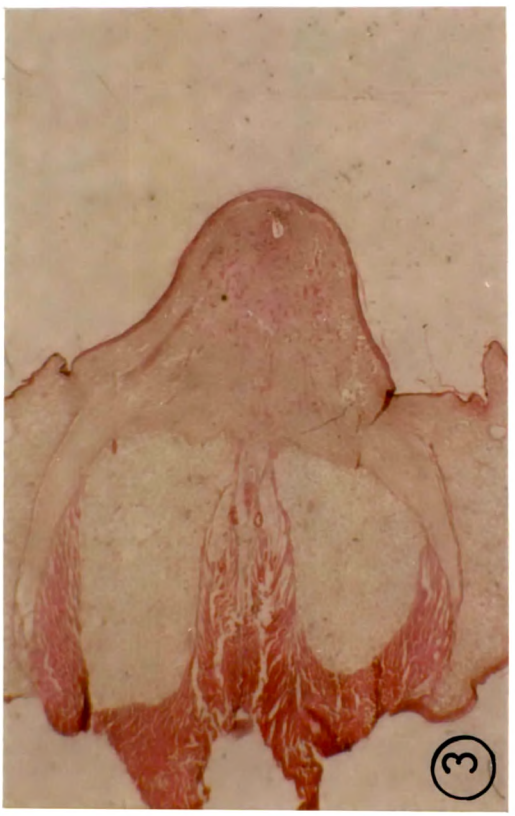
Explanation for Figures

Fig.3. Blastema stage(48 hrs after wound epithelium formation). X 11.5.

Fig.4. 96 hrs. The regenerate elongates and early-differentiation sets in. Chondrogenesis and myogenesis are evident. X 11.5.

Fig.5. Distal tip of the tail regenerate (96 hrs). X 124.

Fig.6. Calcium channel blockage with diltiazem in creased thickness of the apical epidermal cap. The outer epidermal layers are keratinized. (96 hrs). X 108.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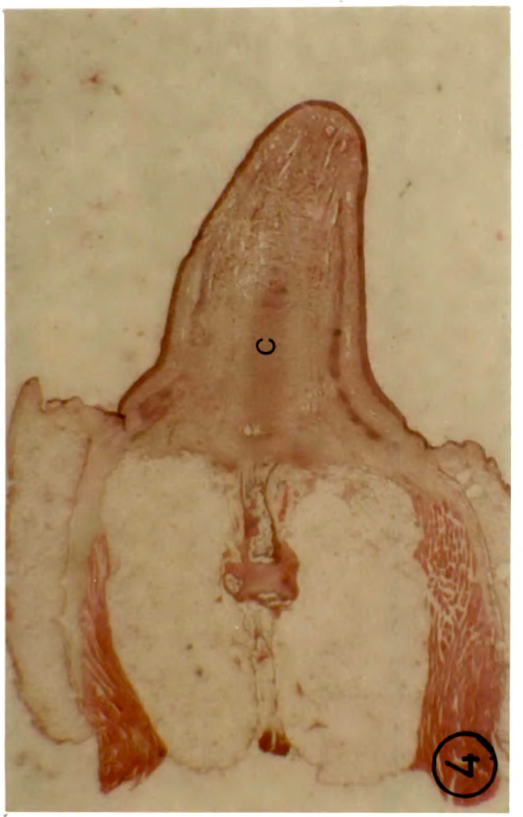
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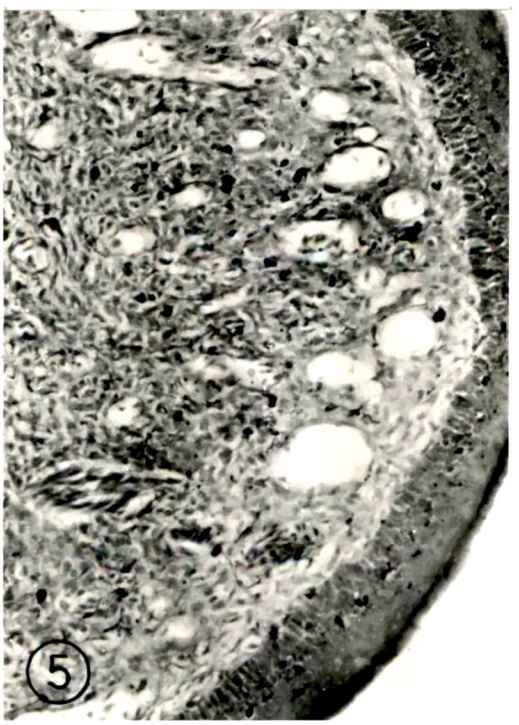
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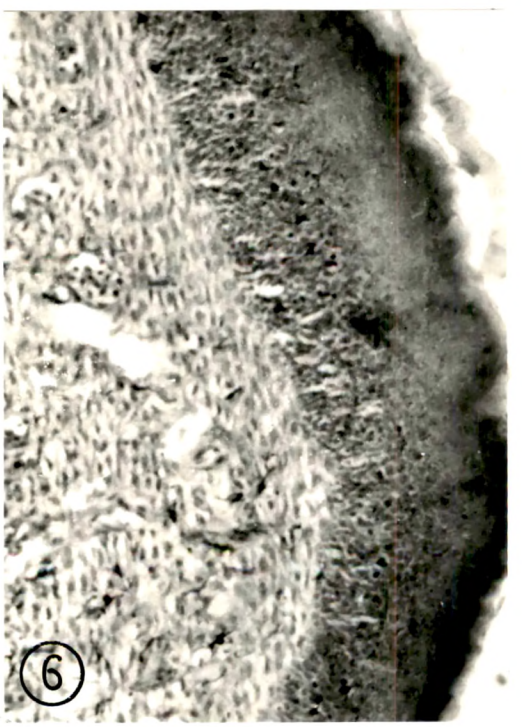
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Explanation for Figures

Figs.7-11. Calmodulin inhibition with trifluoperazine (TFP) at WE stage.

