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Nerve Extracts and Substance P Activate the Phosphatidylinositol Signaling Pathway and Mitogenesis in Newt Forelimb Regenerates

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We investigated the inositol phospholipid transmembrane signaling pathway as a possible mediator of neurotrophic (mitogenic) signals in the newt limb regeneration blastema. Blastema mesoderm tissues were prelabeled with myo-[^{3}H]inositol, treated with 10 m*M* LiCl and then exposed to substance P or to extracts of spinal ganglia, brain, or spinal cord. Stimulation with substance P resulted in a rapid dose-dependent reduction of [^{3}H]phosphatidylinositol 4,5-bisphosphate and [^{3}H]phosphatidylinositol 4-phosphate, correlated with a rapid accumulation of inositol 1,4,5-triphosphate. This effect was inhibited when the blastema tissue was treated with neomycin, a known inhibitor of inositol phospholipid turnover. In addition, substance P stimulated the incorporation of [^{3}H]thymidine into DNA of blastema mesoderm cells, and this effect was also suppressed by neomycin, at a dose corresponding to that required to inhibit inositol phosphate accumulation. Extracts of neural tissues, especially spinal ganglia, induced the formation of inositol phosphates and extract activity was attenuated following treatment with heat or trypsin. These findings suggest a role for mitogen-activated inositol phospholipid signaling, initiating events that ultimately lead to cell proliferation.

INTRODUCTION

Limb regeneration in both larval and adult urodele amphibians is dependent upon the respective contributions of nerves, hormones, and an intact apical epidermal cap at the site of injury (reviews, Singer, 1978; Globus and Vethamany-Globus, 1985; Stocum, 1985; Tassava and Olsen, 1985). Each of these elements contributes to a multifactorial chain of events that culminates in overt mitosis and the accumulation of a critical mass of cells, the blastema, at the wound surface. The influence conferred by the nerve is generally acknowledged to promote blastema cell proliferation (Singer and Craven, 1948; Globus, 1978; Tassava and McCullough, 1978) and macromolecular synthesis (Mescher, 1983). This has been demonstrated utilizing a number of replacement strategies, such as implantation of spinal ganglia into the regenerate of a denervated limb (Kamrin and Singer, 1959), organ culture of blastemas transfilter to spinal ganglia (Globus and Vethamany-Globus, 1977), juxtaposed monolayers of blastemal and neuronal cells (Smith and Globus, 1989), infusion into the blastema of crude nerve homogenates or brain synaptosomal concentrates (Singer *et al.*, 1976; Jabaily and Singer, 1977), and addition of partially purified nerve extracts (Choo *et al.*, 1981) to the culture medium of explanted blastemas. To date, identification of the neurotrophic factors involved and the mechanisms by which these extracellular signals are translated into intracellular events that ultimately lead to proliferation, has not yet been realized.

In many biological systems, an elevation of cytosolic calcium (Ca^{2+}) levels has been associated with early events in the initiation of cell proliferation (Berridge, 1988, 1993; Whitfield, 1982; Whitfield *et al.*, 1986). In the regenerating newt forelimb, a correlation between altered cytosolic calcium levels and increased blastema cell mitosis has been demonstrated (Globus *et al.*, 1987, 1993). Studies of many cellular processes show that the regulation of intracellular Ca^{2+} can be linked to the second-messenger function of inositol 1,4,5-triphosphate (IP_3) (Putney, 1992; Berridge, 1993). In response to many extracellular signals (such as neurotransmitters, hormones, growth modulators), second messengers IP_3 and 1,2-diacylglycerol (1,2-DAG) are formed by receptor-mediated hydrolysis of a polyphosphoinositide precursor (phosphatidylinositol 4,5-bisphosphate) stored in the plasma membrane. The IP_3 released into the cytosol mobilizes calcium from internal stores (Berridge and Irvine, 1984; Berridge, 1993), whereas DAG activates protein kinase C (Nishizuka, 1984). Two major pathways stimulate the formation of IP_3 and DAG, one initiated by a family of G-protein-linked receptors and the other by tyrosine kinase-linked receptors, which activate specific phospholipase C isoforms (Rhee and Choi, 1992). Early responses to growth modulators also include acti-

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vation of mitogen-activated protein (MAP) kinases by both calcium-dependent and calcium-independent pathways (Chao *et al.*, 1992).

We have been exploring the possible involvement in limb regeneration of the neuropeptide substance P (SP), the most widely known and thoroughly studied member of the tachykinin family of bioactive peptides. SP is widely distributed in both the peripheral and central nervous systems (see reviews Leeman and Carraway, 1977; Pernow, 1983) and evokes a variety of biological and behavioral responses. Among its diverse functional capabilities, SP causes intestinal smooth muscle contraction, is a potent secretagogue and vasodilator (Leeman and Carraway, 1977), a neurotransmitter (Otsuka and Konishi, 1975), and a putative neuromitogenic agent (Globus *et al.*, 1983; Globus, 1988; Globus and Alles, 1990; Nilsson *et al.*, 1985; Salo and Baguna, 1986). Substance P, an excitatory neurotransmitter of primary sensory neurons (Otsuka and Konishi, 1975), has been identified as a candidate for the mediation of the neurotrophic effect in urodele limb regeneration (Globus *et al.*, 1983). SP has been immunocytochemically localized in newt spinal ganglia and in the limb blastema (Globus and Alles, 1990), and it exhibits mitogenic activity *in vitro* that is suppressed by anti-SP antiserum (Globus *et al.*, 1983). The current study has been undertaken to extend our findings (Globus *et al.*, 1991) that the transduction of neuromitogenic signals, such as SP, may be mediated, at least in part, by the inositol phospholipid transmembrane signaling pathway.

MATERIALS AND METHODS

Animal Care

Adult newts, *Notophthalmus viridescens*, obtained from Mr. M. Tolley, Donnelson, Tennessee, were kept in deionized water and maintained in an atmospherically controlled incubator at 21 ± 1°C, on a constant 12-hr photoperiod. They were fed frozen brine shrimp twice weekly and the water was changed daily.

Forelimb Amputation and Excision of the Blastema Tissue

Prior to forelimb amputation, the animals were anesthetized in 0.15% ethyl *m*-aminobenzoate methanesulfonic acid (MS-222, Sandoz) neutralized to pH 7.0 by addition of Na_2PO_4 (Vethamany-Globus *et al.*, 1977). Bilateral amputations were performed through the distal third of the forelimb. The protruding radius and ulna as well as the skin were trimmed level with the wound surface following retraction of the soft tissues of the limb.

When regeneration had proceeded to the early cone stage, 19–22 days following amputation, blastema tissue was removed under anesthesia from approximately 35 newts (70 blastemas) at the original level of amputation and placed in sterile amphibian saline (SAS) containing D-glucose (2.22 m*M*). Protease inhibitors (bacitracin, 70 μM; benzamidine HCl, 1 m*M*; and pepstatin, 2 μM) were included in all incubation and rinse solutions.

Blastemas were then incubated in SAS for 1.5 hr at 22 ± 1°C in an atmosphere of 5% CO_2 in air in order to promote detachment of the epidermal cell layer from the underlying mesodermal tissue. Mesodermal cores of the blastemas were separated from their epidermal hulls with fine forceps, and the wet weight of the mesodermal tissue was determined. Tissues were sliced with a razor blade into fine fragments and placed in fresh SAS.

