

Monoclonal Antibody 8A2-Induced Retraction Appears to Be Mediated by Protein Phosphorylation in Goldfish Retinal Ganglion Cell Axons

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We have recently demonstrated that binding by monoclonal antibody (mAb) 8A2 to regenerating retinal ganglion cell axons in goldfish explants specifically induces a sustained, actin-based retraction response that is similar in most respects to a spontaneous retraction (S.G. Finnegan, V. Lemmon, and E. Koenig, *Cell Motil. Cytoskeleton*, 1992). Experiments were conducted to evaluate potential signal transduction pathways that may play a role in mediating retraction, using the mAb 8A2 retraction model system. Potential roles of cAMP, elevated intracellular calcium, or calmodulin-dependent processes were probed and the results did not appear to implicate them in either the induction or the maintenance of the axon retraction response. In contrast, treatment with phorbol 12-myristate 13-acetate, but not with inactive phorbol esters, induced a retraction response, although the response was more variable and less robust than that produced by mAb 8A2. However, both forms of induction were blocked by staurosporine, a nonspecific kinase inhibitor. Okadaic acid, a potent serine/threonine phosphatase inhibitor produced a very robust retraction response, and subthreshold doses significantly potentiated the retraction response induced by mAb 8A2. Genistein inhibited the mAb 8A2-induced retraction response at concentrations selective for tyrosine kinase activity in a dose-dependent manner. These findings are consistent with the hypothesis that an augmented phosphorylation state of one or more axonal proteins, perhaps catalyzed in part by protein kinase C, produces a sustained physiological retraction. In addition, tyrosine kinase may be involved in transducing surface-mediated interactions that trigger retraction, including the binding reaction signal of mAb 8A2. © 1993 Academic Press, Inc.

the growth cone play an important role in restricting the random growth of axons while seeking out appropriate targets (Tosney and Landmesser, 1984; Silver *et al.*, 1987; Snow *et al.*, 1990; Tosney and Oakley, 1990); moreover, retraction is a mode by which axons that form inappropriate connections are eliminated during maturation (Korneliusson and Jansen, 1976; Bixby, 1981; Riley, 1981; Morrison-Graham, 1983; Cowan *et al.*, 1984). Thus, retraction of axons plays an integral role in facilitating the emergence of stable neuronal networks (Letourneau, 1987; Patterson, 1988; Keynes and Cook, 1990; Walter *et al.*, 1990).

Retraction can be reliably triggered in young, vigorously growing goldfish retinal ganglion cell (RGC)¹ axons regenerating explant culture by exposure to monoclonal antibody (mAb) 8A2 (Drazba *et al.*, 1991; Finnegan *et al.*, 1989, 1990; Finnegan *et al.*, 1992). The retraction response evoked by surface binding of mAb 8A2 closely resembles the spontaneous retraction of RGC axons, which signals the onset of senescence (i.e., cessation of axonal growth). Spontaneous retraction involves the formation of thin evacuated distal strands produced by the retrograde translocation of axoplasm. The morphological and dynamic changes associated with retraction induced by mAb 8A2 have been characterized and described in detail recently (Finnegan *et al.*, 1992). Because of the outward similarity between the retraction induced by mAb 8A2 and that occurring spontaneously in senescent RGC axons, we regard the former as a suitable model to study underlying mechanisms of retraction. Some of the salient findings (Finnegan *et al.*, 1992) can be briefly summarized as follows.

INTRODUCTION

Regulation of axonal growth has been the subject of many studies and has largely focused on axon elongation. Much less attention has been given to the phenomenon of retraction because of a lack of a suitable model. It is now clear, however, that cues which are repulsive to

¹ Abbreviations used: 8-bromo-adenosine 3':5'-cyclic monophosphate, 8-bromo-cAMP; ethylenedis(oxyethylenenitrilo)tetraacetic acid, EGTA; monoclonal antibody, mAb; 4 α -phorbol, 4 α P; 4 α -phorbol 12,13-didecanoate, PDC; phorbol 12-myristate 13-acetate, TPA; protein kinase C, PKC; retinal ganglion cell, RGC.