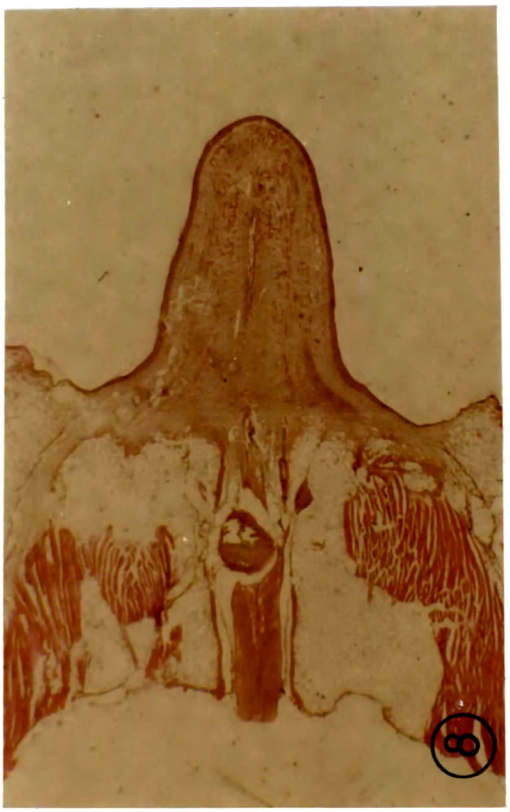
Fig.7. 48 hrs. A poor blastema is formed; cell proliferation and growth of the blastema are suppressed. Chondrogenesis is inhibited. Myogenesis is visible in proximal areas. X 11.5.

Fig.8. The process of differentiation is suppressed. Chondrogenesis is inhibited. Myogenesis is visible in proximal areas. X 11.5.

Fig.9. 96 hrs. The apical epidermal cap is abnormally thickened. The melanocytes are increased in number within the mesenchyme. X 187.

Fig.10. Mesenchyme showing the promuscles. Though the myoblasts differentiates, the cells are unable to fuse to form the myomeres. X 187.

Fig.11. The mesenchyme of TFP treated regenerate. No signs of differentiation. X 187.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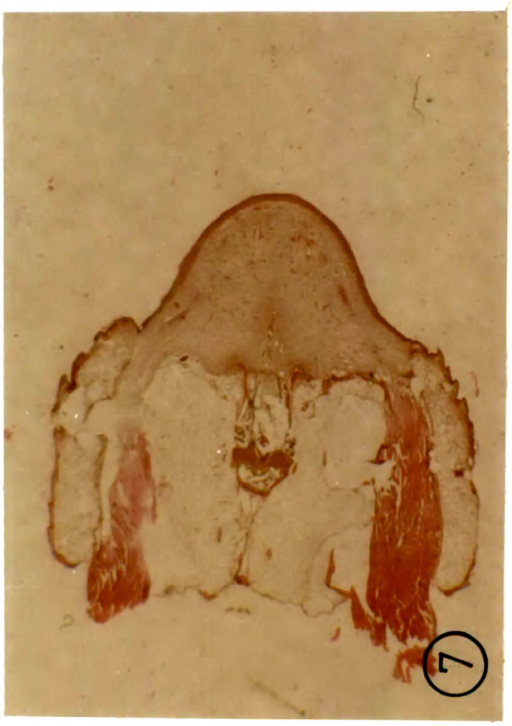
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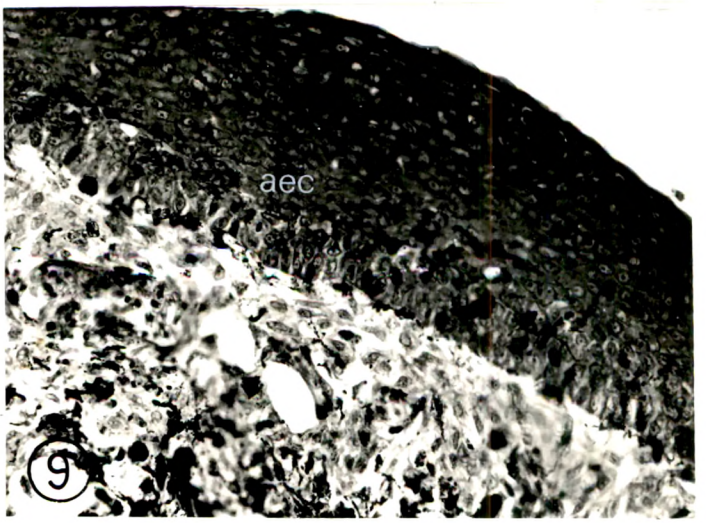
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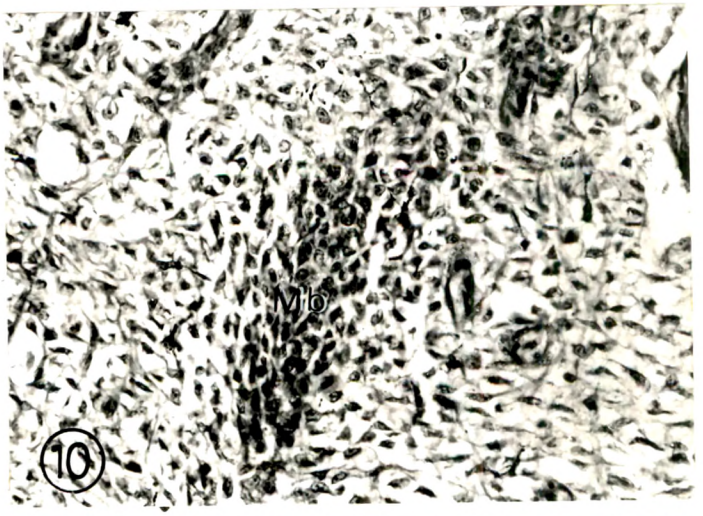
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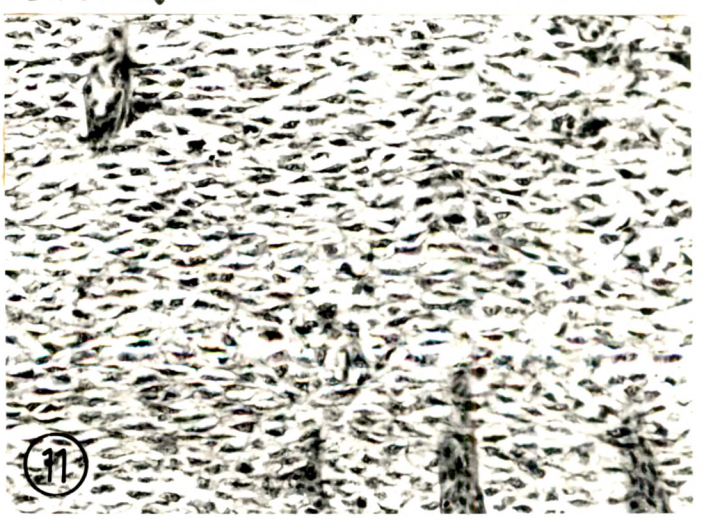
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Explanation for Figures

Figs. 12-15. Papaverine induced Ca^{2+} depletion (WE stage 48 hrs and 96 hrs).

