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A Serine Proteinase Involved in Contact Mediated Repulsion of Retinal Growth Cones by DRG Neurites

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Cultured retinal ganglion cell growth cones avoid neurites extending from PNS explants. Here we characterize a growth cone collapsing activity in detergent extracts of newborn calf adrenal plasma membranes that has characteristics expected for an avoidance cue on peripheral neurites. This adrenal derived activity induces the rapid and reversible collapse of retinal growth cones grown on either of two distinct adhesion substrata, mouse laminin or the chick cell surface axonal glycoprotein G4/NgCAM. The collapsing activity is inhibited by several different types of serine proteinase inhibitors, including the irreversible inhibitor PPACK (D-phenylalanyl-prolyl-arginine chloromethyl ketone). The activity is not inhibited by the specific thrombin inhibitor, hirudin. We have named the adrenal derived collapsing activity erase. PPACK blocks the collapse of temporal retinal growth cones on contact with DRG neurites, but does not block the collapse of the same growth cones on contact with nasal retinal neurites. These results support the hypothesis that a serine proteinase on peripheral axons serves as an avoidance cue which induces contact mediated collapse of retinal growth cones.

[Key words: serine protease, serine proteinase, growth cone guidance, thrombin, growth cone collapse, growth cone repulsion, neurite retraction, protease inhibitor, proteinase inhibitor, inhibition of locomotion, inhibition of motility]

There is now considerable evidence that growth cones are sometimes guided away from inappropriate regions or pathways by specific avoidance cues. Although a wide variety of these repulsive activities have been defined using *in vitro* assays, it has been more difficult to characterize them at a molecular level. Here we show that an avoidance cue on peripheral axons that induces retinal ganglion cell growth cones to collapse (Kapfhammer and Raper, 1987b) is likely to be a serine proteinase that we have named erase.

The growth cones of retinal ganglion cells respond to at least two distinct avoidance cues. One cue specifically affects ganglion cell growth cones that originate from the temporal half of the retina, while the second affects ganglion cell growth cones that originate from both halves of the retina.

A temporal specific cue was first demonstrated by Bonhoffer and Huf (1985) who used an *in vitro* assay to show that chick temporal retinal ganglion cells prefer to extend on temporal as compared to nasal retinal axons. This selective fasciculation may be explained by the observation that temporal retinal ganglion cell growth cones collapse on contact and are repelled by nasal but not temporal retinal axons in culture (Raper and Grunewald, 1990). Similiarly, chick temporal retinal growth cones, but not nasal growth cones, avoid stripes of plasma membranes prepared from the posterior optic tectum (Walter et al., 1987). Temporal but not nasal growth cones collapse and become paralyzed when exposed to extracts of posterior tectal membranes (Cox et al., 1990). The function of this posterior avoidance cue may be to discourage temporal retinal growth cones from extending posteriorly beyond their normal anterior target region in the optic tectum. Finally, retinal growth cones from the ventral temporal region in mouse retina, whose axons are the only retinal axons to project within the ipsilateral optic tract, appear to be specificaly repelled by the midline region of the optic chiasm (Godement et al., 1990; Sretavan, 1990; Sretavan and Reichardt, 1993; Wizenmann et al., 1993). This interaction apparently prevents these growth cones from crossing over into the contralateral optic tract. The selective sensitivity of temporal retinal ganglion cell growth cones to inhibitory stimuli in each of these assays suggests that the same avoidance cue could be responsible for each of the observed behaviors.

A second avoidance cue that retinal ganglion cell growth cones respond to has been described by Kapfhammer and Raper (1987b). Both nasal and temporal retinal ganglion cell growth cones collapse and become temporarilly paralyzed on contact with neurites from cultured dorsal root ganglia (DRG), sympathetic ganglia, or ciliary ganglia (Kapfhammer and Raper, 1987a,b). Since there is no difference in the relative sensitivity of nasal and temporal growth cones to contact with peripheral neurites, it is reasonable to assume that the avoidance cue involved is distinct from the tectal, chiasmatic, or nasal cues just described. In contrast to the collapse of retinal growth cones on contact with peripheral axons, growth cones from any of the peripheral tissues do not collapse when they contact any of the other peripheral neurites. Kapfhammer and Raper (1987b) therefore proposed that peripheral neurites express an avoidance cue that all retinal ganglion cell growth cones respond to and that peripheral growth cones ignore.

The membranes of PC12 cells have been found to express a growth cone collapsing activity that collapses retinal growth cones but not DRG growth cones (Raper and Kapfhammer,

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1990). Since PC12 cells are derived from an adrenal tumor, a tissue derived from neural crest and closely related to sympathetic neurons, adrenals are a potential plentiful source of the cue on peripheral axons that repulses retinal growth cones.

In this study we have used plasma membranes prepared from newborn calf adrenals as a source of growth cone collapsing activity. We have found that they contain an activity, like the PC12 derived activity, that is more effective in collapsing CNS growth cones as compared to PNS growth cones. It induces rapid and reversible collapse that does not depend on the substratum on which the test growth cones extend. The adrenal derived collapsing activity is probably a serine proteinase because its effects are inhibited by a number of different classes of serine proteinase inhibitors. We have named this activity erase.

Moreover, the collapse of retinal ganglion cell growth cones on contact with DRG neurites is blocked by the most selective of the irreversible serine proteinase inhibitors that blocks erase activity. This inhibitor does not simply interfere with the mechanism of growth cone collapse since it has no effect on the collapse of temporal retinal growth cones that contact nasal retinal neurites. These results suggest that the avoidance cue on DRG axons which induces the collapse of retinal ganglion cell growth cones is a serine proteinase similar or identical to erase.

Materials and Methods

Materials. Human two-chain tissue plasminogen activator (78,000 IU/mg protein) and one chain urokinase (80,000 IU/mg protein) were obtained from American Diagnostica, PPACK from Aminobiotech Ltd., mouse laminin from GIBCO, and nerve growth factor and ITS premix from Collaborative Research. Bovine thrombin had a specific activity of 1630–2100 NIH U/mg protein (Sigma #T 7513). Bovine Factor Xa had a specific activity of 500 U/mg, and bovine Factor II had a specific activity of 5 U/mg. The specific activity of bovine Factor Xa (Sigma #B 6003) was defined by the supplier as the amount of activity, measured by substrate clotting time, that would be seen with one unit of Factor X activated in the presence of Russel's viper venom. One unit of either Factor X or Factor II was defined as the amount of that factor found in 1 ml of normal human plasma. TLCK, TPCK, soybean trypsin inhibitor, aprotinin (9700 KIU/ml), leupeptin, antithrombin III (360 U/mg protein), recombinant leech hirudin (8500 U/mg protein), and *N*-*p*-tosyl-Gly-Pro-Arg *p*-nitroanilide were all obtained from Sigma. PMSF was a gift from Randall Pittman. It was originally obtained from Sigma and was further purified by ethanol precipitation before use. DFP was obtained from Aldrich. Specific activities of all proteinases are reported as provided by the suppliers and were not independently verified. A monoclonal antibody that recognizes chick G4 was a gift of Fritz Rathjen. The CSAT antibody that recognizes the integrin β_1 subunit was a gift of David Boettinger.

Cultured tissues. Dorsal root ganglia were dissected from embryonic day 7 (E7) chick embryos. Neural retinae from the temporal or nasal halves of the eye and ciliary ganglia were dissected from E6 chick embryos. Diencephalic and tectal explants were made from E5 embryos. Sympathetic ganglia were dissected from E9 embryos. Ganglia were cut into halves or thirds. Retinal, diencephalic and tectal explants were approximately 0.5 mm^2 in size. The optic fissure was used as a landmark to separate temporal and nasal halves of the retina. The middle one-third of the retina was discarded and only the lateral most thirds of the nasal or temporal retinae were used to make explants. For the video recorded encounters between temporal retinal growth cones and various neurites, explants were spaced 2–3 mm apart from one another. Cultures were incubated at 37°C in 5% CO_2. All videos were made between 16–20 hr after explantation.

Culture substrata. Ten millimeter glass coverslips were washed with nitric acid, silanized, and coated with EHS laminin at a concentration of 40 μg/ml for 1 hr as described previously (Kapfhammer et al., 1986). Chick G4 was isolated from plasma membranes of E16 and E17 chick brains (Rathjen et al., 1987) and 45 μg/ml applied to silanized coverslips for 1 hr at room temperature. In the experiments with G4, both G4 and laminin coated coverslips were blocked with 3 mg/ml hemoglobin and then extensively washed with Ca^{2+} Mg^{+2} free Hanks' Balanced Salt Solution (CMF-HBSS).

Culture media. Explants were cultured 18–24 hr in F-12 medium (J R H Biosciences) supplemented as in Bottenstein et al. (1980) plus 100 μg/ml bovine pituitary extract, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 ng/ml mouse 7S NGF. Because serum contains antithrombin III and other serine proteinase inhibitors that may affect the adrenal derived activity, we have replaced serum in our culture medium with bovine pituitary extract. All data presented here, with the exception of Figure 2*B*, are from experiments performed in the absence of serum. Bovine pituitary extract was prepared from mature bovine pituitaries (Pelfreez) as described by Tsao et al. (1982) with minor modifications. Briefly, pituitaries were thawed in water, washed, and trimmed. They were then extracted in 2.4 ml of CMF-HBSS per gram of tissue by homogenization in a blender at moderate speed for a total of 10 min using five pulses with brief rests between pulses to allow the extract to cool. The extract was stirred for 90 min at 4°C and then centrifuged at 24,000 × *g* for 30 min. The resulting supernatant was dialysed against 20 volumes of PBS twice for 4 or more hours and then additionally dialyzed against 10 volumes of F-12 medium overnight. The bovine pituitary extract was spun at 100,000 × *g* for 1 hr prior to filtration through 0.2 μm cellulose acetate. Extraction, centrifugation, and dialysis were done at 4°C. Aliquots of bovine pituitary extract were frozen at −20°C and retained growth supporting activity for a minimum of 3 months. Protein concentrations of bovine pituitary extracts and adrenal extracts were determined according to Bradford (1976).