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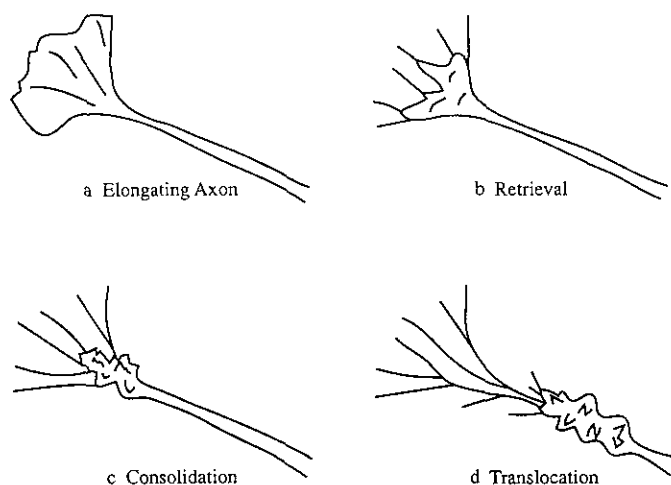


FIG. 1. A cartoon depicting (a) an elongating axon and (b-d) the three overlapping interspersed functional phases of the mAb 8A2 retraction response, as described in Finnegan *et al.* (1992). The drawings were constructed by tracing the phase-contrast images of a retracting goldfish RGC axon displayed on the videomonitor. After exposure to the antibody, the first sign of retraction is a retrieval of axoplasmic material in the form of "packets" from the lamellipodial veil, which translocate to the base of the growth cone concomitant with persistent ruffling. (b) An advanced stage of retrieval with material collecting to form an aggregate mass. The latter is referred to as the "motile mass" because it exhibits growth cone-like motility with protrusive activity and strong phalloidin staining and appears to retain the capacity to translocate as a discrete entity. (c) Consolidation of the motile mass overlaps with the early phase of retrograde translocation. (d) Bulk retrograde translocation of the motile mass and contiguous column of axoplasm leaves long, thin residual strands behind. Note that the third phase is sustained in nature and that the column of axoplasm typically moves proximally about 100 μm in 2 hr on polylysine (rate is approximately three times greater on laminin (Finnegan *et al.*, 1992)).

The retraction response induced by mAb 8A2 is specific because a different monoclonal antibody which reacts specifically with another antigen of the same membrane constituent class is ineffective in producing a retraction. Focal application of the antibody to the growth cone by micropipet is sufficient to elicit it, which indicates that the response is induced locally in the distal axon. The retraction response can be subdivided into three overlapping and interspersed functional phases (see Fig. 1); viz., (i) elongation ceases and "packets" of lamellipodial contents are shuttled toward the growth cone base; (ii) the "packets" collect and ultimately consolidate into a "motile mass" with intrinsic growth cone-like motility directed in the retrograde direction; and (iii) the motile mass, in conjunction with the contiguous column of axoplasm, undergoes retrograde translocation. The bulk retrograde translocation of the distal column of axoplasm yields evacuated distal strands, which remain attached at former sites of growth cone adhesion (see Fig. 2).

Typically, the bulk retrograde translocation is initiated after a latency of 10 to 20 min and may continue for hours. The use of various cytoskeletal probes and immunofluorescence analysis indicates that the transformation of the growth cone into a motile mass and the retrograde translocation of the motile mass and distal column of axoplasm are driven by actomyosin interactions; microtubules appear to play a subordinate, passive role. Experiments further indicate that F-actin becomes reorganized in the transition from growth cone to a motile mass and that the preservation of F-actin in the growth cone is required for inducing retraction by mAb 8A2.

Monoclonal Ab 8A2 binds to the surface membrane of the growing axon RGC axons (see Fig. 2A), probably to a ganglioside (Drazba *et al.*, 1991), and presumably, via a transmembrane signaling mechanism, mobilizes the cytoskeleton and triggers retraction. Transmembrane pathways, responding to extracellular signals, probably play an important role in axon guidance. Activation of such pathways may control changes in phosphorylation states of relevant effectors that modulate growth cone motility. Several serine/threonine protein kinases, including protein kinase A (PKA), Ca^{2+} /calmodulin-dependent protein kinase, and protein kinase C (PKC) (Pfenninger *et al.*, 1983; Ellis *et al.*, 1985; Hyman and Pfenninger, 1985; Katz *et al.*, 1985), in addition to tyrosine kinase (Cheng and Sahyoun, 1990), have been identified in growth cone-enriched fractions. The various signaling pathways that modulate elongation and retraction, however, need to be identified and their significance evaluated.