Fig.12. 48 hrs. The apical epidermal cap is thickened; myogenesis and chondrogenesis are inhibited. X 11.5.

Fig.13. 96 hrs. No growth enhancement is evident. X 11.5.

Fig.14. 96 hrs. The epidermis is thickened. Collagen deposits are visible in the distal area. X 120.

Fig.15. Mesenchyme showing the promuscle aggregates. The myoblasts are unable to fuse to form the myomeres. X 187.


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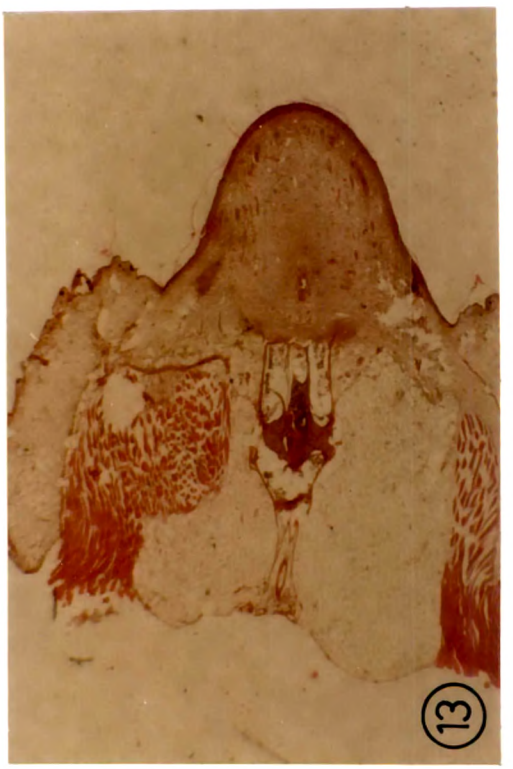
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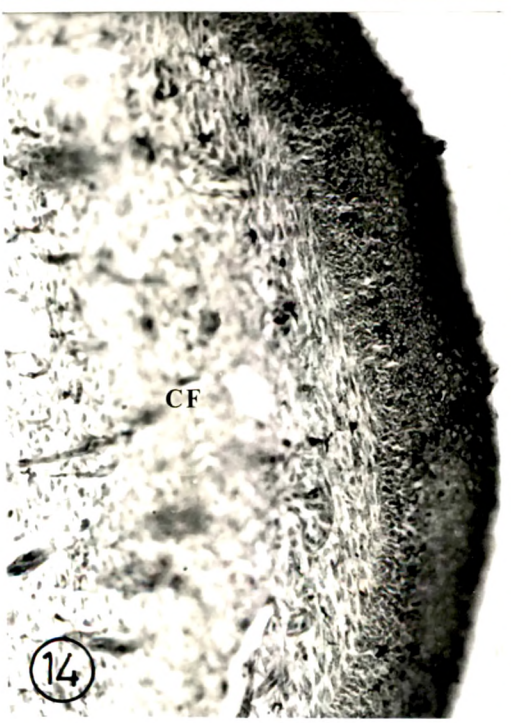
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Explanation for Figures

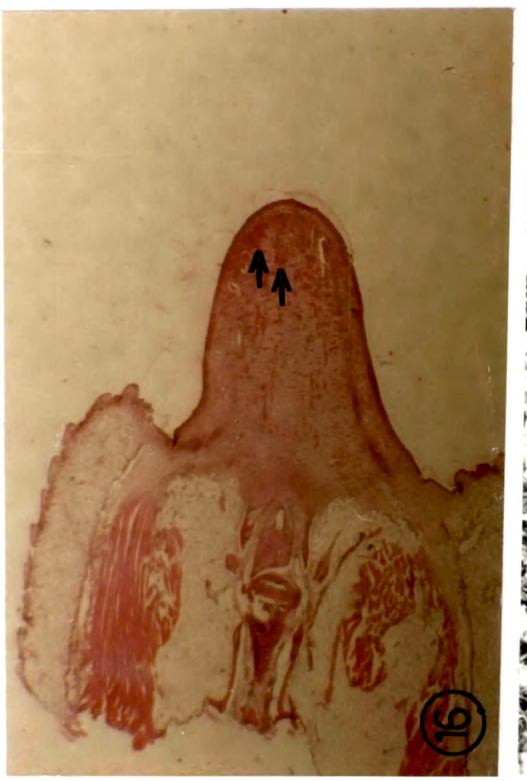
Figs. 16-19. Ionophore-induced changes in the tail regenerate(WE stage, 96 hrs).

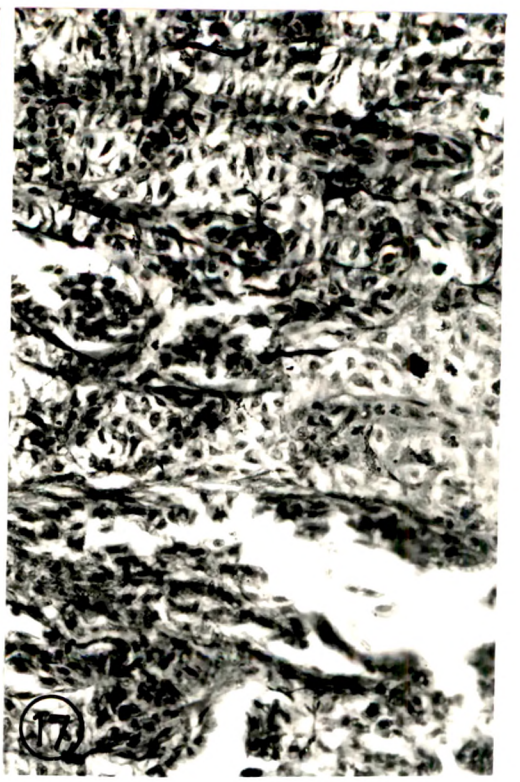
Fig. 16. Regenerate at 96 hrs. The proportion of myogenic cells are increased (arrows) towards the distal area. X 11.5.

Fig. 17. Mesenchyme showing the myoblast cells. The myoblasts are differentiating to form the pro muscle aggregates. X 187.

Fig. 18. The myogenic cells invading into the epidermis X 187.

Fig. 19. Precaucious differentiation of the epidermis induced by ionophore A23187. The epidermis invaginate to form the scale ridges. X 187.



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showed the aggregation of pro-muscle cells.

Verapamil: Verapamil injection had no adverse effect on the process of blastema formation or differentiation.

Chlorpromazine: Though CPZ treatment suppressed the blastema formation, by 96 hrs the signs of differentiation were evident.