Preparation of adrenal plasma membrane extract. Fresh adrenal glands from newborn calfs (≤6 d old) were packed on ice and obtained the day after slaughter from Pelfreez. Fat and connective tissue were trimmed away, the glands were minced with razor blades and then homogenized in a dounce homogenizer with CMF-HBSS containing 20 mM HEPES pH 7.4, 2 mM EDTA, 50 μM leupeptin, and 200 KIU/ml aprotinin. A fraction enriched for plasma membranes was prepared by discontinuous sucrose density centrifugation (Raper and Kapfhammer, 1990). Briefly, the adrenal homogenate was centrifuged at 16,000 × *g* and washed twice with CMF-HBSS containing 20 mM HEPES pH 7.4 and 2 mM EDTA. The resulting pellet was resuspended in 2 volumes of 2.25 M sucrose in PBS to give a final concentration of 1.5 M sucrose. This was layered onto a 2.25 M sucrose cushion, overlayed with CMF-HBSS, 20 mM HEPES pH 7.4, and 2 mM EDTA, and spun at 16,000 × *g* for 30 min. The membranes were removed from the interface of the CMF-HBSS and 1.5 M sucrose layers and washed three times with CMF-HBSS containing 20 mM HEPES pH 7.4 and 2 mM EDTA. Extracts were prepared by thawing an aliquot of packed membranes at room temperature and homogenizing them in twice their volume of PBS containing 5% Chaps and 20 mM HEPES pH 7.4. The extracts were centrifuged at 100,000 × *g* for 1 hr and the pellets discarded. Detergent was removed from the extracts before use in the collapse assay by dialyzing the supernatant twice against 500 volumes of PBS for 4–16 hr each (20 hr total) and then against 100 volumes of F-12 medium for at least 4 hr.

Collapse assay. Dialyzed extracts were added to explant cultures in volumes not exceeding 10% of the total volume of the medium (≤50 μl in 500 μl). Cultures were incubated with the adrenal extract for 1 hr and then fixed with 4% paraformaldehyde in PBS pH 7.4 containing 10% sucrose. Coverslips were mounted onto petriperm dishes (Heraeus) and viewed with a 40× phase objective. Growth cones at the periphery of explants were scored for collapse if they were not in contact or close proximity to other growth cones or neurites. Growth cones contacting other growth cones or neurites were not scored. A growth cone without lamellipodia was scored as collapsed. In comparing the activity between different conditions the protein concentrations that caused 50% of the test growth cones to collapse were compared. In those cases where one test group never achieved a level of 50% collapse, the maximal level of collapse achieved by the least affected sample was used as the comparison point.

Treatment with proteinase inhibitors. For the irreversible inhibitors, 10 μM *N*-*p*-tosyl L-lysine chloromethyl ketone (TLCK), 10 μM *N*-tosyl L-phenylalanine chloromethyl ketone (TPCK), 10–100 μM *p*-nitrophenyl *p*′-guanidinobenzoate (NPGB), 10 μM diisopropyl fluorophosphate (DFP), 10 μM phenylmethylsulfonyl fluoride (PMSF), and 1 nM-10 μM D-phenylalanyl L-prolyl L-arginyl chloromethyl ketone (PPACK), inhibitor was incubated with the extract for one hour at room temperature prior to removal of detergent from the extract. Control extracts were

treated identically to inhibitor treated extracts with vehicle buffer in place of inhibitor. Both detergent and excess inhibitor were then removed by dialyzing the supernatant twice against 500 volumes of PBS for 4–16 hr each (20 hr total) and then against 100 volumes of F-12 medium for at least 4 more hours before using the extracts in the collapse assay. Each extract was dialyzed seperately from all others. The medium against which each treated extract was dialyzed was also tested for collapsing activity. A collapsing response from this control would suggest that trace amounts of inhibitor left in the matching inhibitor treated extract could induce a confounding collapse response of its own. For the reversible inhibitors, aprotinin (200 KIU/ml), leupeptin (50 μM), soybean trypsin inhibitor (200 μg/ml), antithrombin III (1.25 U/ml), or hirudin (5 U/ml) were added to both the cultured explants and the test extract 15 min before using the extract in the collapse assay. The control for these experiments was to add the appropriate buffer without inhibitors to both the adrenal extract and the test culture.

The effectiveness of added inhibitors are expressed as the amount by which they shifted the concentration of adrenal extract that yielded a given level of growth cone collapse. Inhibitors that induced shifts of less than 20% were considered to be ineffective.

Enrichment of activity by anion exchange chromatography. For the experiment shown in Figure 6, we used adrenal membrane derived activity that had been enriched by anion exchange chromatography. This was necessary because the crude extract contained particulate matter that partially obscured the test growth cones being videorecorded. Detergent extracts of adrenal membranes were prepared as before except that membranes were first washed with a low salt buffer of 10 mM Tris and 10 mM HEPES pH 7.65, and then extracted in PBS containing 5% sodium cholate and 20 mM HEPES pH 7.4. Fresh Q sepharose (fast flow, Sigma) was alternately washed with 0.5 M NaCl containing 0.1% sodium cholate and 20 mM HEPES pH 7.4, then 10 mM Tris and 10 mM HEPES pH 7.65, followed by 0.5 M NaCl containing 0.1% sodium cholate and 20 mM HEPES pH 7.4 and finally preequilibrated with extraction buffer. Column bed volume was 1/4 to 1/3 extract volume. Extract was centrifuged at 100,000 × *g* for 1 hr at 4°C and the supernatant was then applied to the Q column. The column was washed with 10 column volumes of PBS containing 0.1% sodium cholate and 20 mM HEPES pH 7.4 and eluted with 0.5 M NaCl containing 0.1% sodium cholate and 20 mM HEPES pH 7.4. The first 0.3 column volumes of eluate was discarded and 3 column volumes of eluate were retained. Q eluate was dialyzed twice against 500 volumes of PBS for a total of 20 hr, once against 100 volumes of F-12 for a miniumum of 4 hr, and then applied to explant cultures which had been prepared for video recording. Q eluates prepared in this manner were enriched for retinal growth cone collapsing activity 30–140-fold as measured separately by the usual collapse assay.

Video microscopy. Two to six hours prior to being moved to a heated stage for viewing, the culture medium was replaced by 2.5 ml of CO_2-independent medium identical to the growth medium with the exception that 20 mM HEPES pH 7.4 replaced the $NaHCO_3$. The culture medium was overlaid with 1 ml of mineral oil preequilibrated with F12 medium to prevent evaporation of the medium during filming. Images were recorded at 1–5 min intervals as described in Fan et al. (1993). Growth cones were viewed by phase contrast through a 40× Plan lens and the image further magnified by 1.6× Optovar on an Axiovert 35 (Zeiss). For the experiments shown in Figure 6, thrombin (120 μl at 50 U/ml) or 120 μl of Q eluate (dialyzed against F12) was added to 380 μl of the CO_2 independent medium preequilibrated at 37°C and then introduced to the culture dish at several points. A solution of 0.05% Coomassie brilliant blue takes about 10 min to completely disperse when added in a similar fashion.

Collection of data and treatment with inhibitors for video recorded encounters between temporal retinal growth cones and various neurites. Fields were chosen for video recording that were as free as possible from additional interfering growth cones or neurites. The angle of approach between growth cones and axons was chosen to be 45° or greater because shallow angle encounters often do not lead to full collapse (Fan and Raper, 1995). Data from encounters in which a test growth cone contacted another growth cone were discarded. Data were also discarded if the test growth cone encountered a second neurite before reaching the arbitrary fixed crossover point of 8 μm. Only one crossover per test growth cone was used in the analysis of data even for growth cones that crossed additional neurites. The one exception to this rule was made for some temporal retinal growth cones that crossed temporal neurites well before encountering test DRG or nasal retinal neurites. In these few cases, the encounter with the temporal neurite was also analyzed for inclusion in the controls. The growth cone was filmed for a minimum of 60 min beyond the predicted time of crossover, or until crossover itself, whichever occurred first.

For encounters recorded in the presence of 50 μM PPACK, 15 μl of 10 mM PPACK (Aminobiotech) in 1 mM HCl was added to 485 μl of the CO_2 independent medium preequilibrated at 37°C and introduced to the culture dish at several places. For encounters in the presence of 5 U/ml hirudin, a 6× solution of hirudin was prepared from 30 μl of a 500 U/ml stock of lyophilized hirudin (Sigma) dissolved in 470 μl F12 medium. We determined that PPACK must be added a minimum of 20 min prior to contact with the test neurite to be effective. Early data collected with insufficient incubation time were discarded. A 10 min minimum preincubation time was used for encounters recorded in the presence of hirudin since longer preincubation times were found to give the same results as shorter times. The half lifes of PPACK and hirudin under these culture conditions were determined by assaying their ability to inhibit thrombin in an *in vitro* colorometric assay using *N-p*-tosyl-Gly-Pro-Arg *p*-nitroanilide as a substrate (Lottenberg et al., 1981). PPACK had a half-life of between 1 and 2 hr. Hirudin retained 75% of its thrombin inhibiting activity for up to 4 hr. Erase is very effectively inhibited by incubating it for 1 hr at room temperature with 10 μM PPACK. We used 50 μM PPACK in these experiments to assure that a sufficient concentration of PPACK was present for the duration of the filming period. Aliquots of inhibitor stocks were made and kept frozen at −70°C and thawed immediately before their use.

Analysis of encounters. Each video recording was converted into a graph relating the distance in mirometers between the growth cone and the neurite in its path as a function of time in minutes. Distance was measured from a point at the approximate center of the lamellipodia of the growth cone to the point on the neurite where the growth cone eventually contacts it. To reduce the contribution of variations in growth cone speed and size to the analysis, we compared how long each growth cone was delayed by contact with a neurite. The average speed of the growth cone at the time of contact was estimated from the position versus time plot. The time at which the center of the growth cone would be predicted to pass 8 μm past the neurite was estimated from the average speed of advancement before contact. The predicted time of crossover was compared to the actual time of crossover to determine how long the growth cone was delayed by contact with the neurite. In measuring the lagtime between contact and the initiation of collapse, the time at which the lamellipodia made contact with the neurite was considered the time of contact because filapodial contact alone was seldom a sufficient stimulus to induce collapse.