Preparation of Brain, Spinal Cord, and Spinal Ganglia Extracts

Brains from 12 adult newts were removed following decapitation, frozen immediately in liquid nitrogen, and weighed (approximately 100 mg in total). The tissue was then pulverized in a ground glass homogenizer (Bellco) in 400 μl of sterile distilled water containing protease inhibitors (bacitracin, 70 μM; benzamidine HCl, 1 m*M*; pepstatin, 2 μM) and transferred to an Eppendorf centrifuge tube. All solutions and apparatus were kept on ice throughout the procedure. The glass homogenizer was then rinsed twice with 200 μl of sterile distilled water containing the peptidase inhibitors, and when the rinse volumes were pooled the combined volume was approximately 1 ml. The homogenate was sonicated and then centrifuged (12,000*g*; 20 min; 4°C); the supernatant was withdrawn and the pellet was discarded. The supernatant was centrifuged for 12 hr at 140,000*g* (4°C) in a Beckman L8 55 ultracentrifuge with a fixed angle rotor. The supernatant was transferred to a clean Eppendorf tube, and aliquots were removed for protein determination. The remainder was stored at −20°C until used. Periods of storage for up to 4 weeks produced no detectable loss of activity in the protein extracts. Extracts of newt spinal cord (12 animals), spinal ganglia (brachial and crural, 30 animals), and skeletal muscle (forelimb) were prepared in the same manner as for brain. Protein concentrations of all extracts were determined by a microassay procedure for the Bradford reagent (Bio-Rad) and read on a Shimadzu UV-160 spectrophotometer using BSA standards.

Prelabeling Blastemal Mesoderm with [^{3}H]Inositol and Treatment with Substance P or Nerve Tissue Extract

Blastemas were placed in 1 ml SAS containing 3–5 μCi myo-2-[^{3}H]inositol (20 Ci/m*M*, Amersham) and incu-

bated for 20 hr at 22 ± 1°C in an atmosphere of 5% CO_2 in air. Five minutes prior to the addition of SP, the SAS was withdrawn and replaced with fresh saline without the radiolabel. LiCl was then added to a final concentration of 10 m*M* in order to inhibit inositol-1-phosphatase activity (Allison and Blisner, 1976). SP was introduced at the concentrations and periods of time indicated in the results. A volume of SAS equivalent to that used for the addition of SP was added to the control tissues. Incubations were terminated by the addition of 3 ml ice-cold chloroform–methanol (1:2 v/v) in 0.5% HCl. The blastema tissues were homogenized, following which an additional 1 ml of chloroform was added. Homogenates were incubated for 1 hr at 4°C and then centrifuged at 1000*g* for 15 min. The supernatant was collected, transferred to clean test tubes, and phase separation was achieved by addition of 2 ml of sterile distilled H_2O. Aqueous and organic phases were separated and transferred to clean tubes. The aqueous phase was extracted with an additional 2 ml of chloroform and withdrawn. The two organic phases were pooled, dried under a stream of nitrogen, and resuspended in 50 μl of chloroform.

Separation of Inositol Phosphates by Anion-Exchange Chromatography

The aqueous phase was neutralized with 0.1 *M* NaOH and applied to an anion-exchange resin (Bio-Rad AG 1 × 8 ammonium formate, formate form) with a bed volume of 1 ml. Columns were eluted with 50 ml of 5 m*M* myo-inositol to remove free [^{3}H]inositol, 6 ml of 5 m*M* sodium tetraborate/60 m*M* ammonium formate to elute glycerophosphoinositol (GPI), and 6 ml each of 0.1 *M* formic acid containing 0.2, 0.4, 0.8, and 1.0 *M* ammonium formate in order to sequentially elute mono-, bis-, tris-, and tetrakisphosphate forms of the inositol molecule, respectively. A 1-ml aliquot of each eluent was taken for determination of radioactivity in a Beckman LS 7000 liquid scintillation counter. The formation of radiolabeled inositol phosphates was measured in response to agonist-stimulated hydrolosis of the radiolabeled inositol phospholipids and expressed as cpm per 100 mg of blastemal mesoderm tissue (wet weight). Significant differences between the means of experimental and control values were determined by Student's *t* test and were considered to be significant if $P < 0.05$. The method used for separation of inositol phosphates was modified after the method of Abdel-Latif (1986).

Separation of Inositol Phospholipids by Thin-Layer Chromatography

TLC plates (Linear K, Whatman) were predeveloped in 1% potassium oxalate, dried, and activated at 115°C for 15 min. The lipids were then spotted onto the TLC plates, which were developed in chloroform–methanol–4 *M* NH_4OH (9:7:2) after Gonzales-Sastre and Folch-Pi (1968). Phosphoinositides were visualized by exposure to iodine vapor, identified by their comigration with standards, and scraped into liquid scintillation vials for the determination of their total radioactivity.

Incorporation of [^{3}H]Thymidine in Cultured Blastema Cell Monolayers

The culture method followed was essentially that of Smith and Globus (1989), derived from the earlier work of Globus and Vethamany-Globus (1977). Mesodermal cores of the blastemas were sterilized by treatment for 5 min in 5 ml chloramine T (10 mg/ml; Aldrich), rinsed in sterile SAS, and dissociated after the method of Prop and Weipjes (1973). Tissues were incubated in 1% testicular hyaluronidase (Worthington)/1.5% type IA collagenase (Sigma) in phosphate-buffered saline (PBS) on a gyratory shaker (22°C ± 1, 80 rpm) for 1 hr. The enzyme solution was then removed, replaced with 10 ml of 2.5% protease (Type XXI, Sigma) in calcium- and magnesium-free PBS (CMF-PBS), gyrated for another 45 min, and washed several times with fresh CMF-PBS. The cells were dispersed by gentle pipetting and centrifuged at 150 × g for 5 min. The resulting pellet was resuspended in minimum essential medium (MEM), supplemented with fetal bovine serum (FBS, 2.8%), gentamycin (50 μg/ml), L-thyroxine (10^{-8} mg/ml), purchased from Gibco (Grand Island, NY), as well as hydrocortisone (0.2 μg/ml), somatotropin (0.2 μg/ml), and bovine insulin (0.14 IU/ml) purchased from Sigma (St. Louis, MO). The cell suspension was passed through Nitex cloth (20 μm mesh size; B & SH Thompson, Montreal); the cell density was determined by counting on a hemocytometer and then adjusted to 10^6 cells/ml. The percentage of viability was determined by the trypan blue dye exclusion test. Mesodermal cells were applied to culture plate inserts (12-mm Millicell-HA, 0.45-μm pore size; Millipore Corp.) at 10^5 cells/insert, and maintained in 24-well culture dishes (22°C, 5% CO_2) for 24 hr and then a further 24 hr in fresh medium containing 3.0 μCi/ml [^{3}H]thymidine (77.2 mCi/m*M;* NEN). The medium was withdrawn and two cold 1-ml thymidine chases (6.6 μg/ml; Sigma) were performed. The inserts were removed, washed for 30 min in 5% trichloroacetic acid, rinsed twice with PBS, filled with 0.2 *M* NaOH for 30 min, rinsed with ethanol–ether (3:1) and air dried. Counts per million [^{3}H]thymidine was determined in a Beckman 7000 liquid scintillation counter. Significant differences between the means of experimental and control values were determined by Student's *t* test and considered to be significant if $P < 0.05$.