Trifluoperazine: Calmodulin inhibition with TFP markedly affected the process of regeneration at all stages. Injection at preblastemic level did not inhibit the blastema formation, but cell proliferation and differentiation of the blastemal cells were greatly hampered (fig.7). Even at 96 hrs, no signs of differentiation were observed (fig.8). Myoblast differentiation and chondrogenesis were completely inhibited. Extensive collagen deposition was observed in distal areas of the regenerate. Though early signs of myoblast differentiation were evident, these cells were unable to fuse to form the myotubes (figs. 9-11).

Papaverine: Papaverine-induced depletion of intracellular Ca^{2+} level, totally inhibited the growth and differentiation of the regenerate. A poor blastema was observable by 48 hrs after treatment (fig.12). Myogenesis and chondrogenesis were adversely affected. Notable changes were observed within the epidermis of the regenerate. The apical areas of the epidermis showed thickening of the epithelium, probably these epidermal cells were originated from the distal mesenchymal cells. Promuscle aggregates were observed towards the distal part of the regenerate in late stages (figs. 13-15).

Ionophore A23187: Ca^{2+} influx with ionophore increased the proportion of myogenic cells within the regenerate (fig.16). These cells were in clusters within the regenerate (figs 17-19). The enhancement in the process of regeneration was evident by 96 hrs.

Series - B, (Treatment at blastemic stages): The results of tail growth rate are presented in table-2 and fig.2. Calcium blockade with verapamil had no significant effect on the growth of the blastema at any intervals. All other treatment groups significantly retarded the growth of the regenerate. At 96 hrs, the animals in the treatment groups of diltiazem, trifluoperazine, and papaverine showed significant retardation in the tail growth rate. In all other treatment groups, the growth in tail length reached almost comparable to that of control.

Table - 2. Length of tail regenerated in lizards after treatment with calcium channel blockers, calmodulin inhibitors and calcium flux modifiers in blastema (BL) stage. The growth was measured at 48 hrs and 96 hrs after treatment. The tail length is presented (in mm) as mean ± SD.

Treatment	Length of tail regenerate	
	48 hrs	96 hrs
Control	5.15 ± 1.05	7.30 ± 1.13
	*	*
Diltiazem	3.18 ± 0.45	5.62 ± 0.69
	NS	NS
Verapamil	4.94 ± 0.77	7.65 ± 0.82
	*	NS
Chloropromazine	3.64 ± 0.49	6.25 ± 0.75
	*	*
Trifluoperazine	2.75 ± 0.26	4.25 ± 0.70
	*	*
Papaverine	2.88 ± 0.52	4.94 ± 0.73
	*	NS
Ionophore A23187	4.00 ± 0.54	6.75 ± 0.70

* $P < 0.050$; NS - Nonsignificant

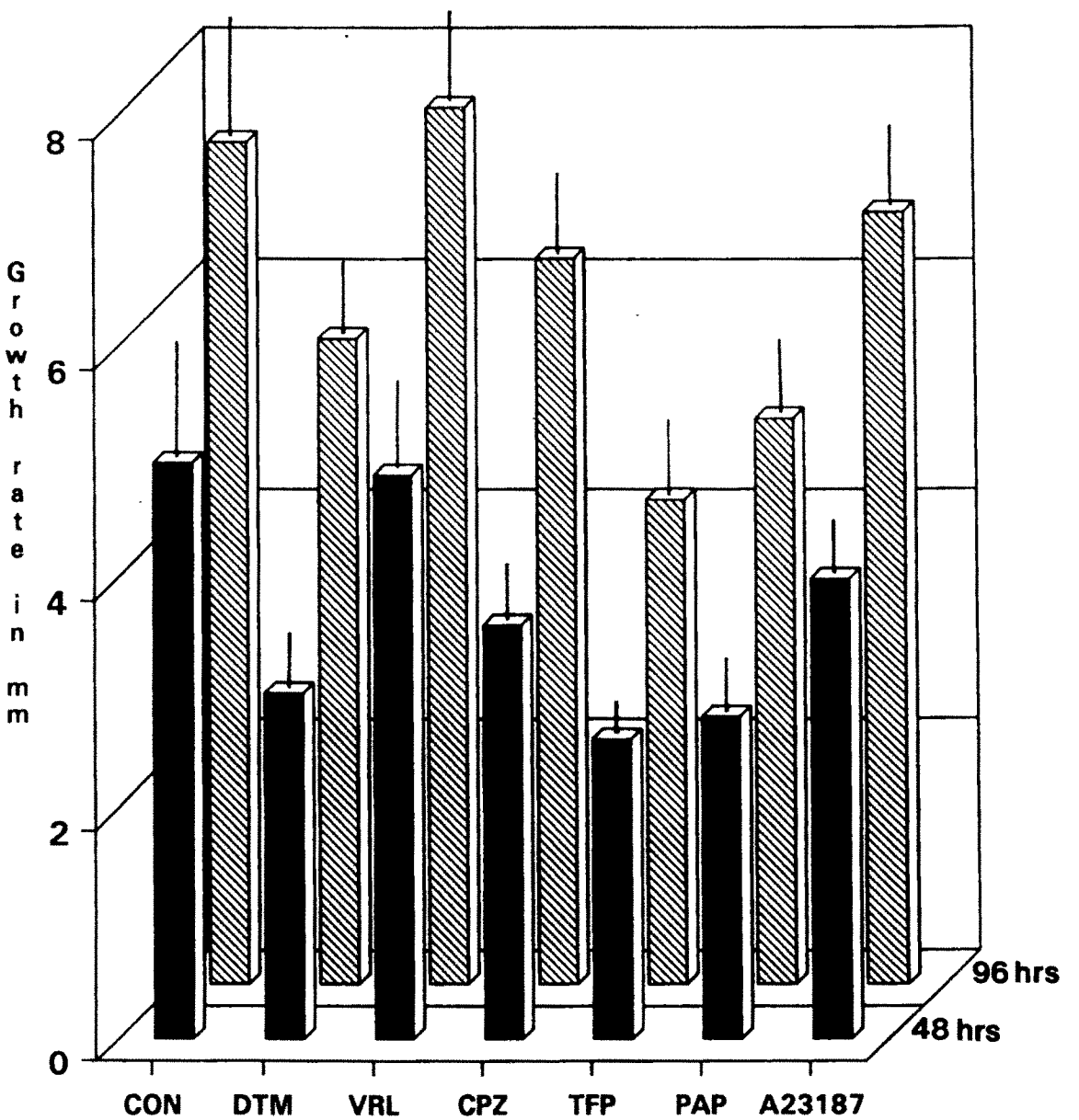


Fig.2. Length of tail regenerated in lizards treated with Ca^{++} channel blockers, CaM inhibitors and Ca^{++} flux modifiers at BL stage. N-6-8 animals per group.