Results

A serine proteinase collapses CNS growth cones

Extracts from adrenal membranes collapse CNS growth cones better than PNS growth cones. Detergent extracts of newborn calf adrenal plasma membranes that have had the detergent removed by dialysis, induce the collapse of retinal growth cones but have little effect on DRG growth cones (Fig. 1). Retinal growth cones are at least 5–15-fold more sensitive to the adrenal extract than are DRG growth cones. Adrenal membrane extracts also effectively collapse growth cones from both chick diencephalic and tectal explants but have little effect on either ciliary or sympathetic growth cones (data not shown). The ability of adrenal derived collapsing activity to affect CNS growth cones in preference to PNS growth cones is similar to that expected for the hypothetical peripheral axonal label.

Adrenal derived collapsing activity acts rapidly and is reversible. Near maximal levels of retinal growth cone collapse are seen within 15 min of exposure to the adrenal extract (Fig. 2*A*). The percentage of collapsed growth cones remains constant for at least 4 hr. The assay does not distinguish between growth cones that have remained collapsed for 4 hr and those which have collapsed more recently, so we cannot conclude that a given growth cone remains collapsed for the entire 4 hr period, only that the percent of affected growth cones remains stable. If the extract is applied for 30 min and then washed out with three

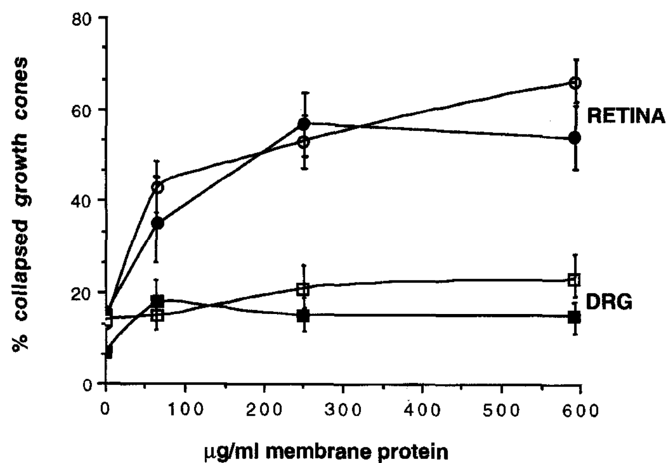

Figure 1. Adrenal extract collapses retinal ganglion cell growth cones better than DRG growth cones. Retinal and DRG explants were grown on silanized coverslips that had been incubated for 1 hr at room temperature with either 40 µg/ml mouse laminin or 45 µg/ml chick G4 protein and then blocked with 3 mg/ml hemoglobin. Detergent extracts of plasma membranes isolated form newborn bovine adrenals were applied to serum-free chick retina (*circles*) or DRG (*squares*) explant cultures after removal of detergent by dialysis. Cultures were fixed one hour after addition of extract and scored for collapsed and intact growth cones. The adrenal extract has the same level of collapsing activity towards retinal growth cones extending on either laminin (*open circles*) or G4 protein (*solid circles*). It has little collapsing activity towards DRG growth cones extending on either laminin (*open squares*) or G4 protein (*solid squares*). Error bars indicate 95% confidence limits of the means estimated using the assumption that the data are distributed according to a binomial distribution.

changes of medium, collapsed growth cones recover their spread morphology. The percentage of collapsed growth cones approaches control levels within 30 min after washout (Fig. 2*B*). The response of growth cones to the adrenal extract is therefore rapid, long lasting, and reversible.

The adrenal derived collapsing activity is reduced by serine proteinase inhibitors. Collapsing activity was lost when 10 µM of the irreversible serine hydrolase inhibitor *p*-nitro *p'*-guanidinobenzoate (NPGB) was added to the cocktail of proteinase inhibitors present during the preparation of adrenal membranes. Treatment of the adrenal membrane extract with 100 µM NPGB for 1 hr at room temperature, followed by dialysis to remove excess inhibitor, inhibited 95% of the growth cone collapsing activity of the extract (Fig. 3*A*). Similar treatment with 10 µM diisopropyl fluorophosphate (DFP) decreased the collapsing activity of the adrenal extract to 46% of control values, and treatment with 10 µM phenylmethylsulfonyl fluoride (PMSF) decreased collapsing activity to 29% of control values (data not shown). Inhibition of growth cone collapsing activity by three chemically distinct serine hydrolase inhibitors suggests that the adrenal derived activity is a serine hydrolase and could be either a proteinase or an esterase.

In order to identify the class of serine hydrolase that the adrenal derived collapsing activity belongs to, we screened a number of serine proteinase inhibitors for their ability to decrease the growth cone collapsing activity of the adrenal extract (Table 1). The inhibitors selected are effective against different subsets of serine proteinases. For example *N*-tosyl phenylalanine chloromethyl ketone (TPCK) is an active site directed inhibitor that has Phenylalanine at the amino side of the mock scissile bond (the P_1 site by the nomenclature of Schechter and Berger, 1967).

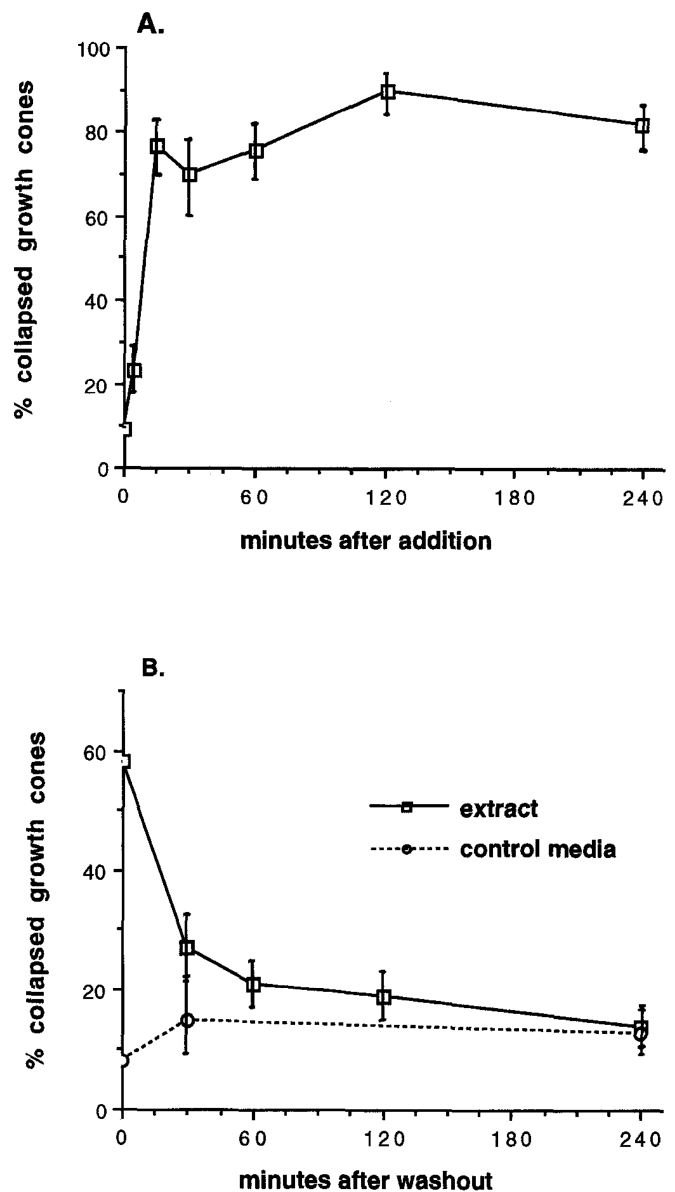

Figure 2. Adrenal extract causes rapid and reversible collapse. *A*, Adrenal extract (430 µg/ml) was added to retinal explant cultures at various time points prior to fixation and the fixed cultures were then scored for collapse. *B*, Retinal cultures were treated with 1.15 mg/ml adrenal extract for 30 min (*solid squares*) or F12 medium (*open circles*). Extract was removed from the cultures by washing them three times with serum-containing media. Explants were fixed at various time points after washing and then scored for collapse. Control cultures were subjected to the same washes as extract treated cultures. The zero time points are from treated but unwashed wells. Error bars indicate 95% confidence limits of the means estimated using the assumption that the data are distributed according to a binomial distribution.

TPCK inhibits chymotrypsin-like serine proteinases but not trypsin-like or elastase-like serine proteinases. PPACK and *N*-tosyl lysine chloromethyl ketone (TLCK) are active site directed irreversible inhibitors with the basic amino acids arginine or lysine, respectively, in the P_1 site. They inhibit trypsin-like but not chymotrypsin-like or elastase-like serine proteinases.

We found that pretreatment of the adrenal extract with the irreversible inhibitor PPACK at 1 nM, 100 nM, and 10 µM for 1 hr at room temperature inhibited the adrenal derived collapsing