Although changes in intracellular calcium concentration are believed to modulate growth cone motility (Kater and Mills, 1991), not all available studies are consistent with this inference. An influx of calcium ions was correlated with an inhibition of neuritic outgrowth and growth cone motility of specific neurons in *Heliosoma in vitro* upon application of serotonin or generation of action potentials (Haydon *et al.*, 1984; Cohan *et al.*, 1987; Cohan and Kater, 1986). Phasic electrical stimulation of dorsal root ganglion cells, which may have increased calcium conductance, also caused occasional retraction (Fields *et al.*, 1990). In contrast, electrical stimulation of cultured rat superior cervical ganglion (SCG) neurons, which also increased intracellular calcium concentration, did not retard outgrowth (Garyantes and Regehr, 1992). In one case in which retraction was induced in chick dorsal root ganglion (DRG) axons by contact with retinal ganglion cells *in vitro* (Kapfhammer and Raper, 1987), intracellular calcium levels also did not appear to change (Ivins *et al.*, 1991).

Kinases that have been implicated in growth-associated functions include PKC, PKA, and protein tyro-

sine kinases, but effects on various substrata reflect an apparent complexity of regulation. For example, activation of PKC potentiated neurite outgrowth on suboptimal but not on optimal laminin concentrations, fibronectin, and collagen (Bixby, 1989), as well as on cell adhesion molecules (CAMs) L1 or N-cadherin (Bixby and Jhabvala, 1990). Although inhibition of PKC inhibited growth on laminin, fibronectin, or collagen, it potentiated the initial growth response on the CAMs. In the case of PKA, activation by dibutyryl cAMP suppressed growth cone motility in *Helisoma* neurons (Mattson *et al.*, 1988), but 8-bromo-cAMP treatment of cultured rat sympathetic and sensory neurons promoted neurite outgrowth and survival (Rydel and Greene, 1988).

Protein tyrosine kinases are also enriched in growth cone fractions as compared to adult synaptosomes (Cheng and Sahyoun, 1990) and have been implicated in neuritogenesis and adhesion. The protooncogene product tyrosine kinase, pp60^{c-src} (Maness *et al.*, 1988), and an adhesion plaque protein, vinculin (Igarashi *et al.*, 1990), are enriched in growth cone fractions and tyrosine phosphorylated in growth cone membranes in a developmentally regulated way. Also, α -, β -tubulin, enriched in a growth cone membrane fraction, are tyrosine phosphorylated by pp60^{c-src} (Matten *et al.*, 1990), although the significance of the modification in assembly and/or growth cone function is unknown.

A homologous protein, the retroviral transforming pp60^{v-src}, has been shown to remain associated with detergent extracted cytoskeletons, suggesting that cellular transformation is achieved, in part, by way of cytoskeletal changes (Burr *et al.*, 1980). Chicken embryo fibroblasts, transformed by the Rous sarcoma virus, show morphological and cytoskeletal changes such as retraction of long fibroblastic processes, rounding up, and formation of surface ruffles, as well as changes in the organization and assembly of microfilament bundles (Ambros *et al.*, 1975; Wang and Goldberg, 1976). Also, a role for pp60^{v-src} in cell adhesion is supported by its localization in adhesion plaques of transformed cells and its ability to cause a loss of zonula adherens junctions between epithelial cells (Maness *et al.*, 1988). Finally, pp60^{v-src} induces neurite outgrowth in PC12 cells, which is consistent with a general role for tyrosine kinases in growth processes (Alema *et al.*, 1985).