Histological observations. *Control 48 hrs*: The process of differentiation was already set in and proximal areas showed differentiation where the myomeres were found arranged; condrogenesis was initiated.

Control 96 hrs: This stage demarcated the early differentiating stages with rapid elongation in the tail regenerate (fig.20). The process of differentiation was extending proximo-distally at a faster rate. Cartilage differentiation and myogenesis were also seen as proceeding at a fast rate. Myomers were visible in the proximal areas. The arrangement of dermal layers was observable. Only the tail tip contained proliferating cells.

Diltiazem: Though the growth rate decreased in DTM-treated lizards, the proximal areas showed the signs of differentiation with prominent muscle aggregates. The process of chondrogenesis considerably affected along with the inhibition of myogenesis in distal areas. Myoblast fusion and myotube formation were found to be inhibited in the blastema of DTM-treated lizards (figs. 21-23).

Verapamil: Calcium channel blockade with VRL had no apparent effect on the process of differentiation in BL stage.

Chlorpromazine: Chlorpromazine treatment had no inhibitory effects on the process of differentiation of the regenerate.

Trifluoperazine: Inhibition of calmodulin in BL stage depressed the growth rate and differentiation of the blastema (figs. 24,25). Promuscle aggregates could be seen proximo-distally. The epidermis showed thickening and hyperplasia in the apical areas. A high rate of cell prolilferation and increase in the number of epidermal layers were observable (figs. 26,27). The chondrogenesis was also found to be inhibited.

Papaverine: A poor growth rate was observable in the papaverine treated blastema (figs. 28,29). No signs of differentiation was evident. The processes of myogenesis and chondrogenesis were totally inhibited in the regenerate. In contrast to this, the epidermal cells showed high rate of cell prolilferation and abnormal thickening in the distal areas. The apical epidermis showed invagination into the mesenchyme. These highly proliferating cells were immediately differentiated, resulting in epidermal hyperplasia (figs. 30,31). Though myoblast cells could be seen, these cells were unable to fuse to form the

Explanation for Figures

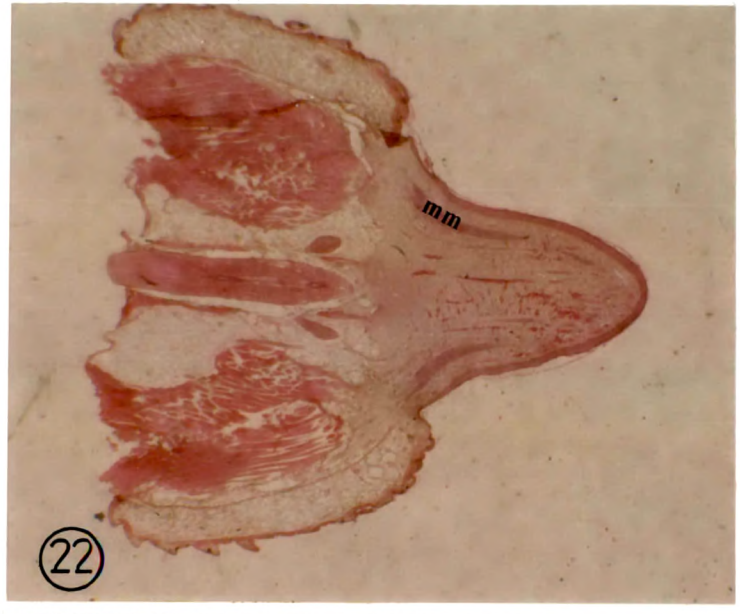
Fig.20. Control-96 hrs(from BL stage). Early-differentiation. The proximal area comprises proliferating cells. X 9.

Fig.21-23 Calcium channel blockage with diltiazem in BL stage.

Fig.21. 48 hrs. The apical epidermis is thickened. The process of differentiation is inhibited. X 11.5.

Fig.22. 96 hrs. Growth of the regenerate is totally inhibited. In proximal areas myomeres are present, chondrogenesis is inhibited. X 11.5.

Fig.23. 96 hrs. Tip of the regenerate. the number of melanocytes are increased. Apical area shows collagen deposition. X 120.



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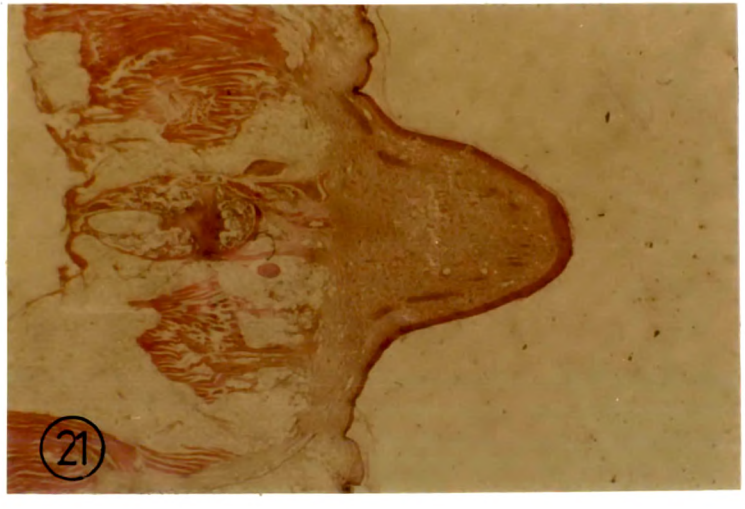

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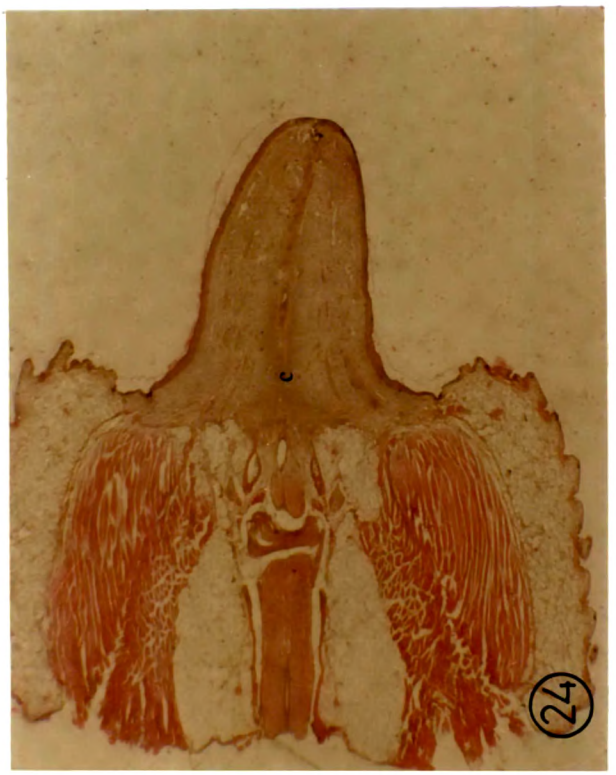
Explanation for Figures