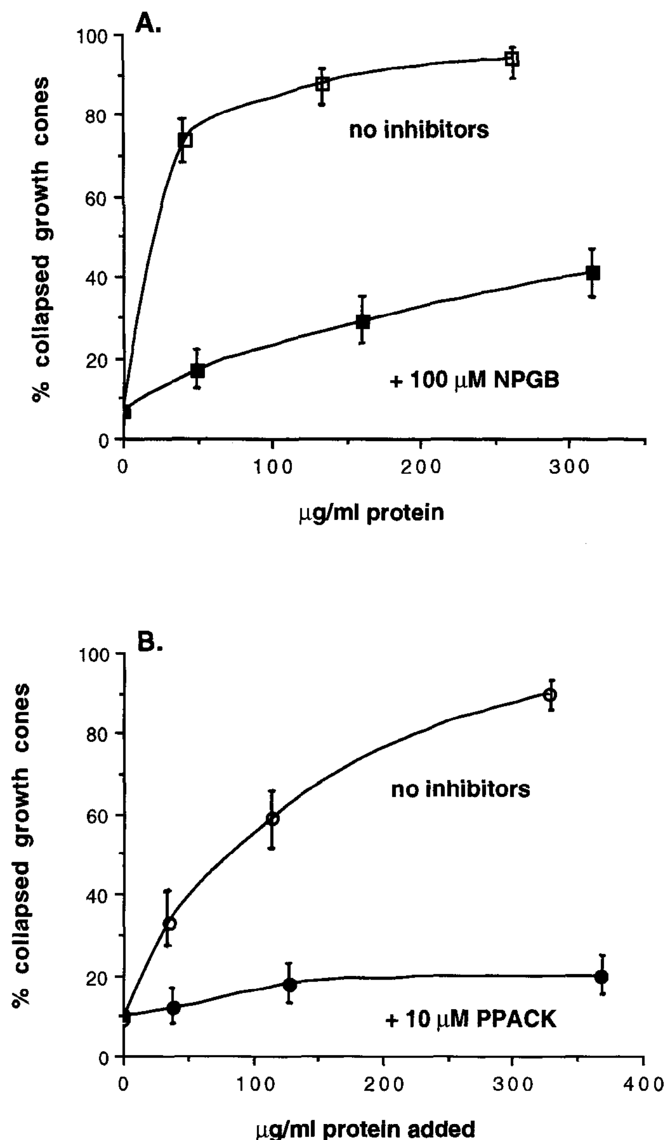

Figure 3. Adrenal derived collapsing activity is inhibited by serine proteinase inhibitors. *A*, Adrenal extract was incubated for 1 hr at room temperature with 100 μM NPGB (*solid squares*) or F12 medium (*open squares*) prior to dialysis and then applied to retinal explant cultures. *B*, Adrenal extract was incubated for 1 hr at room temperature with 10 μM PPACK (*solid circles*) or F12 (*open circles*) prior to dialysis and then applied to retinal explant cultures. Error bars indicate 95% confidence limits of the means estimated using the assumption that the data are distributed according to a binomial distribution.

activity 59%, 90%, and 96% (Fig. 3*B*). This suggests that the adrenal derived collapsing activity belongs to the trypsin-like subclass of serine proteinases. Since the irreversible inhibitor *N*-tosyl lysine chloromethyl ketone had no effect on the adrenal derived collapsing activity (Table 1), the adrenal activity is probably a trypsin-like serine proteinase with a preference for arginine in the P_1 site. While PMSF and PPACK will also inhibit some cysteine proteinases, DFP is thought to only inhibit serine proteinases (Salvesen and Nagase, 1989). Therefore, it is unlikely that the adrenal derived activity is a cysteine proteinase. Several common reversible inhibitors of trypsin-like serine proteinases were also tried. Antithrombin III, a reversible proteinase inhibitor that is found in serum, decreased the adrenal derived activity 50% when present at a concentration of 1.25 U/ml (data not shown).

The collapsing activity of the adrenal extract is substratum independent. The adrenal derived collapsing activity was tested for its ability to affect growth cones extending on two different substrata. The extracellular matrix protein EHS laminin was used in our standard assay. In addition, we used as an alternative substratum the cell surface axonal glycoprotein G4/NgCAM (Rathjen et al., 1987). CSAT antibody (Neff et al., 1982), a monoclonal antibody directed against the β_1 subunit of integrin, detaches both retinal and DRG growth cones and axons from laminin but not from G4. A monoclonal antibody directed against G4 (Rathjen et al., 1987) detaches retinal growth cones and axons from G4 but not from laminin. We therefore infer that both retinal and DRG growth cones grow on these two substrata using independent cell surface receptors. The adrenal derived collapsing activity is equally effective in inducing the collapse of retinal growth cones extending on either laminin or G4 (Fig. 1). It induces little collapse of DRG growth cones extending on either substrate.

A comparison of the adrenal derived collapsing activity with the blood coagulation enzyme thrombin. The adrenal derived collapsing activity can be distinguished from thrombin, a serine proteinase that causes neurite retraction in both CNS and PNS neurons and neuronal cell lines (Hawkins and Seeds, 1986; Gurwitz and Cunningham, 1988; Grand et al., 1989; Jalink and Moolenaar, 1992). We found that the effects of the adrenal derived collapsing activity and thrombin differed in several respects. *First*, they affected different neural cell types. The adrenal derived activity is more effective in collapsing retinal (Fig. 4*B*). as compared to DRG growth cones (Fig. 4*E*). In contrast, thrombin affects both equally (Fig. 4*C,F*). Thrombin also causes retraction of ciliary ganglia neurites while the adrenal derived activity has little effect on these peripherally derived growth cones (not shown). These differences between the effects of the adrenal derived activity and thrombin are not simply due to differences in the potencies of the two proteinases. The same specificities are apparent when using concentrations of thrombin that cause only half of the test neurites to respond. *Second*, thrombin and the adrenal derived activity differ in their susceptibility to hirudin, a selective inhibitor of thrombin. The addition of 5 U/ml of hirudin to the culture medium inhibits thrombin induced neurite retraction (Fig. 5*A*) but has no effect on the adrenal derived collapsing activity (Fig. 5*B*). *Third*, the degree of neurite retraction is significantly greater for thrombin as compared to adrenal extract treated neurites.

Time lapse video microscopy was used to follow the reaction of retinal growth cones to either the adrenal derived collapsing activity or thrombin. Collapsing activity enriched on an anion-exchange column was used in this series of experiments. This enriched activity, like that in the crude adrenal extracts, collapses retinal but not DRG growth cones and is blocked by PPACK. The adrenal derived collapsing activity causes retinal growth cones to collapse in place while inducing minimal shortening of neurites (Fig. 6*A–C*). The median retraction of retinal neurites treated with adrenal extract was 18 μm in 31 trials (for comparison, the median length of the growth cones is 14 μm). In contrast, thrombin frequently causes extensive neurite retraction (Fig. 6*D–F*; note especially the retraction of the bottom neurite of the pair). The median retraction of thrombin treated retinal neurites was 44 μm in 15 trials. The probability that the measured thrombin and adrenal extract induced retractions are drawn

Table 1. Serine proteinase inhibitors assayed for inhibition of adrenal derived collapsing activity

Type of inhibitor	Inhibitors that decrease adrenal derived collapsing activity	Inhibitors with no effect on adrenal derived collapsing activity
General irreversible inhibitors of serine hydrolases	NPGB DFP[a] PMSF[a,b]	
Active site directed irreversible inhibitors of serine proteinases	PPACK[b]	TLCK[b] TPCK[a,b]
Reversible inhibitors of some serine proteinases	Antithrombin III	Hirudin Aprotinin Soybean trypsin inhibitor[a] Leupeptin[b]

NPGB (*p*-nitrophenyl *p'*-guanidinobenzoate) is effective at concentrations of 10–100 μM. PPACK (*N*-tosyl L-phenylalanine chloromethyl ketone) is effective at concentrations of 1 nM–10 μM. DFP (diisopropyl fluorophosphate), PMSF (phenylmethylsulfonyl fluoride), TLCK (*N-p*-tosyl L-lysine chloromethyl ketone), and TPCK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone) were tested at a single dose of 10 μM. These irreversible inhibitors were added to adrenal extract prior to dialysis and incubated at room temperature for 1 h. Unreacted inhibitor was removed by dialysis. Controls for each inhibitor were treated with the buffer vehicle identically to inhibitor treated samples. For the reversible inhibitors aprotinin (200 KIU/ml), leupeptin (50 μM), soybean trypsin inhibitor (200 μg/ml), antithrombin III (1.25 U/ml), and hirudin (5 U/ml), inhibitor was added to both the cultured explants and the test extracts 15 minutes prior to their use in the collapse assay. Inhibitors were considered ineffective if the specific activities of the inhibitor treated and control extracts differed by less than 20% (the specific activity is defined as the protein concentration of the extract causing 50% of the growth cones to collapse).

[a] Have only been assayed in presence of serum.

[b] Will also inhibit some cysteine proteinases.

from a similar population is estimated to be $p < 0.001$ by the nonparametric Wilcoxan rank test. The concentrations of thrombin and adrenal extract used in these measurements cause similar levels of collapse in the collapse assay (60–80%, not shown), so the difference in retraction is not simply due to differences in the potencies of the two enzymes.

We considered the possibility that the adrenal derived activity was prothrombin and that retinal growth cones or explants, but not the DRG growth cones or explants, are able to convert prothrombin to thrombin. In this case, the addition of prothrombin to the culture medium should collapse retinal but not DRG growth cones. We found that prothrombin had little effect on either type of growth cone (data not shown). We considered the possibility that retinal explants but not DRG explants produced prothrombin and that the adrenal derived extract was the prothrombin converting proteinase, Factor Xa. In this case, the addition of Factor Xa should collapse retinal but not DRG growth cones. Factor Xa has little collapsing activity towards either type of growth cone (data not shown).

Cultured rat sympathetic neurons and mouse sensory neurons release both tissue-type and urokinase-type plasminogen activators (Pittman et al., 1989; Seeds et al., 1992). Inhibitors of the plasminogen activators, as well as inhibitory antibodies to urokinase increase neurite outgrowth in serum-free culture (Zurn et al., 1988; Pittman et al., 1989). We therefore tested plasminogen activators for growth cone collapsing activity. Neither human two chain tissue plasminogen activator nor human one-chain urokinase affected retinal or DRG growth cones in the collapse assay at the concentrations tested (data not shown).

Relationship between adrenal derived collapsing activity and a previously descibed peripheral axon label

Peripheral axons express a label that induces retinal ganglion cell growth cones to collapse on contact (Kapfhammer and Raper, 1987b). If the adrenal derived activity is the same as the avoidance cue associated with peripheral neurites, then PPACK should prevent retinal growth cones from collapsing on contact with peripheral neurites, but should not interfere with their response to neurites that express other avoidance cues.

Temporal retinal growth cones cross DRG neurites in the presence of the proteinase inhibitor PPACK. Data were collected by video microscopy from temporal retinal ganglion growth cones that contact DRG or nasal retinal neurites in the absence of proteinase inhibitors (control condition) or in the presence of 50 μM PPACK. Under control conditions, temporal retinal growth cones generally collapsed on contact with DRG neurites and were therefore delayed in crossing them by 60 min or more (second column, Table 2). An example of a typical encounter between a retinal growth cone and a DRG neurite in the absence of PPACK is shown in Figure 7*A–D*. The growth cone was spread and advanced at a steady pace as it approached a DRG neurite (Fig. 7*A*). The lamellae of the retinal growth cone contacted the DRG neurite 22 min later (Fig. 7*B*). Collapse of the retinal growth cone was initiated 2 min after lamellipodial contact (Fig. 7*C*). The retinal growth cone collapsed and remained so for more than 2 hr after contact (Fig. 7*D*). Approximately half of the growth cones that collapsed on contact with DRG axons remained so for more than the minimal 80 min observation period. The remainder reassembled after 15–75 min but were rarely seen to cross DRG neurites within the same observation period.