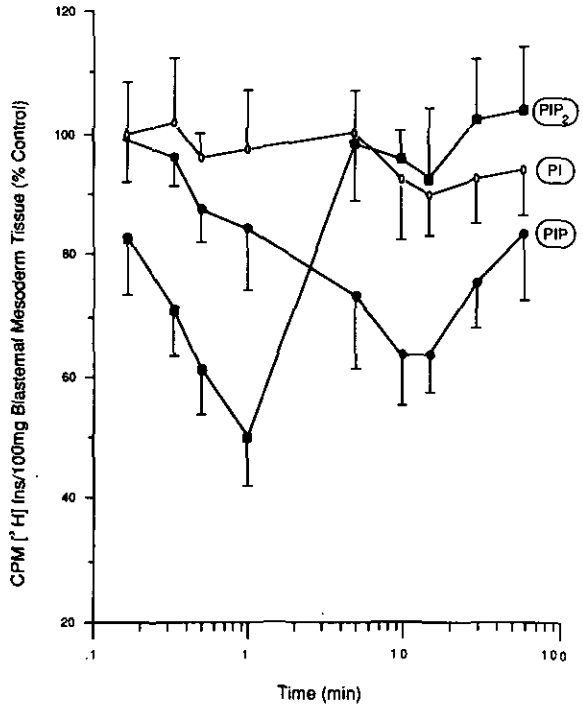

FIG. 1. Substance P-induced inositol phospholipid hydrolysis. Blastemal mesoderm tissue was prelabeled with [^{3}H]inositol for 20 hr, followed by incubation with 10 mM LiCl and 10^{-6} M SP for the indicated time. Control tissues were prelabeled and treated with LiCl but SP was not provided. Protease inhibitors (bacitracin, 70 μM; benzamidine HCl, 1 mM; pepstatin, 2 μM) were included in all incubation solutions. Agonist stimulation was terminated and inositol phospholipids (PI, PIP, and PIP_2) were isolated by thin-layer chromatography. Radiolabel was quantitated by liquid scintillation counting and reported as a percentage of the control. Each experimental point represents the mean of four separate experiments. Bars indicate the standard error of the mean (SEM).

RESULTS

In order to determine whether the inositol phospholipid signaling pathway is involved in the transduction of mitogenic signals across the blastema cell membrane, the hydrolysis of polyphosphoinositides PIP_2, PIP, and PI, as well as the resultant formation of inositol phosphates IP_1, IP_2, IP_3, were monitored in response to SP and neural tissue extracts.

Effect of Substance P on the Hydrolysis of Inositol Phospholipids and DNA Synthesis in Blastemal Mesoderm Tissue

When blastemal mesoderm tissue was prelabeled with [^{3}H]inositol and exposed to substance P, a significant decrease in the amount of labeled PIP_2 and PIP was observed, both as a function of time and the dose of SP (Figs. 1 and 2); the level of radioactivity in the PI fraction remained unchanged. The PIP_2 value decreased rapidly but reestablished its initial level within 5 min of the onset of hydrolysis, i.e., the moment of addition of SP. PIP levels decreased somewhat more slowly and to a lesser extent than PIP_2, and a longer period of recovery was observed. SP-induced increases in [^{3}H]thymidine incorporation into DNA were observed to occur at concentrations greater than 10^{-14} M, with half-maximal stimulation resulting at approximately 10^{-9} M substance P (Fig. 3).

Formation of Inositol Phosphates in Response to Stimulation by Substance P

Anion-exchange chromatography of the aqueous component of the blastemal mesoderm tissue resulted in the resolution of six distinct fractions of radioactivity (Fig. 4). These peaks correspond to [^{3}H]inositol, glycerophosphoinositol (GPI), IP, IP_2, IP_3, and IP_4, as determined by comparison with the elution of commercially available standards. Elution profiles were prepared from SP-

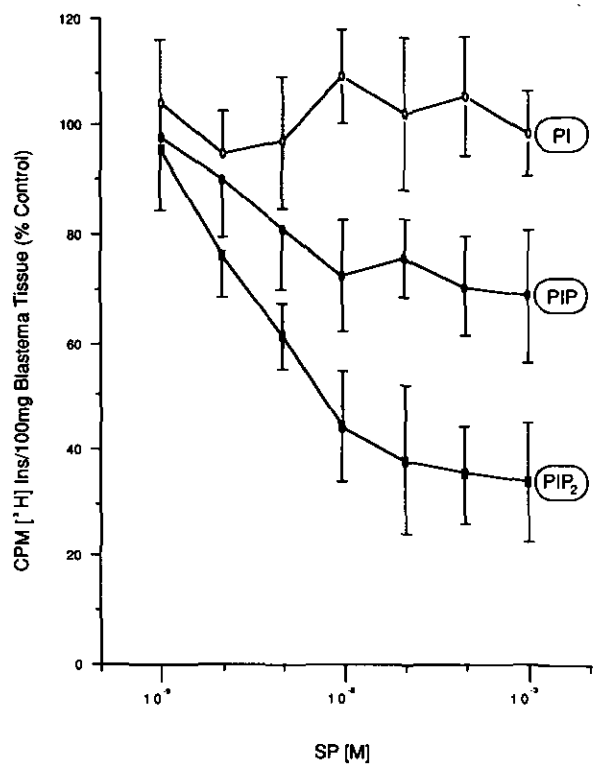

FIG. 2. Changes in radiolabeled inositol phospholipid levels in the mesoderm of the regeneration blastema after stimulation with different concentrations of SP. Experimental and control groups consisted of blastemal mesoderm tissue prelabeled with [^{3}H]inositol and treated with LiCl. Protease inhibitors (bacitracin, 70 μM; benzamidine HCl, 1 mM; pepstatin, 2 μM) were included in all incubation solutions. SP was added to the experimental tissues and the incubation was terminated after 1 min; controls did not receive SP. [^{3}H]Inositol levels reported are the percentage of the control values. Each point represents the mean of four separate experiments, and bars illustrate SEM.

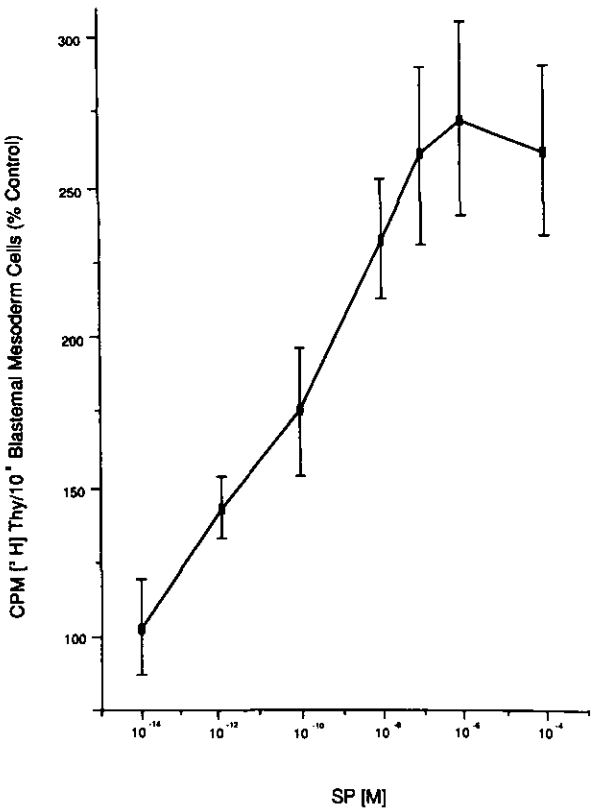

FIG. 3. SP-induced incorporation of [^{3}H]Thy into DNA of blastemal mesoderm cells. Blastemal mesoderm was dissociated into single cells and plated in 24-well multiwell culture dishes at a density of 10^5 cells/well. Cells were exposed to [^{3}H]Thy (3 μCi/ml) for 48 hr in either the presence or absence of SP. Protease inhibitors (bacitracin, 70 μM; benzamidine HCl, 1 mM; pepstatin, 2 μM) were included in all incubation solutions. Radiolabel was quantitated by liquid scintillation counting and reported as a percentage of the control. Data points represent the mean value of four separate experiments and bars indicate the SEM.

treated and untreated blastemal mesoderm tissue in both the presence and absence of 10 mM LiCl. In the presence of SP, an increase in product formation was observed in the latter four peaks with IP and IP_2 enhancement recorded in the presence of LiCl. Inhibiting the dephosphorylation of IP, and the subsequent reincorporation into the PI fraction, greatly enhances the sensitivity of detection of IPs (Berridge *et al.*, 1982).