Figs.24-27.	Calmodulin inhibition by trifluoperazine(TFP) at BL stage.
Fig.24.	48 hrs. No sign of differentiation is evident. X 11.5.
Fig.25.	The distal tip of the regenerate showing collagen deposition and increase in the number of melanocytes. X 120.
Fig.26.	96 hrs. The growth of the regenerate is suppressed. Promuscle aggregates are visible, epidermis is highly thickened. X 11.5.
Fig.27.	The AEC showing hyperplasia, outer layers are keratinized. X 120.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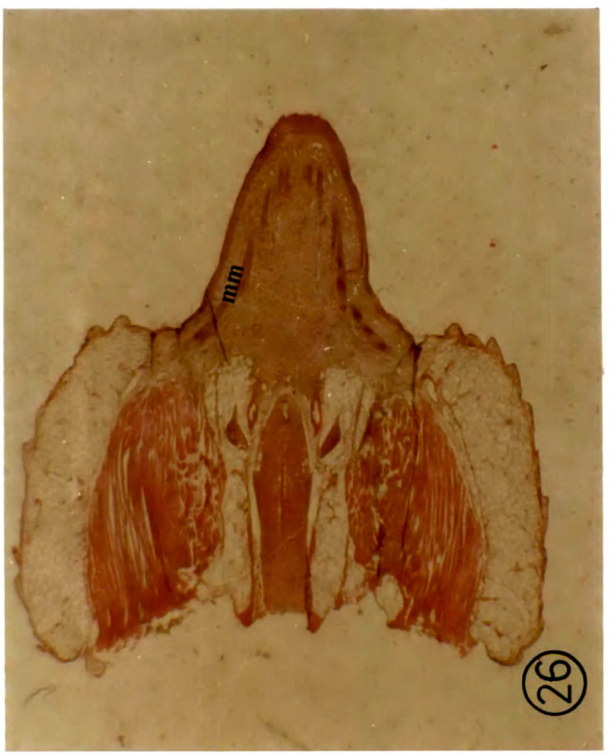
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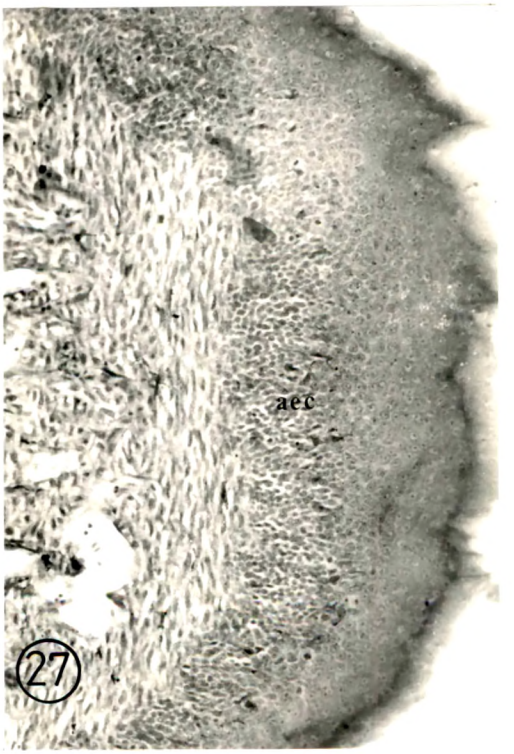
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Explanation for Figures

Figs.28-33. Papaverine-induced Ca^{2+} efflux and its effect on the tail regenerate (BL stage, 48 hrs and 96 hrs).

Fig.28. 48 hrs. Apical epidermal cap shows hyper-proliferation and invade into the mesenchyme. X 11.5.

Fig.29. 96 hrs. The process of differentiation is to tally inhibited. The epidermis shows hyperplasia. promuscle aggregates are visible in the proximal area. X 11.5.

Fig.30. 48 hrs. Hyperplasia of the apical epidermal cap X 120.

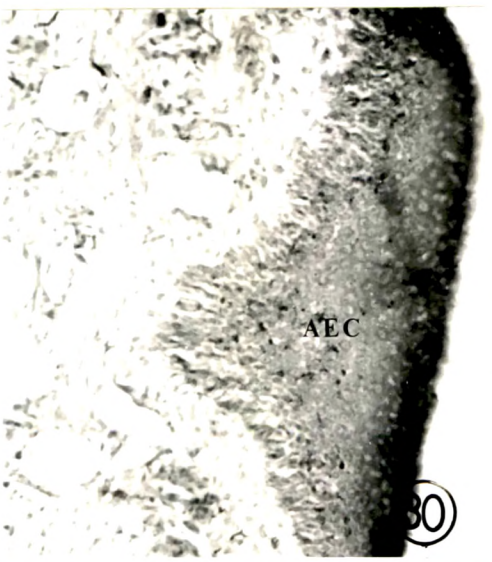
Fig.31. 96 hrs. Epidermal layers are increased. X 120.

Fig.32. Myoblast cells within the mesenchyme, the cells are unable to fuse to form the myomers. X 187.

Fig.33. Mesenchymal cells in the distal area of the regenerate. X 187.