In the presence of 50 μM PPACK, almost all temporal retinal growth cones were able to cross DRG neurites within 60 minutes of lamellipodial contact (Table 2). Temporal growth cones crossed DRG neurites without collapsing in more than half of the encounters. An example of this type of response is shown in Figure 7*E–H*. A retinal growth cone approached a DRG neurite (Fig. 7*E*). Eleven minutes later it made lamellipodial contact with the neurite (Fig. 7*F*). The growth cone did not collapse and continued to advance steadily, crossing the DRG neurite within

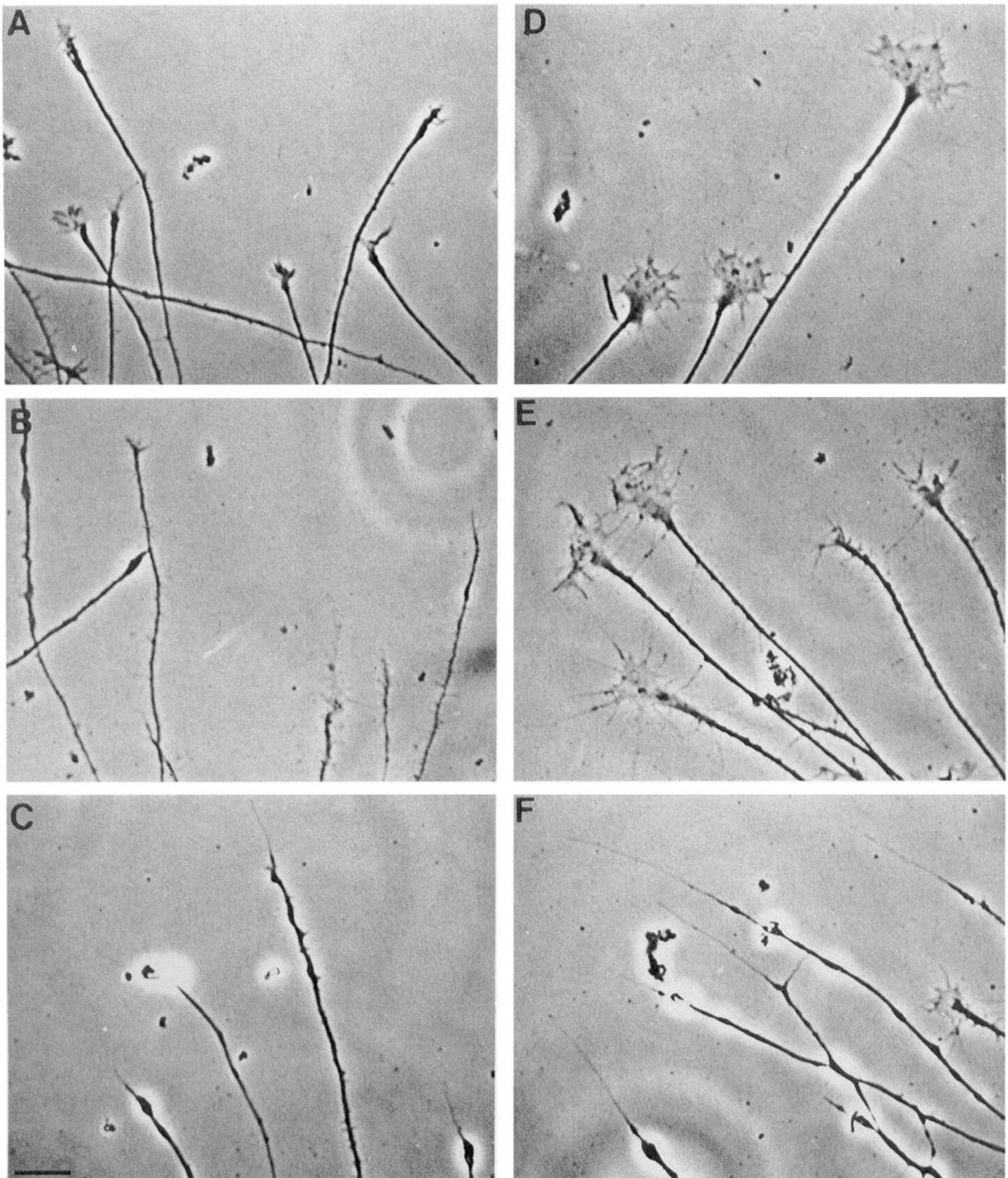

Figure 4. Adrenal extract collapses only retinal growth cones while thrombin affects both retinal and DRG neurites. Retinal explants (*A–C*) and DRG explants (*D–F*) were treated with control F12 medium (*A, D*), 782 μg/ml of adrenal extract (*B, E*), or 1 U/ml thrombin for 1 hr prior to fixation. The total percent collapse for each condition was 13% for *A*, 57% for *B*, 61% for *C*, 3% for *D*, 11% for *E*, and 57% for *F*. Scale bar, 20 μm.

17 min after first contact (Fig. 2*G,H*). Some temporal retinal growth cones encountering a DRG neurite in the presence of PPACK collapsed for a brief interval, reassembled, and then crossed the neurite on the second attempt. An example of this kind of interaction is shown in Figure 8. A retinal growth cone collapsed when first contacting a DRG neurite, but later crossed over on a subsequent approach.

PPACK decreases collapse frequency and duration. Since it

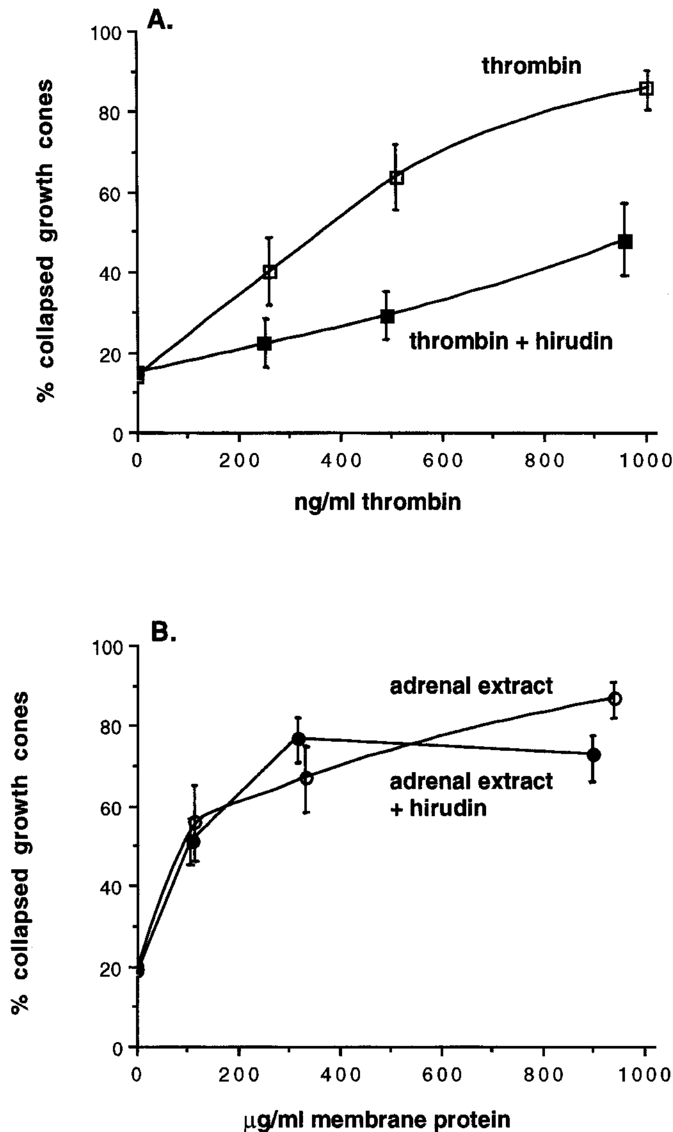

Figure 5. The thrombin inhibitor hirudin inhibits thrombin's action on retinal growth cones but does not inhibit the collapsing activity of adrenal extract on retinal growth cones. *A*, Thrombin's action on retinal neurites is substantially reduced in the presence of 5 U/ml hirudin. Explants and thrombin were pretreated with hirudin for 15 min prior to the addition of thrombin. Controls were mock treated with F12 medium. The maximum concentration of thrombin used in the assay was equivalent to 2 coagulation units per ml (manufacturer's measurement). One unit of hirudin is defined as the amount necessary to inhibit 1 unit of thrombin. *B*, 5 U/ml hirudin has no inhibitory effect on the collapsing activity of the adrenal extract on retinal growth cones. Error bars indicate 95% confidence limits of the means estimated using the assumption that the data are distributed according to a binomial distribution.

is collapse that prevents growth cones from crossing DRG axons, we examined the effect PPACK may have on the frequency and duration of growth cone collapse. If PPACK treatment completely neutralizes the retinal avoidance cue on DRG neurites, the collapse of retinal growth cones that normally occurs on contact with DRG neurites should be completely abolished. Other more subtle aspects of collapse might be affected by incomplete neutralization. As the avoidance cue is weakened, the delay between a growth cone's contact with a DRG neurite and the start of collapse might be expected to lengthen, the duration of collapse might also be expected to decrease, and the likelihood that a collapsed and reformed growth cone crosses the DRG neurite on a second attempt might be expected to increase.

Temporal retinal growth cones encountering DRG neurites in the absence of PPACK generally collapsed on first contact, remained collapsed for a long time, and if they recovered, generally collapsed on a second contact (Table 2, Fig. 9*A*). They therefore were significantly delayed in crossing DRG neurites. Temporal retinal growth cones encountering DRG neurites in the presence of PPACK usually did not collapse on first contact. Those that did collapse were slower to initiate collapse, remained collasped for a relatively brief period of time, and were unlikely to collapse on second contact (Table 2, Fig. 9*A*). They therefore were likely to experience modest delays when crossing DRG neurites.