Kinase inhibitors, such as H-7 and staurosporine, promote neurite outgrowth in PC12 cells (Hashimoto and Hagino, 1989), as well as in mouse neuroblastoma and cerebellar cells (Tsuda *et al.*, 1989). A recent finding noteworthy for its significance for the present work is that these same kinase inhibitors also block thrombin-induced neurite retraction in neuroblastoma cells (Suidan *et al.*, 1992), indicating that protein phosphorylation may play a role in mediating the retraction response.

In the present study, we have utilized the goldfish retinal explant preparation (Heacock and Agranoff, 1977; Koenig and Adams, 1982) to evaluate potential involvement of calcium, PKA, and PKC, as well as tyrosine kinases in the retraction response induced by mAb 8A2 in regenerating RGC axons in explant culture. The experimental effects were characterized on the basis of a quantitative analysis of the retraction response magnitude, as measured by the length of distal strands evacuated of axoplasm. Some of the results have previously been presented in abstract form (Finnegan *et al.*, 1991). We have found that experimental manipulations expected to increase the level of protein phosphorylation by stimulation of kinase activity or by inhibition of serine/threonine phosphoprotein phosphatases in these axons cause retraction, while those that presumably reduce the activity of one or more kinases inhibit the retraction response.

MATERIALS AND METHODS

Chemicals. Poly-L-lysine, 5-fluorodeoxyuridine, gentamycin sulfate, uridine, methyl cellulose, staurosporine, phorbol 12-myristate 13-acetate (TPA), 4 α -phorbol 12,13-didecanoate (PDC), 4 α -phorbol (4 α P), and 8-bromoadenosine 3':5'-cyclic monophosphate (8-bromo-cAMP) were purchased from Sigma Chemical Co. (St. Louis, MO). Okadaic acid was obtained from L. C. Services (Woburn, MA). Genistein was purchased from Calbiochem (San Diego, CA). Ethylenebis(oxyethylene-nitrilo)tetraacetic acid (EGTA) was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). Rhodamine isothiocyanate (RITC)-conjugated phalloidin was obtained from Molecular Probes, Inc. (Eugene, OR).

Retinal explant preparation. Goldfish retinal explants were prepared as described elsewhere (Koenig and Adams, 1982; Koenig *et al.*, 1985) and were used for experimental observations after 3–5 days in culture. Briefly, 2–4 weeks after optic nerve crush, the retina was isolated, chopped into squares (0.65 \times 0.65 mm), plated onto polylysine-coated No. 1.5 circular coverslips and cultured in L-15 (GIBCO Laboratories, Grand Island, NY) medium supplemented with 10% fetal calf serum (Flow Laboratories, Inc., McLean, VA), 0.02 M HEPES, 0.1 mM 5-fluorodeoxyuridine, 0.1 mg/ml gentamycin sulfate, 0.2 mM uridine, and 0.6% methyl cellulose. Explants were cultured in humid air atmosphere at 27°C.

Videomicroscopy. For viewing, the circular coverslip was inverted over a 35 \times 50-mm No. 2 coverslip supported by 0.5- to 1-mm-thick spacers polymerized and trimmed from Silastic medical elastomer (Dow). The chamber permitted total exchange of bathing media in less than a minute. The standard bathing medium was a

modified Cortland physiological fish saline (Koenig and Adams, 1982) composed of (in mM): 132 NaCl, 5 KCl, 1.6 MgCl₂, 1.8 CaCl₂, 5.5 glucose, 20 Hepes, adjusted to pH 7.2 with Tris. The calcium-free medium was prepared in the same way except MgCl₂ was substituted for CaCl₂ and 1 mM EGTA was added. Calcium-containing solutions buffered to specific calcium concentrations with EGTA were formulated using a computer program provided by Dr. Zahur Ahmed based on the method of Beers (1982). All experiments were conducted at 22–24°C.