Abbreviations

aec - apical epidermal cap
c - cartilage tube
cf - collagen fibres
e - ependyma
mb - myoblasts
me - melanocytes
mm - myomeres
pm - promuscles
sp - spinal ganglia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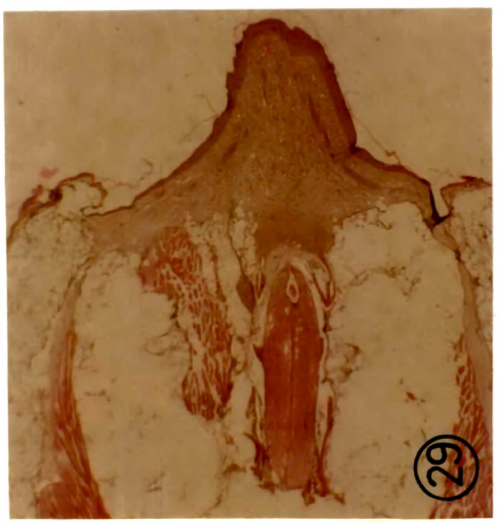
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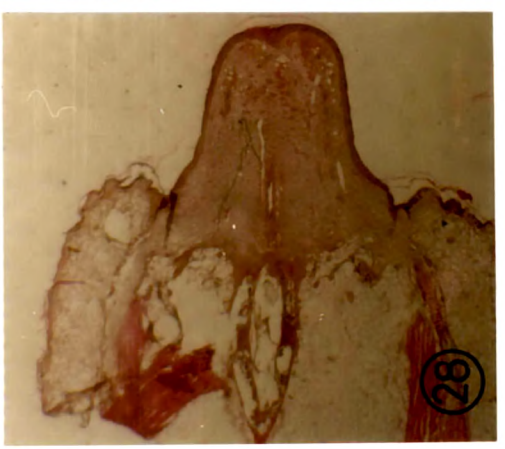
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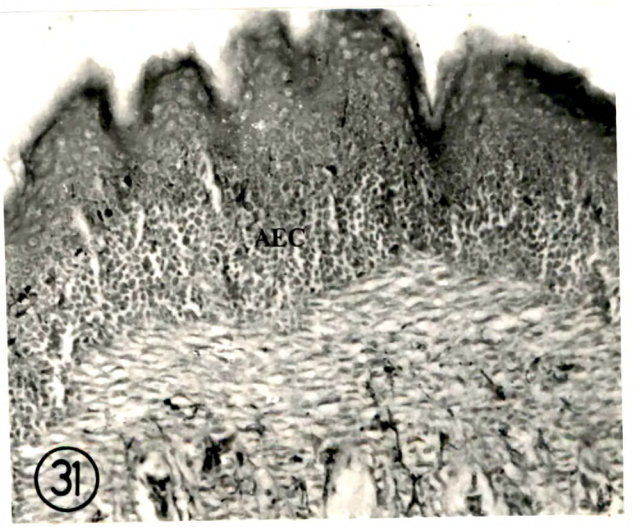
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myotubes. Promuscle cells showed degeneration (figs. 32,33).

Ionophore A23187: Ionophore treatment significantly increased the number of myogenic cells within the regenerate. These cells immediately differentiated to form the promuscle cells. The epidermal cells showed signs of early differentiation as revealed by the invagination of epidermis to form the scale ridges.

DISCUSSION

The results obtained from the experiments suggest that an active Ca^{2+} flux occur through the cell membrane during the process of regeneration. Within the cellular environment, the calcium ions may be coupled to the calcium receptor protein - calmodulin, initiating regulatory influence on cell proliferation and differentiation.

The specificity of various calcium channel antagonists are well studied. Verapamil is considered as a full spectrum calcium channel blocker (Guerrero and Martin, 1984; Godfraind *et al.*, 1986). In the present experiments, verapamil administration exerted inhibitory effects on regenerative events only at preblastemic stage. Verapamil did not evoke any adverse effect on the process of differentiation; only a marginal suppression on tail growth was observed. On the contrary, diltiazem suppressed the cell proliferation and differentiation. The process of chondrogenesis and myogenesis were also greatly hampered. The dosage of drugs administered to the animals were restricted to level that did not affect cardiac functions, food intake or water consumption. Verapamil above 5 mg/kg body wt was found to affect the cardiac functions, as it is a potent vasodialator. Diltiazem upto 100 mg/kg body wt does not have any adverse effect on the animals. It is reasonable to believe that the noted inhibitory effects on the process of regeneration is essentially due to the blockade of calcium channels. Another aspect which should be considered is the calcium channel blockage on the release of neurotransmitters and hormones. The calcium channel blockers are known to suppress the release of many hormones. However it is suggestive that, basal level of pituitary hormone release are not altered by calcium entry blockers (Godfraind *et al.*, 1986). Also, calcium channel antagonists do not affect the glucose homeostasis (Trost and Weidmann, 1984).

Calcium channel antagonists are found to inhibit the cell proliferation in a wide variety of cells both *in vivo* and *in vitro*(Blitstein-Willinger and Diamantstein, 1978; Kennes *et al*, 1981; Grier and Martin, 1985; Maloney *et al.*, 1991). The observed

inhibitory effects of calcium channel blockers on regeneration may be related to non availability of a sufficient Ca^{2+} pool in the proliferative events. Diltiazem is also known to have several intracellular actions, many of which remain unknown(Nayler and Dillion, 1986). Verapamil may not be completely inhibiting the voltage dependent calcium channels at the given dosage, as it is having initial effects on the cardiac functions.

Calmodulin antagonists inhibited the tail growth and the process of differentiation. Here also, a divergence in the mode of action was observed among chlorpromazine and trifluoperazine. Trifluoperazine is a specific calmodulin antagonist that binds to calmodulin and inhibits calmodulin dependent processes (Hartshorne, 1985). Antipsychotic drug chlorpromazine has both anti-calmodulin effects and anti-adrenergic properties. This drug is comparatively less specific for calmodulin and is having broad spectrum effects.

Trifluoperazine depressed the process of dedifferentiation and cell proliferation resulting in a poorly formed blastema. The process of myogenesis and chondrogenesis were also inhibited. Calmodulin is directly implicated in a variety of cellular processes. Calmodulin regulates the intracellular Ca^{2+} concentration and of several Ca^{2+} sensitive intracellular enzymes (Means *et al.*, 1982). Calmodulin is also directly associated with the early phases of cell proliferation. Calmodulin levels have been found to increase during G_1 - S transition phases of cell cycle(Boynton *et al.*, 1980; Chafouleus *et al.*, 1982). In cultured CHO-K cells, addition of W_7(a potent calmodulin antagonist) inhibited the cell proliferation, which was due to the block in G_1 - S transition phase(Hidaka and Sasaki, 1985). W_7-induced blockade was not only at G_1-S phase but also at G_2 or M phase with increasing concentration of the drug. Calmodulin plays an integral part of the regulatory events during oocyte development. Trifluoperazine inhibited the molluscan and starfish oocyte maturation (Meiger and Guerrier, 1983). In amphibian oocytes, progesterone-induced maturation was inhibited by TFP and W_{13} (Hollinger and Alveraz, 1982, 1984). In mouse oocytes TFP, W_7 and CPZ inhibited maturation (Jagiello *et al.*, 1980; Bornslanger *et al.*, 1984). Calmodulin antagonists were also found to inhibit regenerative processes. In the ciliate, *Stentor coeruleus*, TFP inhibited the oral regeneration(Maloney *et al.*, 1991). In newt limb regenerate, chlorpromazine suppressed the mitosis while calcium influx with ionophore A23187 reversed these effect (Globus *et al.*, 1987). It is suggestive that the suppression of mitosis is due to an increase in cellular cyclic AMP levels and a corresponding decrease in cyclic GMP levels. A relationship between cyclic AMP/cyclic GMP has been clearly illustrated(Goldberg *et al.*, 1974). The present observations corroborate with these