Temporal retinal growth cones collapse on contact with DRG neurites in the presence of the thrombin hirudin. PPACK is known to be a very effective inhibitor of thrombin (Kettner and Shaw, 1981). To determine whether PPACK's affects on growth cone collapse might be mediated through an inhibition of thrombin activity, we tested a specific thrombin inhibitor, hirudin, to see if its effects on growth cone collapse are similar to those of PPACK. In the presence of 5 U/ml hirudin, most temporal retinal growth cones collapsed and did not cross DRG neurites within 60 min of prediction. There is no statistical difference in the crossover delays of encounters in the absence as compared to the presence of 5 U/ml hirudin (Table 2, Fig. 9*A*). We confirmed that hirudin at this concentration is able to prevent the clotting of chick blood, implying that it also inhibits chick thrombin activity (data not showm). The addition of hirudin also had no effect on the frequency or extent of growth cone collapse. Almost all collapsed growth cones that reassembled, collapsed again when they contacted DRG neurites a second time. Hirudin should affect crossover delays in a manner similar to PPACK, if and only if PPACK exerts its effect by inhibition of thrombin. Our results suggest that PPACK's facilitation of retinal crossovers of DRG neurites cannot be ascribed to an inhibitory effect on thrombin.

PPACK does not prevent temporal retinal gowth cones from collapsing on contact with nasal retinal neurites. Could PPACK have a direct effect on the mechanism of growth cone collapse independent of the specific cue that initiates collapse? Similarly, could PPACK simply increase the "robustness" of retinal growth cones so they are less prone to collapse? In either case, PPACK should interfere with the collapse of temporal retinal growth cones collapsing in response to avoidance cues other than that associated with DRG neurites. To explore this possibility, the collapse of temporal retinal growth cones to contact with nasal retinal neurites was examined in the presence and absence of PPACK.

Temporal retinal growth cones collapsed when they contacted nasal retinal neurites and were subsequently delayed from crossing them by more than 60 min in 73% of the encounters filmed in the absence of PPACK, and in 71% of the encounters filmed in the presence of PPACK. PPACK also had no significant effect on the time between first contact and the initiation of growth cone collapse, or on the duration of collapse (Table 2, Fig. 9*B*). PPACK appears to specifically affect the response of temporal retinal growth cones on contact with DRG neurites. It does not affect the ability of temporal retinal growth cones to collapse in response to the avoidance cue associated with nasal retinal axons.

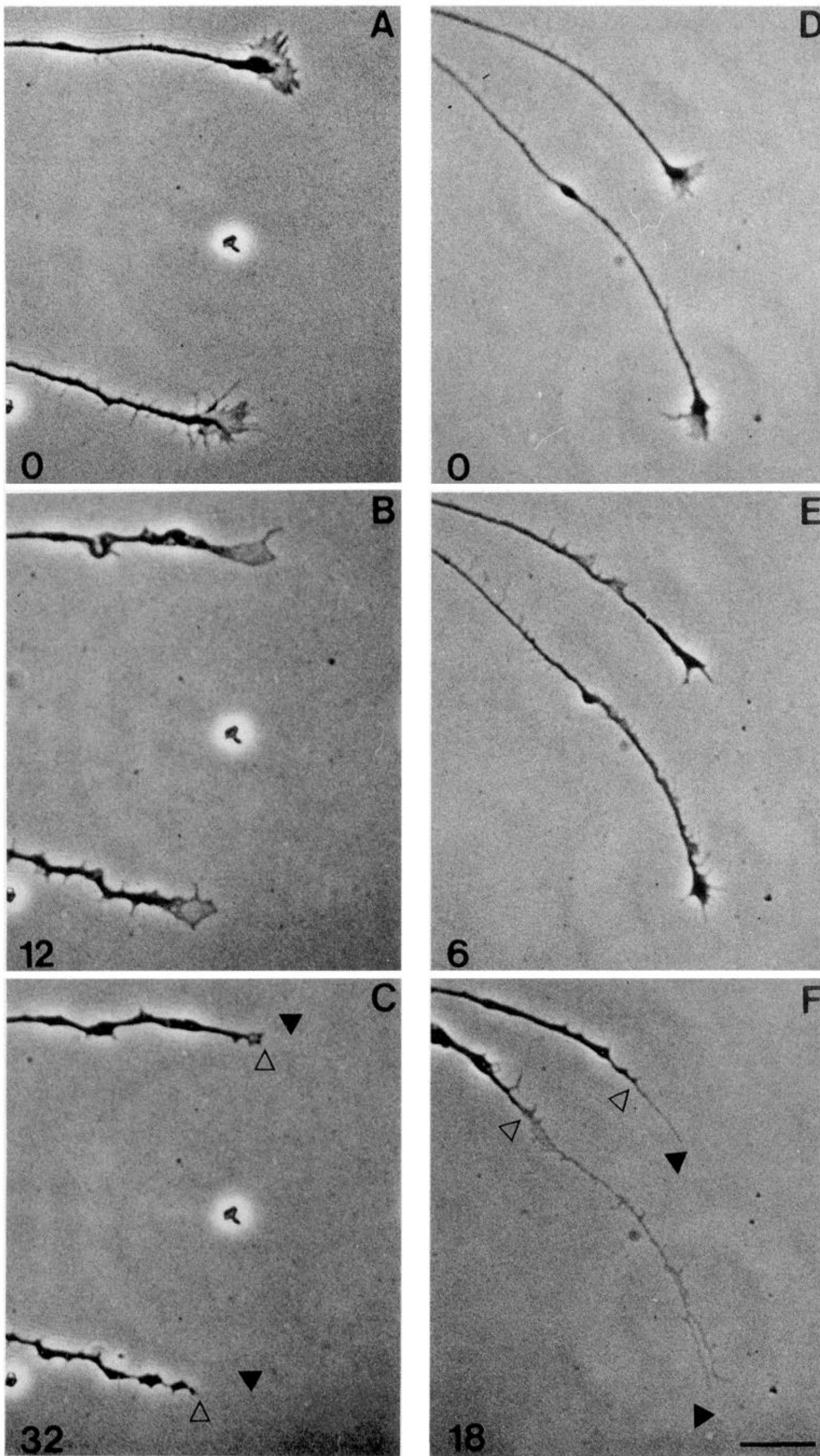

Figure 6. Adrenal extract causes retinal growth cone collapse while thrombin causes collapse and extensive neurite retraction. Retinal explants were treated with adrenal derived collapsing activity enriched ~140-fold by anion exchange chromatography on Q sepharose (*A–C*). *A* shows two growth cones 1 min prior to addition of 22 μg/ml of Q eluate. Twelve minutes later both growth cones neared full collapse (*B*), and 31 min after addition of extract they have fully collapsed and slightly retracted (*C*). Retinal explants were treated with 2 U/ml thrombin (*D–F*). *D* shows a pair of growth cones 1 min prior to addition of thrombin. Six minutes later the growth cones have collapsed but not yet begun to retract (*E*) and 17 min after addition of thrombin they have retracted significantly (*F*). *Solid arrowheads* in *C* and *F* indicate the positions of the growth cones' distal tips in *A* and *D*, respectively. *Open arrowheads* indicate the distal tips of the retracted neurites. Scale bar, 20 μm.

Table 2. Median crossover delays, collapse frequency, and duration of collapse for temporal retinal ganglion cell growth cones meeting DRG or retinal neurites

Type of neurite contacted	Inhibitor added	n	Median predicted time to crossover (min)	Median crossover delay (min)[a]	Frequency of collapse at first contact	Median duration collapse (min)[b,c]	Median lag time to collapse (min)[b]	Frequency of collapse at 2nd contact (n)[d]
DRG	None	19	15	60	95%	63	2	83% (6)
	50 μM PPACK	19	14	7*	41%	6**	7**	17% (6)
	5 U/ml hirudin	19	16	60	100%	16***	3	92% (13)
Nasal RG	None	26	12	60	73%	13	4	n.a.
	50 μM PPACK	24	13	60	71%	8	2	n.a.
Temporal RG	None	17	10	2	18%	n.a.	n.a.	n.a.
	50 μM PPACK	13	9	5	8%	n.a.	n.a.	n.a.
	5 U/ml hirudin	15	10	2	0%	n.a.	n.a.	n.a.

The type of neurite encountered is indicated in the leftmost column and the number of interactions analyzed for each condition under "n". The time that growth cones were predicted to take crossing neurites were compared to the additional delays they experienced during crossing in each condition. The percentage of growth cones that collapsed, the median duration of collapse for those that did, and the median time between first contact and collapse are noted for each condition. Also the percentage of reformed growth cones that collapsed at second contact is noted for encounters with DRG neurites. The Wilcoxon rank test was used to determine if responses in the presence of inhibitor were significantly different than those in its absence. PPACK caused a significant shortening of delay times when temporal retinal ganglion cell growth cones meet DRG neurites. PPACK also decreases several other measures of the strength of the collapsing response including collapse frequency, duration, latency, and frequency of collapse on second contact. n.a. were not analyzed. *, $p < 0.0001$; **, $p < 0.01$; and ***, $p < 0.02$ compared to similar contacts in the absence of inhibitors.

[a] In cases where delay exceeds observation period, the minimum observation period of 60 min beyond predicted time of crossover was used for statistical analysis.

[b] Only growth cones that collapsed are included in determining median values and statistical significance.

[c] For purposes of comparison the duration of collapse for those growth cones which remained collapsed beyond the period of observation was considered to terminate at 60 minutes beyond predicted time of crossover.

[d] (n) are the number of growth cones which made multiple contacts.

Discussion

We have characterized a retinal ganglion cell growth cone collapsing activity derived from the plasma membranes of adrenals. The biological and biochemical profile of this collapsing activity is distinct from those of other known collapse inducing molecules. Luo et al. (1993) recently isolated and sequenced collapsin, a glycoprotein in the embryonic and adult chick brain that induces growth cone collapse. Recombinant collapsin has potent collapsing activity against DRG growth cones but little activity against retinal growth cones, an activity profile opposite from that of the adrenal activity. Collapsing activities associated with oligodendrocytes (Bandtlow et al., 1990) or posterior somites (Davies et al., 1990) are also very effective against DRG growth cones and are therefore different from the adrenal activity. The adrenal derived activity must also be distinct from an avoidance cue associated with membranes from the posterior chick optic tectum (Stahl et al., 1990). This activity collapses temporal but not nasal retinal growth cones, while the adrenal derived activity collapses both temporal and nasal retinal growth cones equally well (data not shown). These considerations indicate that the adrenal activity is a novel collapsing factor.