Axons were viewed under phase-contrast microscopy (Olympus BHS microscope) with a ×100 oil immersion planapochromat objective (Zeiss N.A. = 1.25) combined with an achromat condenser (Olympus, N.A. = 1.4). The microscope stage was isolated from external vibration by a Vibraplane air-suspension table-top platform (Kinetic Systems, Inc.). The phase image was displayed on a videomonitor (Sanyo) by using a DAGE NC-67M video camera with a Newvicon tube (DAGE-MTI, Inc.) mounted on a trinocular head of the microscope. Experiments were recorded in a time-lapse mode with a videorecorder (TLC 2001, GYYR, Inc.), where time was compressed by a factor of 12. Still photographs were taken from the videomonitor screen by using a Polaroid CU-5 Land camera type 665 positive/negative Polaroid film, or directly through the microscope with an attached 35-mm photomicrographic system (Olympus PM-10AD) on T-Max (Kodak) film processed to ASA 400–800 with T-Max developer (Kodak).

Quantification of retraction response. A comparison of dialyzed ascites fluid with that of S-600 column-purified mAb indicated that a dilution of 1:100 of the former was equivalent in response magnitude and in all qualitative respects to approximately 50 µg/ml of the latter. Control experiments were conducted to test for potential effects of the dialyzed ascites fluid on RGC axons. Thus, dialyzed ascites fluid, containing either (i) mAb D1.1, another IgM, which yields surface labeling similar to that of mAb 8A2 (Finnegan *et al.*, 1992), but which is directed against a different set of *O*-acetylated ganglioside antigens (Drazba *et al.*, 1991) expressed on embryonic neurons (Levine *et al.*, 1984), or (ii) mAb JG22, which specifically recognizes the β₁ integrin subunit of chick and not that of goldfish (Grieve and Gottlieb, 1982), was negative from the standpoint of inducing retraction or producing any notable side effects (Finnegan *et al.*, 1992, and unpublished observations). In still other experiments testing for toxicity (Finnegan *et al.*, 1992), mAb 8A2 in dialyzed ascites fluid was added to the culture medium at the time explants were plated. Outgrowth from explants during the first 2 days was not blocked; however, the growth was stunted and abnormally fasciculated. Therefore, for reasons of availability and greater biological activity due to the higher concen-

tration of antibody, mAb 8A2 in dialyzed ascites fluid was used in all experiments described.

A standard 90-min treatment period was used to produce a retraction for the purpose of quantifying the magnitude of the response. Thus, retinal explant sister cultures were treated with mAb 8A2, active or inactive phorbol ester, or okadaic acid in the presence or absence of inhibitors, as indicated in the text, and fixed in preparation for analysis. Typically, 20 to 40 consecutive single axons in distal axonal fields were evaluated for each of 6 explants on a coverslip. Single, isolated axons were displayed on the videomonitor, and the lengths of distal evacuated strands, reflecting the length of axon cleared by retrograde translocation of axoplasm, were measured on the screen, using a pair of calipers. A mean retraction length (µm) was calculated for each coverslip and used for comparison. Statistical difference between retraction responses was determined by using the Mann-Whitney test of median difference as the data appeared to have a nonnormal distribution.

RESULTS

A number of probes and experimental paradigms were employed to evaluate potential signal transduction pathways that may play a role in the initiation and/or mediation of retraction. Second messenger systems investigated included calcium and cAMP, while various enzymes that control the protein phosphorylation states probed included protein kinase A (PKA), protein kinase C (PKC), calcium/calmodulin (CaM)-dependent kinases, tyrosine kinase, and serine/threonine protein phosphatases. In most instances effects of experimental manipulation was assessed on the basis of efficacy of the latter to modify the retraction response of RGC axons specifically induced by mAb 8A2 (see below); however, in some instances, efficacy of a probe in inducing retraction was tested on its own merit. Axonal behavior was monitored by phase-contrast, time-lapse videomicroscopy for a period of 90 min and the preparation was then fixed. Quantitative measurements of the retraction response magnitude were made on fixed preparations from the videomonitor (see Materials and Methods). It is important to note that response magnitude measurements were based on the length of evacuated distal strands (see Fig. 2D); i.e., distance from attachment sites of the strands, representing original adhesion sites of the former growth cone (Finnegan *et al.*, 1992), to the motile mass.