findings. It is plausible that the noted inhibitory effect of TFP on tail regeneration is essentially due to the inhibition in the formation of calcium-calmodulin complex which in turn prevented the progress of regenerative phases. Myoblast differentiation was visible proximo-distally to some extent, but these cells were unable to fuse to form the myomeres. Trifluoperazine specifically inhibited the phosphorylation of 100kDa protein and myoblast fusion in cultured embryonic chick muscle cells probably by inhibiting the protein kinase activity (Sun *et al.*, 1992). Another notable observation was the deposition of collagen fibres at the distal areas of the regenerate. This phenomenon is observed usually in non-regenerating appendages. However, such observations are recorded in denervated limb regenerate of *Xenopus laevis*(Liversage and Khan, 1990). Extensive collagen depositions have also been observed in ricin treated regenerating blastema of lizards(unpublished observations). Tassava and Olsen(1982) hypothesised that such collagen deposition is due to the limited cell cycling occurring in denervated limbs. As TFP suppressed the cell proliferation by inhibiting the cell cycle events, the above hypothesis is in line with the present results. Taken together, the results suggest that calmodulin in association with calcium plays a pivotal role during the proliferative phases of tail regeneration. Inhibition of this protein resulted in decreased cell proliferation and differentiation of the regenerate.

Modification of the Ca^{2+} flux affected all facets of regeneration. Calcium efflux with papaverine totally inhibited the cell prolilferation; however, a poor blastema was formed with relatively less number of mesenchymal cells. Both chondrogenesis and myogenesis were inhibited. A sparse distribution of myoblast cells was visible, but these cells were unable to fuse to form the myotubes. The apical epidermal cap showed hyperproliferation which resulted in a thickened epidermis. Contrary to this, calcium influx promoted the regenerative events. Though no appreciable difference in tail length was recorded between control and ionophore treated tail regenerate, histological features revealed an enhancement in cell proliferation and differentiation. Ionophore injection increased the proportion of highly eosinophilic cells of the mesenchyme, which are the precursors of myogenic cells. Their proportion increased markedly towards the distal end of the regenerate, which indicate that, calcium influx might be influencing the non-committed cells of mesenchyme to differentiate into myoblast cells. In most of the regenerate the epidermal cell layers showed invagination, a process associated with scale ridge formation. Ionophore treatment in BL stages also evoked similar response.

The net effects of papaverine-induced Ca^{2+} efflux demonstrate the importance of

intracellular Ca^{2+} in the proliferation-differentiation stages of regeneration. Papaverine is known to interfere with Ca^{2+} flux (Imai and Takeda, 1967) and inhibit phosphodiesterase activity resulting in an increase in cyclic AMP levels (Markwardt and Hoffman, 1970; Prasad and Sheppard, 1973; Bravo *et al.*, 1978; Duprat and Kan, 1981). Papaverine-induced Ca^{2+} efflux inhibits the neurulation by disrupting microfilament structure while ionophore counteracts these effects and restore microfilament structure (Moran and Rice, 1976). Papaverine also inhibits cell movements by virtue of its ability to affect the Ca^{2+} mobility(Triner *et al.*,1970). The morphogenesis in the mouse salivary gland found to be inhibited by papaverine(Ash *et al.*, 1973). During dedifferentiation cells change their shape and cell movements occurs. An increase in cyclic GMP level has been found to be necessary for these processes(Yamada, 1977; Taban and Cathieni, 1989). Papaverine primarily affects these events by interferring with the increase in intracellular Ca^{2+} and elevating cyclic AMP levels.

The increase in cytoplasmic Ca^{2+} is postulated to be atleast part of mitogenic signal(s) that initiate DNA synthesis and cell division(Luckasen *et al.*, 1974). The divalent transporting ionophore A23187 is reported to increase the cytoplasmic Ca^{2+} concentration in several developing systems. In starfish, amphibian and mammalian oocytes ionophore induces the unfertilized eggs to develop parthenogenetically and considered as a universal activator of unfertilized eggs(Steinhardt *et al.*, 1974). Calcium ionophore stimulated DNA synthesis in quiscent human and pig lymphocytes (Luckasen *et al.*, 1974; Maino *et al.*,1974) and in a variety of invertebrate oocytes(Steihardt and Epel., 1974). It is suggestive that increase in cytosolic Ca^{2+} concentration elevate the cyclic GMP levels and stimulate cell proliferation(Goldberg *et al.*, 1974; Drummond, 1983; Lincoln and Corbin, 1983).

Similar findings have been reported in regenerating limbs of the newt, *Notophthalmus viridescens* where ionophore induced Ca^{2+} influx elevated cyclic GMP levels and increased the mitosis (Globus *et al.*, 1987). Ionophore A23187 elevated cyclic GMP followed by cell proliferation in cultured lymphocytes(Hadden *et al.*, 1976).

An increase in the thickness of the apical epidermal cap(AEC) has been observed within the regenerate in a decreased Ca^{2+} pool. This phenomenon has been observed after calcium channel blockage, calmodulin inhibition and intracellular calcium depletion. In the distal epidermis(AEC) the cell proliferation increased and cells exhibited hyperplasia. Similar results have been observed in actinomycin treated newt limb regenerate, where

a compensatory hyperplasia occurred with increasing concentration of drug (Wolsky, 1988). The AEC is implicated in providing certain regeneration promoting substances; the identity of which remain unknown. Calcium ions are implicated in controlling epidermal cell proliferation and differentiation. The epidermis has its own calcium 'reservoir'(Menon *et al.*, 1985). A low calcium level stimulated the murine epidermal cells to proliferate but they do not differentiate unless the calcium level is further increased(Hennings *et al.*, 1980). A low calcium level inhibited the differentiation of human and murine epithelial cells in culture (Sacks *et al.*, 1985). In the present experiment when Ca^{2+} level was low, the AEC responded with an increase in cell proliferation resulting in hyperplasia. Interestingly, only the apical areas of the epidermis showed this phenomenon. Ionophore treatment induced epidermal differentiation within the tail regenerate. The behaviour of the epidermal cells to modified calcium flux is not fully explainable with the present experimental evidences. Further experiments are required along these lines.