SP-Induced Inositol Phosphate Formation

The accumulation of radiolabeled inositol phosphates in prelabeled blastemal mesoderm tissue increased significantly both as a function of time after addition of 1 μM SP (Fig. 5) and as a function of increasing dosage of SP (Fig. 6). Increased levels of radiolabeled IP_3 were detectable within 10 sec of addition of SP, reached a peak 18× control values within 1 min followed by a decrement, but remained elevated for a period exceeding 60 min; half-maximal stimulation occurred at a concentration of approximately 10^{-8} M SP. The rapid rise in radiolabeled IP_3 levels preceded those observed for IP_2 and IP, respectively. [^{3}H]IP_2 levels also increased rapidly, reached a peak within 1 min, followed by a decrement, but remained elevated for the duration of SP stimulation; half-maximal stimulation (10^{-8} M SP) was similar to that for IP_3. The observed sustained levels of IP_3 and

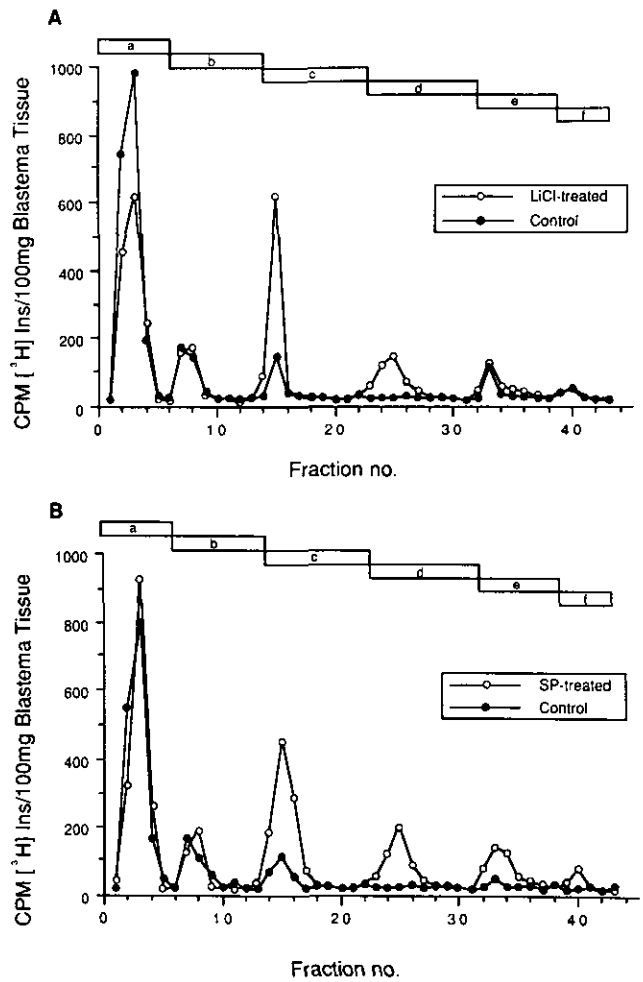

FIG. 4. (A) Anion-exchange elution profile of SP-treated mesoderm in the presence and absence of LiCl. Prelabeled mesoderm, exposed to 10 mM LiCl (○) was treated with 1 μM SP for 5 min. Control tissues (●) were treated with SP but LiCl was not added. Values represent cpm above background. Eluents used were: (a) 5 mM cold myo-inositol; (b) 5 mM sodium tetraborate/60 mM ammonium formate; (c) 0.1 M formic acid/0.2 M ammonium formate; (d) 0.1 M formic acid/0.4 M ammonium formate; (e) 0.1 M formic acid/0.8 M ammonium formate; (f) 0.1 M formic acid/1.0 M ammonium formate. The identity of the peaks from left to right are: free myo-inositol, GPI, IP, IP_2, IP_3, and IP_4. (B) Elution profile of Li^+-treated mesoderm in the presence and absence of SP. Prelabeled mesoderm tissue was exposed to LiCl and SP (○). Control tissues (●) were treated identically except that no SP was added. Values represent cpm above background. Elution buffers are described in A above.

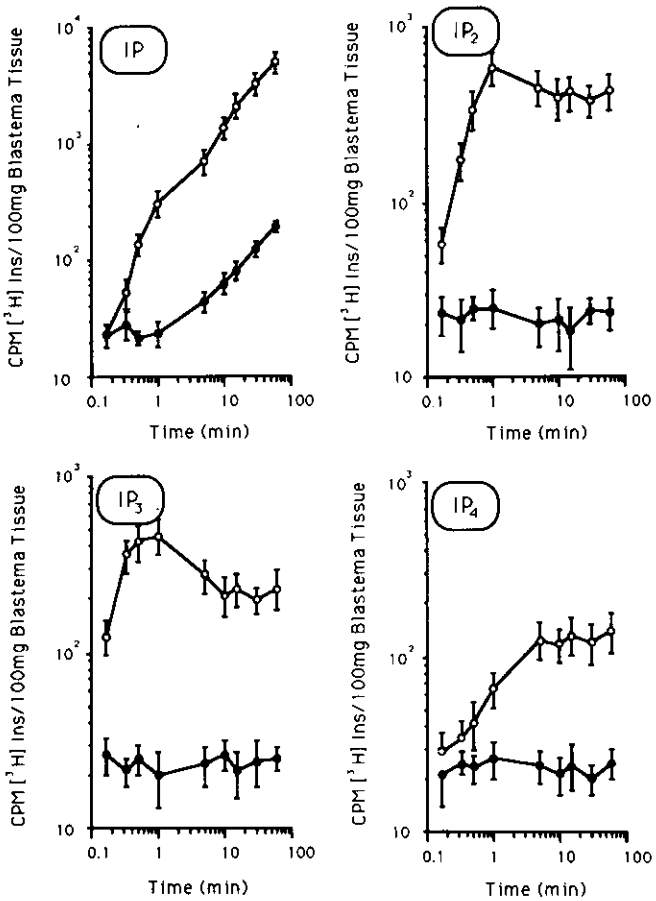

FIG. 5. SP-induced formation of inositol phosphates as a function of time in response to stimulation by 1 μM SP (open symbols) and in the presence of 10 mM LiCl. Controls (closed symbols) were treated with LiCl but SP was not added. Protease inhibitors (bacitracin, benzamidine HCl, pepstatin) were included in all incubation solutions. Radiolabeled inositol phosphates were separated by anion-exchange chromatography and quantitated by liquid scintillation counting. Values represent cpm above background. Each point illustrates the mean value of four separate experiments. Bars represent the SEM.

the concurrent accumulation of [^{3}H]IP may be attributed on the one hand to the rapid hydrolysis of PIP_2, generating IP_3, and on the other hand to the sequential dephosphorylation of Ins (1, 4, 5) P_3 to Ins (1, 4) P_2 and eventually to Ins IP, by specific phosphomonoesterases (Majerus *et al.*, 1986). The accumulation of [^{3}H]IP was facilitated by the presence of 10 mM LiCl (optimal dose, see Fig. 7), which inhibits Ins-1-phosphatase, and thus prevents the recycling of myo-inositol into the Ptd Ins pool (Majerus *et al.*, 1986). The accumulation of [^{3}H]IP was also dose-dependent, with half-maximal stimulation occurring at an SP concentration of approximately 10^{-7} M.