The adrenal derived collapsing activity is likely to be a serine proteinase. Its activity is blocked by incubating adrenal extracts with the general serine hydrolase inhibitors NPGB, DFP, or PMSF, or with the active site directed irreversible inhibitor PPACK. It is formally possible that a serine proteinase activates a pro-collapsing activity in the adrenal extract. If the collapsing activity were generated in the adrenal extract, the late addition of PPACK to extracts preincubated in its absence should fail to block the collapsing activity. We have observed no such effect (data not shown). It is also unlikely that a pro-collapsing activity present in the extract is activated by serine proteinases produced by the retinal explant during the assay itself, since PPACK added to the extract and then removed by dialysis before the assay blocks collapse. The simplest explanation for our observations is that the collapsing activity in the adrenal extract is itself a serine protease.

The sensitivity of the adrenal derived collapsing activity to serine proteinase inhibitors further distinguishes it from other collapsing activities. No other growth cone collapsing activity that we are aware of is known to be similarly affected. The avoidance of posterior tectal membranes by temporal retinal growth cones is not blocked by serine proteinase inhibitors (Schlosshauer et al., 1990). Collapsin remains active after treatment with irreversible general serine proteinase inhibitors like NPGB, and it has no recognized sequence similarity to other serine proteinases (Luo et al., 1993).

We are naming the adrenal derived growth cone collapse inducing proteinase "erase" because it is a serine proteinase that causes growth cones to disappear. By naming this activity we do not mean to imply that erase is necessarily a previously uncharacterized serine proteinase. It is possible that erase's ability to induce the collapse of CNS growth cones is an interesting new activity of a previously characterized serine proteinase.

Although no other collapsing activity is known to be a serine proteinase, there is a serine proteinase, thrombin, that induces a dramatic retraction of neurites from both central and peripheral tissues grown in culture (Hawkins and Seeds, 1986; Gurwitz and Cunningham, 1988; Grand et al., 1989; Jalink and Moolenaar, 1992; data presented here). Like erase, thrombin is also very effectively inhibited by PPACK (Kettner and Shaw, 1981). Furthermore, prothrombin mRNA has been detected in DRGs of E15 rats using *in situ* hybridization (Dihanich et al., 1991). It is possible that erase is related to thrombin, however, several observations indicate that the two are not identical: (1) thrombin affects both central and peripheral growth cones while erase affects only central growth cones, (2) thrombin is inhibited by hirudin while erase is not, and (3) thrombin causes significantly

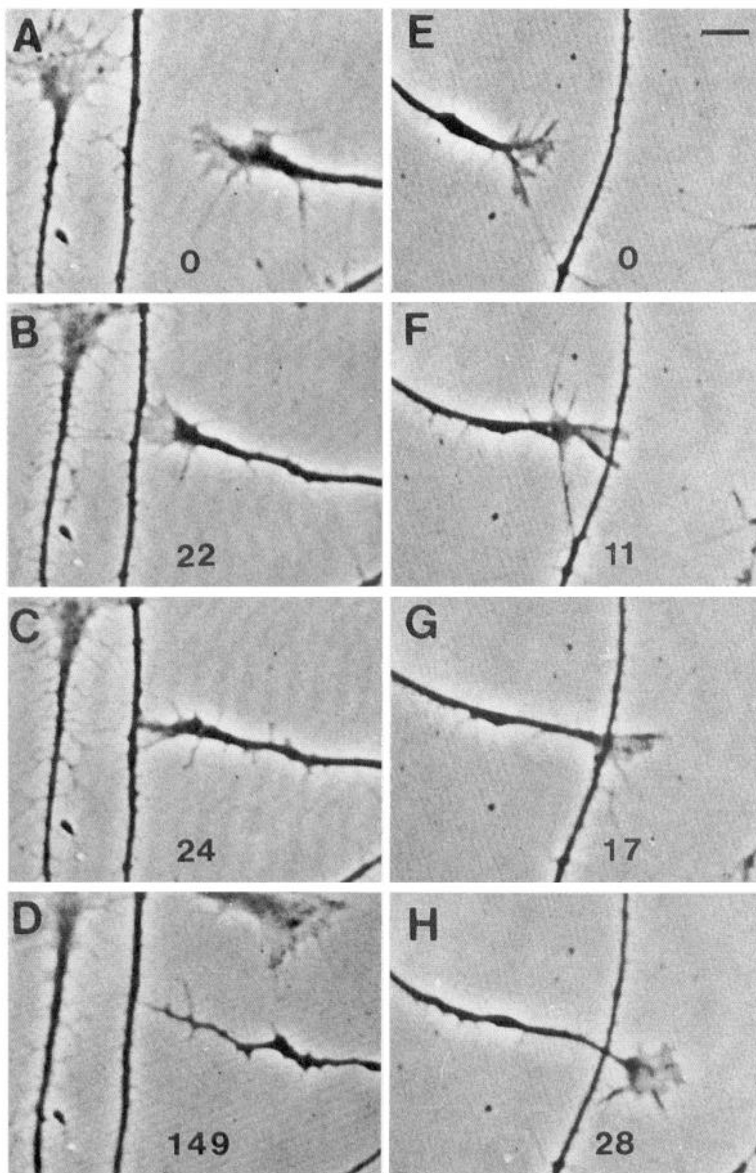

Figure 7. Temporal retinal ganglion cell growth cones contacting DRG neurites are less likely to collapse in the presence of PPACK. A comparison of the responses of temporal retinal growth cones to contact with DRG neurites in the absence (*A–D*) or presence (*E–H*) of 50 μM PPACK. In the absence of PPACK, a retinal growth cone approaching a DRG neurite from the right has a spread and motile morphology when one of its filopodia first touches the DRG neurite (*A*). After making contact with the neurite along most of its leading edge (*B*), the growth cone collapses (*C*), and remains collapsed for more than 2 hr (*D*). In the presence of PPACK, a retinal growth cone approaching a DRG neurite from the left has a spread and motile morphology when one of its filopodia first touches the DRG neurite (*E*), the lamellae remain active and cross the DRG neurite without collapsing (*F–H*). Minutes are indicated at the bottom of each panel. Scale bar, 10 μm.

greater neurite retraction than erase when each is used at concentrations which cause only a portion of tested growth cones to respond. It is possible that some other known proteinase has properties more like those of erase, but our tests with Factor Xa, tissue plasminogen activator, and urokinase have thus far failed to identify a likely match.

How might erase initiate growth cone collapse? One trivial possibility is that its enzymatic activity damages the adhesive substratum on which the growth cones extend or the receptors they use to adhere to the substratum. It is likely that the extension of both retinal and DRG growth cones on laminin is mediated by a β_1 containing integrin since both types of neurites detach from laminin when treated with the CSAT antibody. It is unlikely that proteolysis of a laminin substratum could specifically affect retinal growth cones without having a comparable effect on DRG growth cones, or that the effect could be reversible. Moreover, proteolysis of specific components of the substratum or the receptors used to recognize them are ruled out by the finding that erase collapses retinal but not DRG growth cones extending on two entirely different substrata, laminin and G4, which are recognized by independent receptors (Lemmon et al., 1989; Kleinman et al., 1991; Kuhn et al., 1991; Reichardt and Tomaselli, 1991). It therefore seems likely that erase induces collapse by some other more direct effect on growth cones.

What other ways could a serine proteinase initiate growth cone collapse? The interaction of thrombin with its receptor provides an attractive model for the action of erase on growth cone collapse. The thrombin receptor is a member of the seven transmembrane receptor family. Thrombin is thought to first bind to its receptor and then cleave off a piece of the receptor's N-terminal end. Thrombin proteolysis of the receptor thereby unmasks a portion of the receptor that acts as a tethered ligand to activate the receptor. Peptides corresponding to this region of the receptor can mimic thrombin activation of platelets (Vu et al., 1991). Activation of the thrombin receptor in platelets causes an influx of Ca^{+2} and activation of pertussis sensitive G proteins (Purdon et al., 1984; Brass and Joseph, 1985; Brass et al., 1986). Activation of the thrombin receptor in neuroblastoma cells can be blocked by inhibitors of tryosine kinases and phosphatases (Jalink and Moolenaar, 1992). Both Ca^{+2} and pertussis sensitive

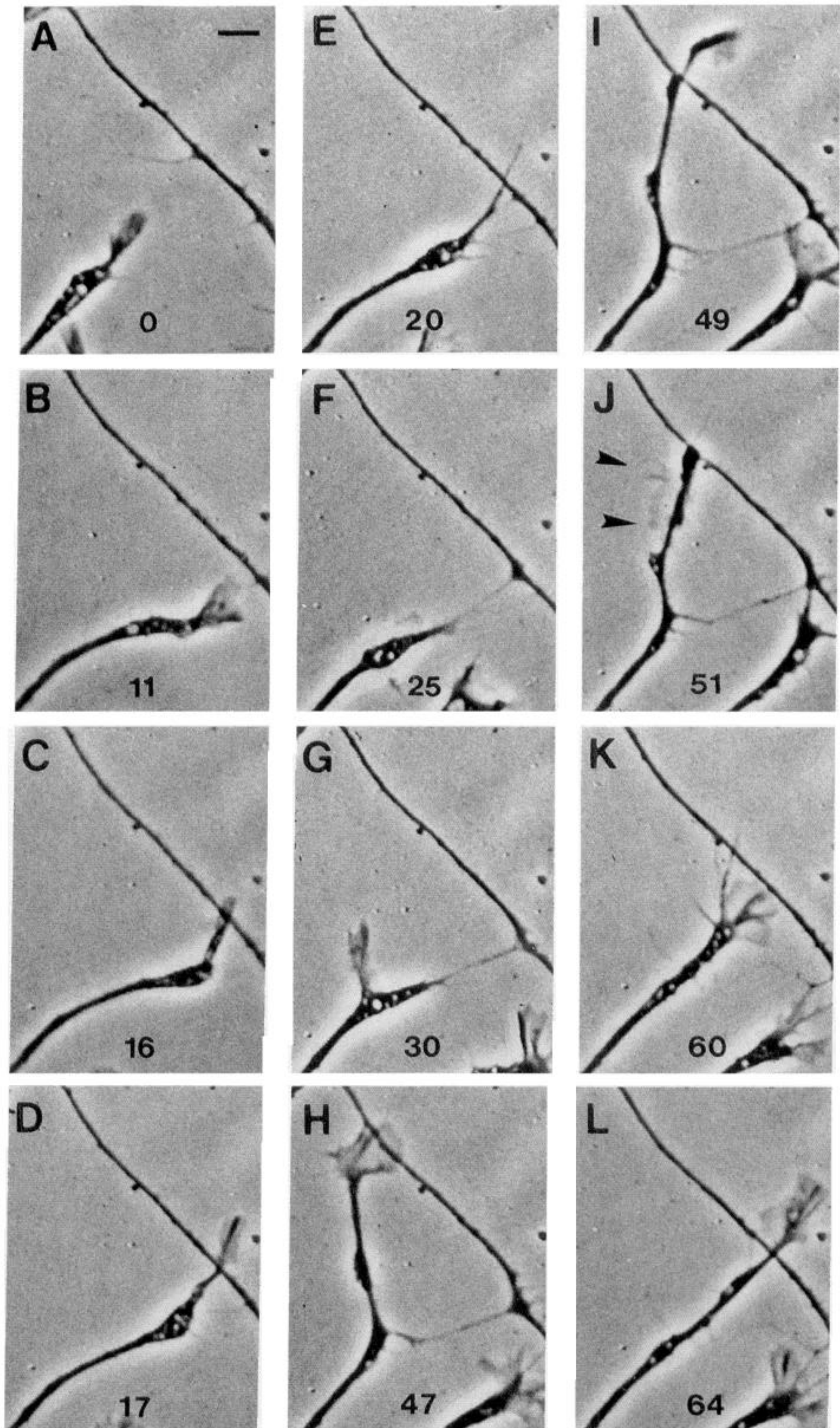

Figure 8. A temporal retinal ganglion cell growth cone in the presence of 50 μM PPACK is delayed in crossing a DRG neurite. Not all temporal growth cones cross DRG neurites without delay in the presence of 50 μM PPACK. A retinal growth cone approaches a DRG neurite from the left (*A–D*) and collapses on contact with the DRG neurite (*E, F*). A new growth cone is assembled (*G*), advances across the neurite (*H, I*), retreats slightly while remaining motile (*J, K*), and then advances across the neurite. The growth cone's lamellae in *J* are out of the plane of focus and are indicated by *arrowheads*. Minutes are indicated at the bottom of each panel. Scale bar, 10 μm.

G proteins have been suggested to play a role in some forms of growth cone collapse (Bandtlow et al., 1993; Igarashi et al., 1993). Neurite retraction in neuroblastoma cells can be initiated by the same peptide fragment of the thrombin receptor that causes platelet activation, suggesting that thrombin causes neurite retraction through activation of its receptor (Jalink and Moolenar, 1992; Suidan et al., 1992). A similar receptor mechanism may mediate erase's induction of growth cone collapse.

A proteinase could also initiate growth cone collapse without directly activating a receptor. Cleavage of another molecule on DRG axons could generate a locally diffusible collapsing molecule. An example of this kind of proteinase action is seen in

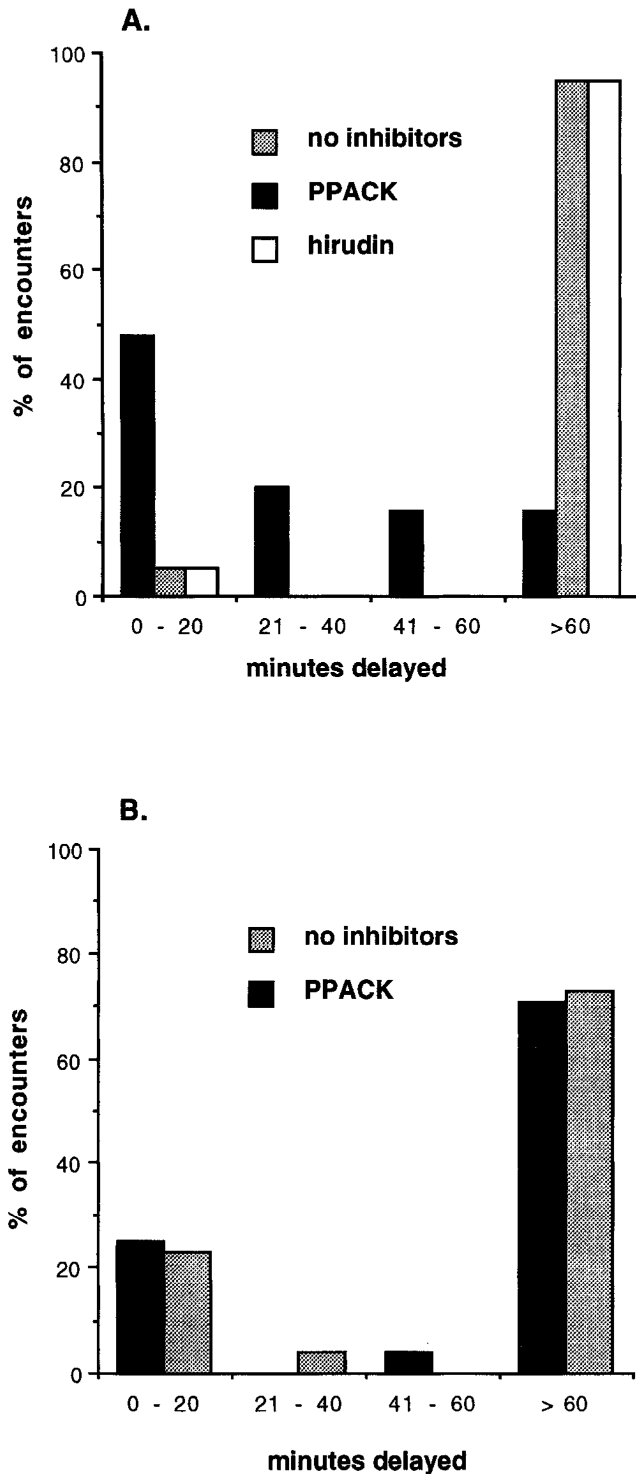

Figure 9. Temporal retinal ganglion cell growth cones cross DRG neurites more rapidly in the presence of 50 μM PPACK. *A*, The crossover delays for encounters with DRG neurites are shortened in the presence of PPACK. The percentage of growth cones that were delayed by the indicated amount are indicated for all encounters in the absence of inhibitors (*stippled bars*), the presence of 50 μM PPACK (*solid bars*), and the presence of 5 U/ml hirudin (*open bars*). In the absence of inhibitors or the presence of hirudin, nearly all growth cones are delayed by 60 min or more. Delays are shifted to shorter values in the presence of PPACK. *B*, The presence of PPACK has no effect on crossover delays in encounters with nasal retinal neurites. The percentage of growth cones that were delayed by the indicated amount are indicated for all encounters in the absence of inhibitors (*stippled bars*) and the presence of 50 μM PPACK (*solid bars*). The majority of growth cones in either condition are delayed by 60 min or more. PPACK does not affect the distribution of delay times.

the generation of peptides chemotactic for neutrophils by neutrophil elastase from heparin cofactor II (Church et al., 1991). Along these lines the existance of diffusible chemorepulsive molecules have been reported (Fitzgerald et al., 1993; Pini, 1993). It is unlikely that erase generates a collapsing activity from some component of the assay medium since the assay works in medium containing serum but no bovine pituitary extract (BPE), BPE but no serum, and neither serum nor BPE (data not shown). It is unlikely that erase generates a collapsing activity from some component of the adrenal extract itself since the addition of PPACK to extract that has been pre-incubated at room temperature for 2 hr in its absence is just as effective in blocking collapse activity as earlier additions without preincubation (our data, not shown).

The adrenal derived collapsing activity is a good candidate for the cue on peripheral axons that induces collapse of retinal ganglion cell growth cones. First, the adrenal activity affects retinal ganglion cell growth cones but not DRG or sympathetic growth cones, just like the peripheral axon activity. Second, the adrenal activity is largely neutralized by treatment with the irreversible and specific tri-peptide serine proteinase inhibitor PPACK, as is the collapse of retinal growth cones on contact with DRG axons. The blocking effect of PPACK on either the adrenal derived or the peripheral axon collapsing activities are not likely to be mediated by the inhibition of thrombin. Hirudin, a specific inhibitor of thrombin action is ineffective in blocking either activity.

PPACK is likely to neutralize the inhibitory cue on DRG axons that induces retinal collapse, and not to simply interfere in a more general way with the ability of retinal growth cones to respond to such cues. An avoidance cue on nasal retinal axons that is different from that on peripheral axons also induces the collapse of temporal growth cones (Raper and Grunewald, 1990). There is no difference between the responses of temporal growth cones to nasal neurites in the presence or absence of PPACK by any of the parameters measured. We conclude that PPACK does not directly affect the ability of temporal retinal growth cones to collapse, but specifically inhibits the ability of DRG neurites to induce collapse. We cannot rule out, however, the possibility that PPACK somehow affects an intracellular pathway that is specifically activated by the peripheral avoidance cue.

The simplest interpretation of our data that is consistent with the specificity with which erase induces collapse of retinal growth cones, and the ability of PPACK to block retinal collapse on contact with DRG neurites, is that a serine proteinase similar or identical to erase induces the collapse of retinal growth cones when they contact DRG neurites.

We do not know the physiological function of erase during normal development. However, because our studies implicate erase as a peripheral axon label that repulses a variety of central growth cones, it is possible that erase plays a repulsive guidance role when outgrowing axons of the PNS and CNS confront one another. One region in which such a confrontation occurs is in the dorsal spinal cord and lateral brainstem as sensory axons invade the CNS. One reasonable hypothesis is that erase associated with peripheral axons prevents the fasciculation of central growth cones onto sensory tracts. Biochemical and molecular studies of erase should ultimately provide the reagents necessary to test this hypothesis.

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