Radiolabeled IP_4 was detected in the aqueous phase following stimulation of blastema tissues with substance P, but its appearance was slower in comparison with the time course of formation of the other inositol phosphates. IP_4 formation was dose-dependent (Fig. 6), but levels plateaued after approximately 10 min (Fig. 5) and remained at a constant level within the experimental time period.

Radiolabeled glycerophosphoinositol (GPI) was detectable in all tissue extracts, although the levels were not responsive to stimulation by substance P (Fig. 8). Although GPI levels did not differ significantly from controls, an increase was observed as a function of time, both in the presence and absence of SP. This result suggests to us, and is substantiated by the work of others (Berridge, 1993), that the observed SP-independent increase in GPI could be the result of inositol phospholipid deacylation, as has been reported by Irvine *et al.* (1982).

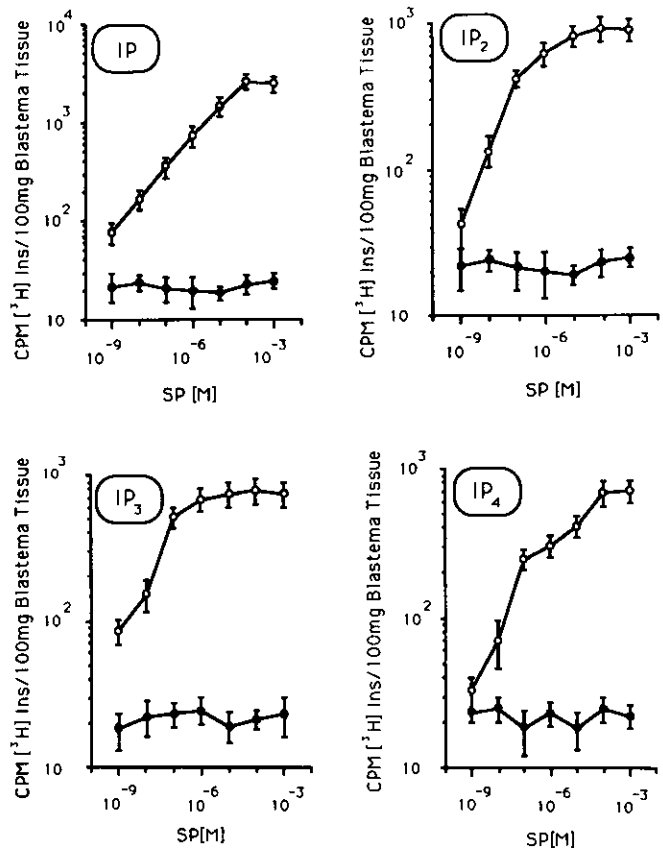

FIG. 6. Dose-dependent formation of inositol phosphates in SP-treated (open symbols) blastemal mesoderm tissue, prelabeled with [^{3}H]inositol, treated with LiCl, and exposed to the indicated concentration of SP for 1 min. Prelabeled, LiCl-treated mesoderm (without SP treatment) served as control (closed symbols). Protease inhibitors (bacitracin, 70 μM; benzamidine HCl, 1 mM; pepstatin, 2 μM) were included in all incubation solutions. Radiolabeled inositol phosphates were separated by anion-exchange chromatography and quantitated by liquid scintillation counting. Values represent cpm above background. Data points are the mean value of four experiments, and bars represent the SEM.

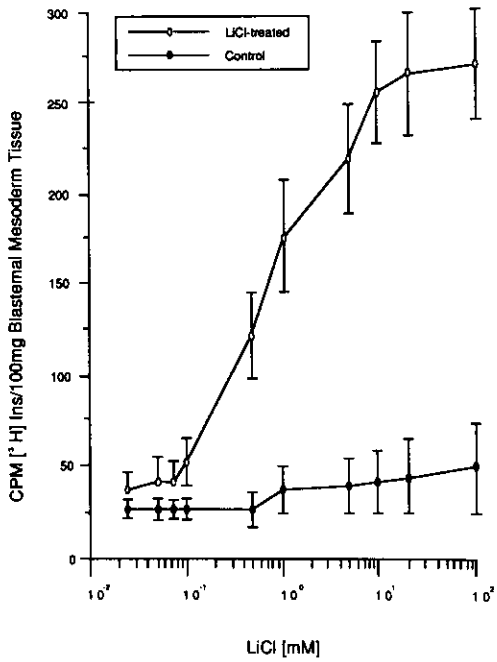

FIG. 7. Effect of lithium on SP-induced IP formation. Blastemal mesoderm tissue was prelabeled with [^{3}H]Ins and exposed to 1 μM SP for 1 min in the presence of increasing concentrations of LiCl (○). Control tissues (●) were treated identically except that LiCl was not added. Protease inhibitors (bacitracin, 70 μM; benzamidine HCl, 1 mM; pepstatin, 2 μM) were included in all incubation solutions. IP was eluted from anion-exchange columns and radioactivity was determined by L.S.C. Values represent cpm above background. Data points represent the mean of four separate experiments and bars indicate SEM.

Inhibition of SP-Stimulated Inositol Phospholipid Hydrolysis and DNA Synthesis by Neomycin

SP-induced hydrolysis of PIP_2 in blastemal mesoderm was inhibited by the aminoglycoside neomycin in a dose-dependant manner (Fig. 9), with a half-maximal effect occurring at a neomycin concentration of 0.5 mM. Blastema mesoderm was exposed to neomycin throughout the 20-hr prelabeling period, prior to exposure to SP. Inositol phosphate formation (Fig. 10) was inhibited by neomycin in the same range of concentration, with the half-maximal effect being observed at 0.5 mM. Neomycin treatment also inhibited the incorporation of [^{3}H]-thymidine into DNA of mesoderm cells, with the half-maximal effect occurring at a concentration of approximately 1.1 mM (Fig. 11). The same half-maximal neomycin concentration was required to inhibit PIP_2 hydrolysis and inhibit DNA synthesis. The concentration of neomycin at which a reduction of [^{3}H]thymidine incorporation was first observed was the same as that required to reduce IP_3 formation by 20%. This apparent close association of dose–response has been noted by others (Carney *et al.*, 1985), which led them to suggest the possibility of a direct correlation between the effects of neomycin on inositol phospholipid hydrolysis and DNA synthesis.

Stimulation of Blastemal Mesoderm Tissue with Extracts of Brain, Spinal Cord, Spinal Ganglia, and Skeletal Muscle

Extracts of brain, spinal cord, spinal ganglia, and skeletal muscle were incubated with blastemal mesoderm cells at concentrations ranging from 10^{-14} to 10^{-4} g/ml and assessed for their ability to stimulate the formation of inosital phosphates (Fig. 12). Spinal ganglia extract effected the most potent stimulation of InsIP

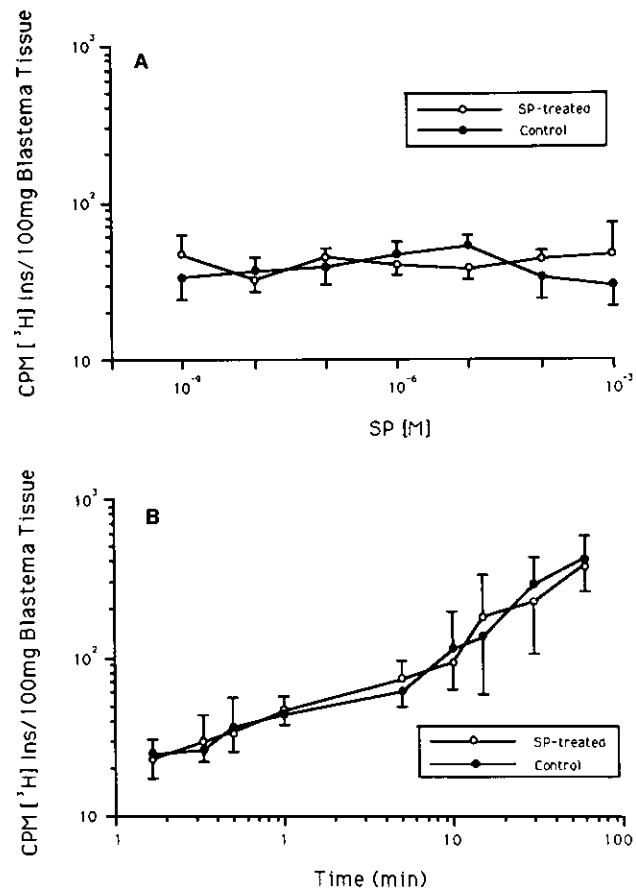

FIG. 8. Determination of glycerophosphoinositol (GPI) formation in response to SP. SP-stimulated inositol phosphate formation (open symbols) was measured in blastemal mesoderm tissues prelabeled with [^{3}H]inositol and exposed to 10 mM LiCl; controls (closed symbols) did not receive SP. In (A), mesoderm was exposed to different concentrations of SP for 1 min; in (B), the tissues were exposed to SP at 10^{-6} M for the indicated length of time. Protease inhibitors (bacitracin, 70 μM; benzamidine HCl, 1 mM; pepstatin, 2 μM) were included in all incubation solutions. Inositol phosphates were separated by anion-exchange chromatography and radioactivity was determined by liquid scintillation counting. Values represent cpm above background. Bars represent SEM.

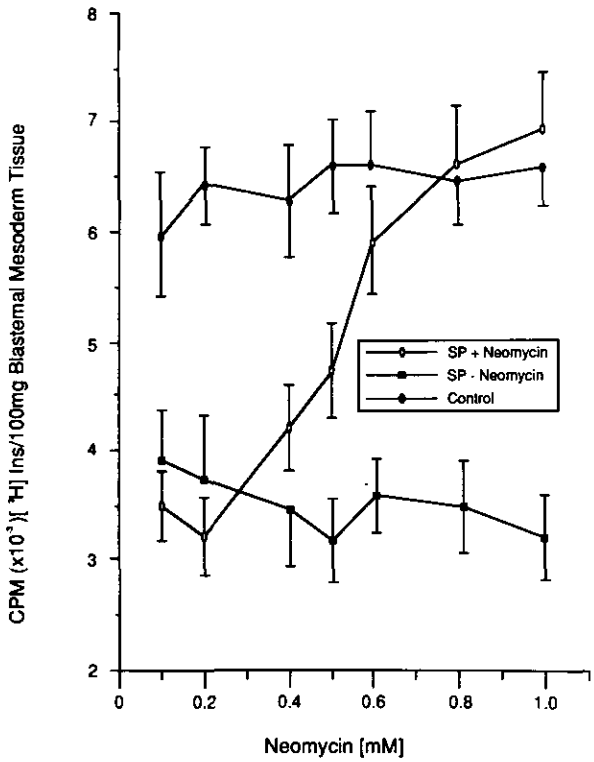

FIG. 9. Reduction of SP-induced PIP_2 hydrolysis by neomycin dose-response. Blastemal mesoderm tissues were prelabeled with [^{3}H]Ins and exposed to neomycin for 20 hr, treated with 10 m*M* LiCl, and exposed to 1 μM SP in either the presence (○) or absence (■) of neomycin at the indicated concentration. Control mesoderm was not exposed to either SP or neomycin (●). The incubation was terminated after 1 min. Protease inhibitors (bacitracin, benzamidine HCl, pepstatin) were included in all incubation solutions. Radiolabeled inositol phospholipids were extracted and separated by thin-layer chromatography and PtdIns (4, 5) P_2 was quantitated by L.S.C. Values represent cpm above background. Data points represent the mean value of four separate experiments and bars are the SEM.

formation, with a measurable response occurring in the presence of 10^{-12} g/ml. Extract from newt brain also caused a dose-dependent increase in inositol phosphate formation, with a half-maximal effect between 10^{-10} and 10^{-8} g/ml and spinal cord extracts were less potent with the half-maximal concentration occurring between 10^{-8} and 10^{-6} g/ml. No significant increase in IP formation above control levels was observed when mesoderm was treated with skeletal muscle extract. These results are interesting when it is considered that innervation by spinal ganglia comprises the major source of nerve fibers to the limb and that spinal ganglia are a rich source of SP in amphibians (Globus and Alles, 1990). We have localized SP in newt brain and spinal cord (Globus, 1988), but the distribution of SP is more sparse than that observed in spinal ganglia. Treatment of neural extracts by exposure to either heat (90°C for 10 min) or to trypsin (Fig. 13) resulted in a sharp reduction in the ability of extracts to induce inositol phosphate formation in blastemal tissue. This finding is consistent with reports (Singer *et al.*, 1976) that newt brain extracts and brachial spinal nerve extracts prevent a denervation-induced decline in protein synthesis, an effect that was abolished by treatment of the extracts with heat (90°C, 10 min) or with trypsin.

DISCUSSION

We previously reported (Globus *et al.*, 1983; Globus and Vethamany-Globus, 1985; Globus, 1988) that substance P was mitogenic at extremely low concentrations (10 pg/ml) in cultured limb regeneration blastemas and that the mitogenic influence of intact nerves was suppressed, in a dose-responsive manner, when sensory ganglia, cocultured transfilter to the blastema, were

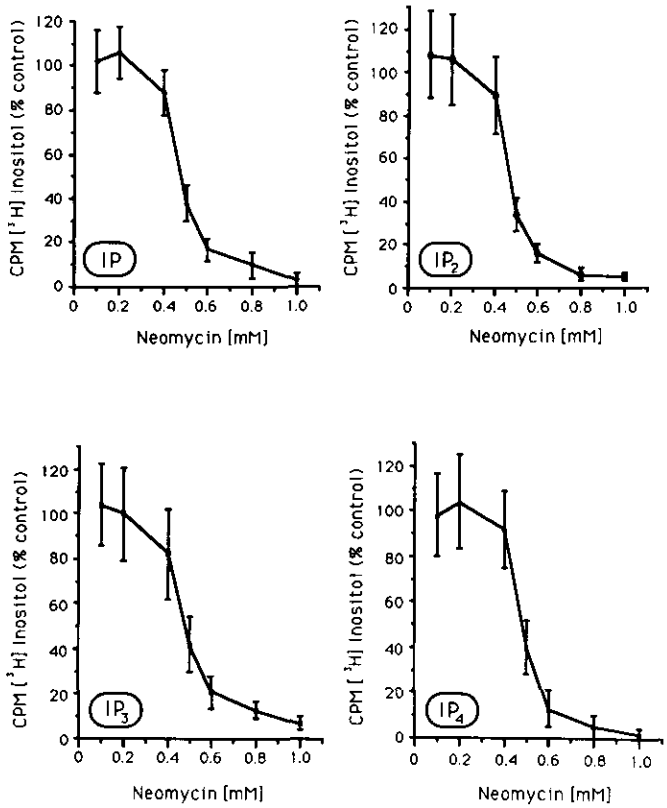

FIG. 10. Reduction of SP-induced inositol phosphate formation in the presence of neomycin. Prelabeled blastemal mesoderm tissues were treated with 10 m*M* LiCl and stimulated with 1 μM SP in either the presence or absence of neomycin. Protease inhibitors (bacitracin, 70 μM; benzamidine HCl, 1 m*M*; pepstatin, 2 μM) were included in all incubation solutions. Inositol phosphates were separated by anion-exchange chromatography and quantitated by liquid scintillation counting. The effect of neomycin on the levels of the individual radiolabeled inositol phosphates was reported as a percentage of the untreated control. Data points represent the mean of four separate experiments and bars indicate the SEM.

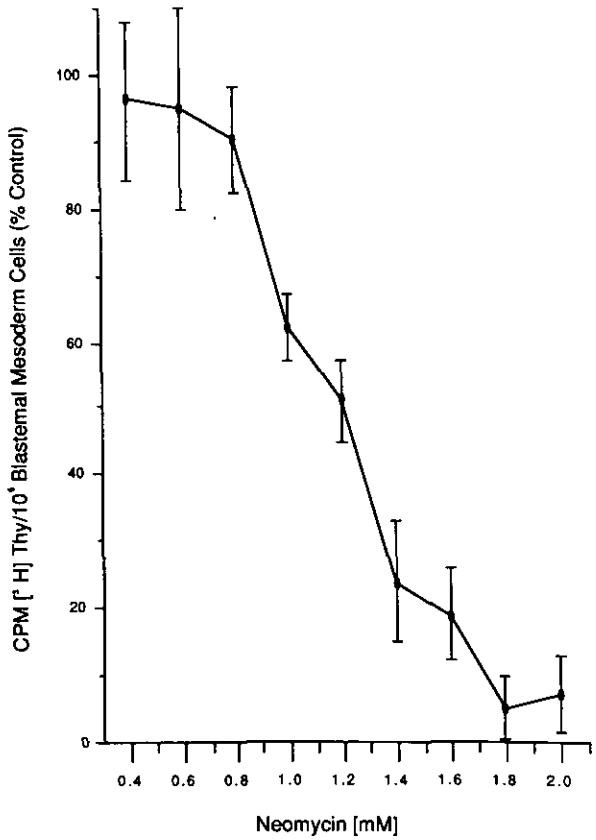

FIG. 11. Reduction of SP-induced [^{3}H]Thy incorporation by neomycin dose-response. Blastemal mesoderm cells were dissociated into single cells, seeded into culture plate inserts, and placed in 24-well multiwell culture dishes at a density of 10^5 cells/well. Cells were incubated for 24 hr in the presence of 1 μM SP and the indicated concentration of neomycin, after which fresh medium containing SP, neomycin, and [^{3}H]Thy was added. Control cultures were treated identically except that no neomycin was present. [^{3}H]Thy incorporation was determined and reported as a percent of the control. Protease inhibitors (bacitracin, benzamidine HCl, pepstatin) were included in all incubation solutions. Data points indicate the mean of four separate experiments. Bars represent the SEM.

treated with increasing concentrations of SP antiserum in the culture medium. Subsequent reports lending support to our findings have appeared, showing that SP stimulates DNA synthesis in cultured smooth muscle cells and human skin fibroblasts (Nilsson *et al.*, 1985; Payan, 1985) and appears to act as a mitogen in regenerating planarians (Salo and Baguna, 1986).

The current study provides the first experimental evidence that the inositol phospholipid signaling pathway is involved in the transduction of neurotrophic signals across the blastemal cell membrane. The neural peptide substance P, as well as extracts of spinal ganglia, brain, and spinal cord, stimulated PIP_2 hydrolysis and [^{3}H]-thymidine incorporation into DNA of blastemal mesoderm cells. It is interesting to note that spinal ganglia are a rich source of SP in the newt (Globus and Alles, 1990) and appear to be a more concentrated source of the peptide than brain or spinal cord. Moreover, the aminoglycoside neomycin, which binds to PIP_2 with high affinity (Gabev *et al.*, 1989) and inhibits its turnover (Downes and Michell, 1981), suppressed both SP-stimulated inositol phosphate accumulation and [^{3}H]-thymidine incorporation into DNA at closely corresponding doses; a dose-dependent reduction of thrombin-stimulated [^{3}H]IP_3 formation and inhibition of mitogenesis by neomycin was previously reported by Carney *et al.* (1985) in cultured Syrian hamster fibroblasts. These results suggest that agonist-stimulated polyphosphoinositide turnover may be essential, albeit not necessarily sufficient for stimulation of blastema cell proliferation. It is also possible that 1,2-DAG, an-

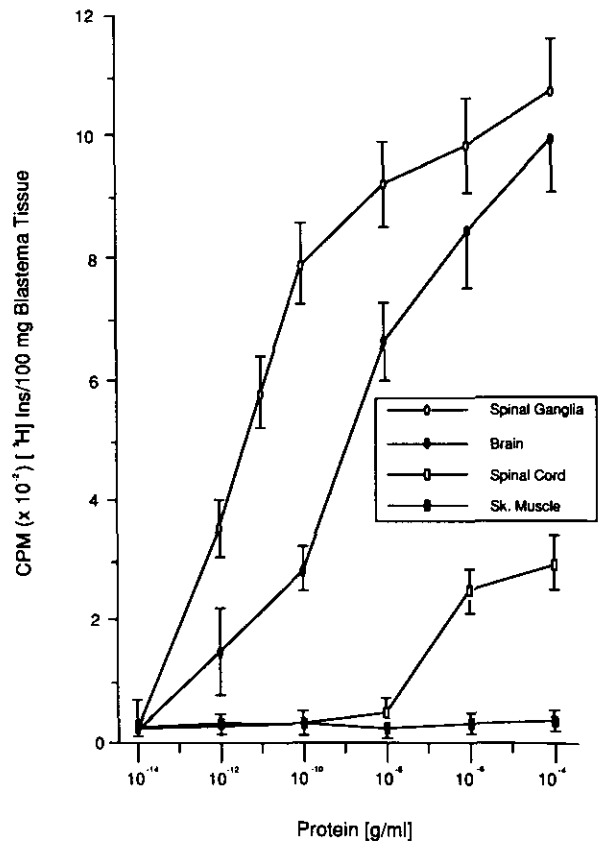

FIG. 12. Extract-induced inositol phosphate (IP) formation in blastemal mesoderm tissue. Extracts of newt spinal ganglia (○), brain (●), spinal cord (□), and skeletal muscle (■) were added to blastemal mesoderm fragments at the indicated concentrations for 1 min, in the presence of 10 mM LiCl. Upon termination of the reaction, the inositol phosphates were extracted and separated by anion-exchange chromatography. Tissue extracts and saline contained protease inhibitors (bacitracin, benzmidine HCl, pepstatin). The radioactivity was determined by L.S.C. Values represent cpm above background. Data points indicate the means of four separate experiments, and bars represent the SEM.

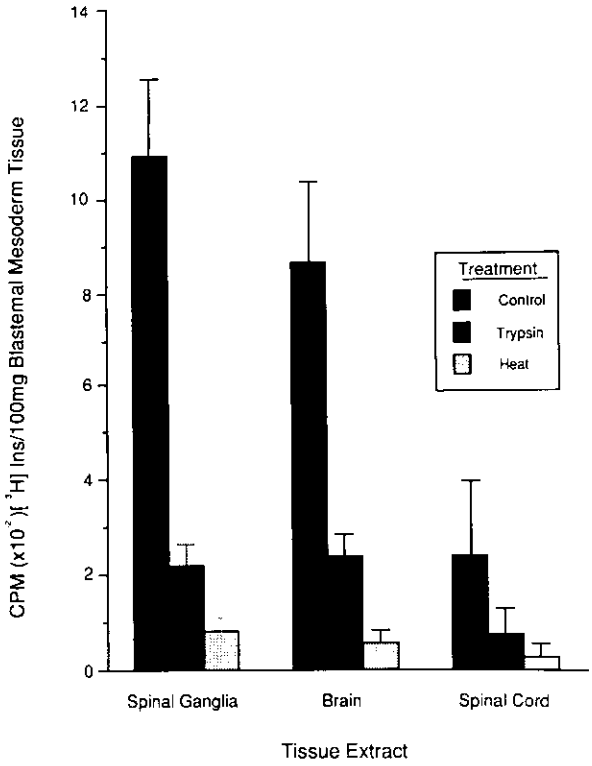

FIG. 13. Reduction of nerve extract-induced IP formation by treatment with heat and trypsin. Nerve extracts were exposed to trypsin (1 mg/ml, 2 hr), or heat (90°C, 10 min), and incubated with the blastemal mesoderm fragments. Inositol phosphates were chromatographically separated on an anion-exchange resin, and radioactivity was determined by L.S.C. Controls consisted of mesoderm exposed to the untreated extracts. Data points represent the means of four separate experiments, and bars indicate the SEM.

other product of the inositol phospholipid bifurcating signal pathway, may synergistically contribute to cell proliferation (Berridge, 1987; Hokin, 1985). In this context, it is recognized that extracts derived from nerves surely contain a vast array of uncharacterized peptides and polypeptides, and the possibility of synergistic influences or the stimulation of multiple pathways cannot be disregarded. The search for neuromitogenic signals in the limb regeneration system is very complex, not only because unique signals may be required in different populations of quiescent cells, but also because in any given population, mitogenesis may require multiple, or even synergistic signals (Globus, 1988) in a multistep or multipathway process (Whitman and Cantley, 1988). Nevertheless, the data provide evidence of a causal link among neural signals, phosphatidylinositol turnover, and mitogenesis in the newt limb regeneration system. Numerous reports consistent with our findings, show that substance P and several of the family of related tachykinins stimulate inositol phospholipid hydrolysis in mammalian tissues; for example, guinea pig ileum muscle, rat hypothalamus (Watson and Downes, 1983), and cultured smooth muscle cells (Hultgårdh-Nilsson *et al.*, 1988; Payan, 1985). In addition, a correlation between SP-induced inositol phospholipid hydrolysis and the number of specific [^{3}H]SP binding sites in rat CNS has been demonstrated by Mantyh *et al.* (1984).

Over the past decade, a model, depicting a putative agonist-activated cascade of cellular events leading to proliferation, has emerged based upon evidence of causal relationships among receptor-activated phosphatidylinositol signaling, elevation of cytosolic calcium levels, and mitogenesis (Abdel-Latif, 1986; Berridge 1981, 1993; Carney *et al.*, 1985; Majerus *et al.*, 1986; Michell, 1989; Putney, 1981, 1986, 1992; Rozengurt, 1986; Whitfield, 1982; Whitman and Cantley, 1988). Several studies indicate that rapid phosphatidylinositol turnover almost always accompanies and is perhaps a prerequisite for cell proliferation in diverse tissue types; for example, T- and B-lymphocytes (Fisher and Meuller, 1971; Nishizuka, 1984) and mammalian fibroblasts *in vitro* (Diringer and Friis, 1977). The latter authors have shown that increased PI turnover accompanies the transition from quiescence to growth induced by decreased cell density, increased serum concentration, or transformation with DNA or RNA viruses. Further evidence is provided by reports that two cellular growth factors PDGF and EGF were stimulating for both inositol phospholipid turnover and cell division in a number of cell types (Habenicht *et al.*, 1981; Sawyer and Cohen, 1981). Transfection of NIH-3T3 cells with N-ras, enhances both growth factor-induced PI turnover and DNA synthesis (Wakelam *et al.*, 1986), and δ-hexachlorocyclohexane, an analogue of myo-inositol, inhibits both serum and growth factor-induced PI turnover and cell cycle traverse in rat fibroblasts (Hoffmann *et al.*, 1980). Also reports (see review Macara, 1985) that two of the cellular oncogenes (src and ros) encoded for protein tyrosine kinases might function as inositol lipid kinases lend support to speculation that the inositol phospholipid signaling pathway might provide a mechanism for oncogene-activated control of cell proliferation in both normal and malignant cells (Michell, 1984).

Inositol phospholipid turnover and an elevation of intracellular calcium have been shown to occur within seconds of agonist stimulation in many cell types (Abdel-Latif, 1986; Morris *et al.*, 1984). One of the initial products of PIP_2 hydrolysis, IP_3, is thought to function by binding to specific receptor proteins (Guillemette *et al.*, 1987), located on portions of the endoplasmic reticulum (Abdel-Latif, 1986; Berridge and Irvine, 1984; Berridge, 1987, 1988; Carsten and Miller, 1985; Ferris and Snyder, 1992; Majerus *et al.*, 1986), and thus opening a Ca^{2+} pore which results in the passive efflux of Ca^{2+} into the cytosol (Berridge, 1988; Ferris and Snyder, 1992). IP_3 binding

sites have been identified (Spät *et al.*, 1986) and it has been reported (Hirata *et al.*, 1985) that IP_3-induced Ca^{2+} release is inhibited by activation of a photoaffinity label to the receptor, previously introduced to permeabilized cells. Formation of Ins (1, 3, 4, 5) P_4 by phosphorylation of IP_3 has also been shown to increase the levels of intracellular Ca^{2+} (Irvine and Moore, 1986; Irvine, 1992) by promoting calcium channel activity.

A link between intracellular calcium levels and mitogenesis is also firmly established (see reviews, Berridge, 1988; Whitfield, 1982) and it is generally recognized that an elevated level of intracellular Ca^{2+} is one of the critical signals initiating events leading to cellular replication. In the context of limb regeneration, we previously reported (Globus *et al.*, 1983, 1987) that exposure of the newt limb blastema to the transmembrane Ca^{2+} carrier ionophore A23187, doubled blastema cell mitotic activity, both *in vivo* and *in vitro*. On the other hand, administration of papaverine (an alkaloid that promotes a net Ca^{2+} efflux) treatment with chlorpromazine (which interferes with the activation of calmodulin) or with chlorpromazine in combination with A23187 resulted in a suppression of mitosis (Globus *et al.*, 1987). In addition, A23187-promoted mitosis was correlated with an elevation of cGMP levels in the blastema and a suppression of cAMP, whereas either papaverin or chlorpromazine-suppressed mitosis was correlated with elevated cAMP and suppressed cGMP levels. Interestingly, activation of receptors that initiate PI signaling and generate calcium signals are often associated with an increase in cGMP levels (Berridge, 1981). Conversely, increased cAMP levels block agonist-stimulated PI hydrolysis, probably preventing a rise in intracellular Ca^{2+} necessary for a response to stimulation (see Whitfield *et al.*, 1986). These data suggest a regulative role for altered Ca^{2+} levels during blastema cell proliferation, underscoring observations in several other biological systems (see Hesketh *et al.*, 1982; Whitfield, 1982).

In summary, we have demonstrated that substance P and probably other neuromitogenic factors present in the nerve extracts tested activate the inositol phospholipid signaling pathway, likely initiating a cascade of intracellular events that involves calcium and ultimately leads to blastema cell divisions and the accumulation of a critical mass of cells required for limb regeneration. The PI signaling system may also serve as a convenient means of screening candidates for mitogenic activity in the limb regeneration system.

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