Effects of changing intracellular calcium concentration on the distal axon. Because changes in intracellular calcium concentration have been implicated in the modulation of growth cone motility (see Kater and Mills, 1991), attempts were made to determine if elevation of

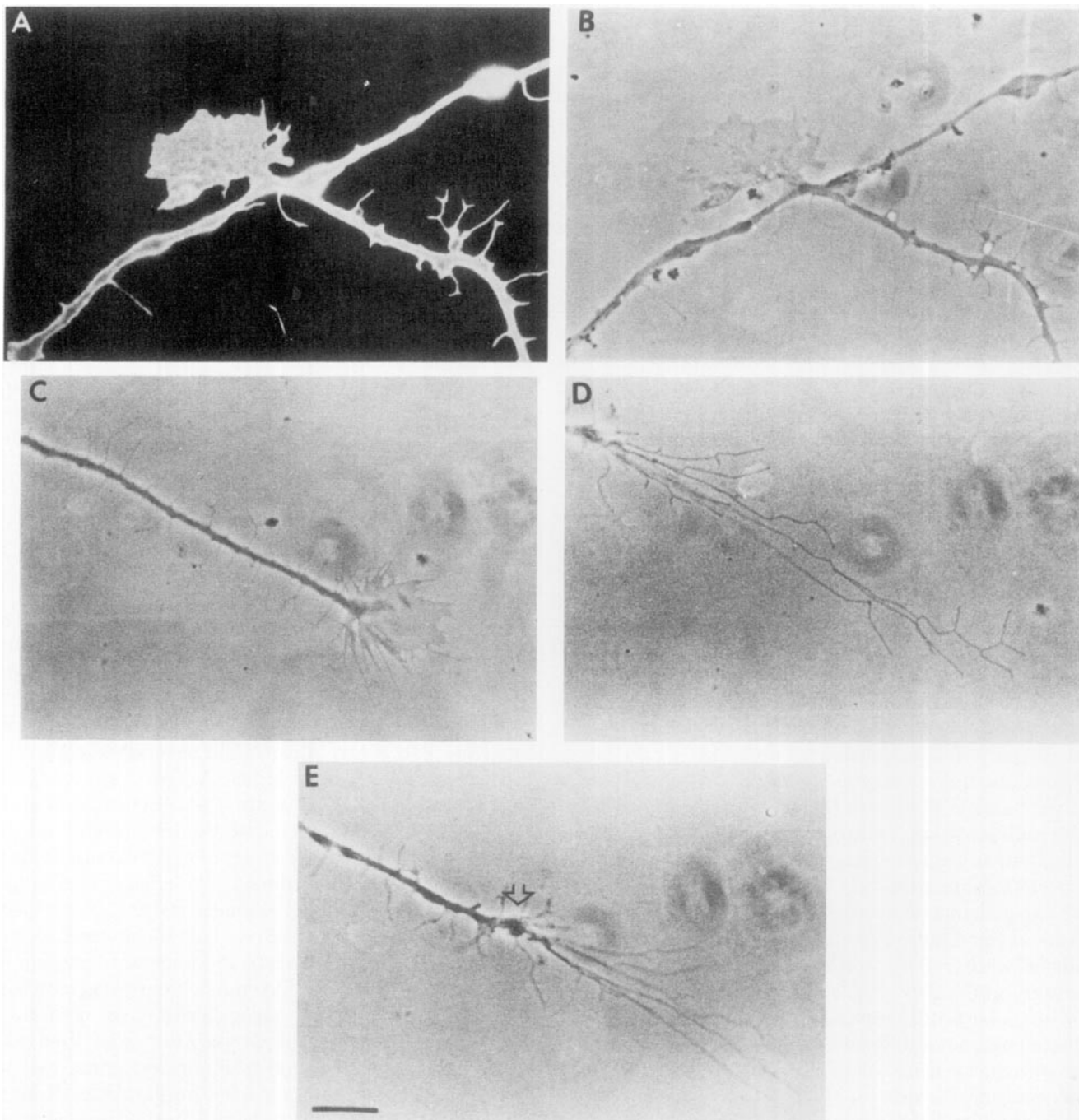


FIG. 2. Immunolabeling by mAb 8A2 and selected photomicrographs of mAb 8A2-induced retraction of RGC axons. Paired (a) fluorescence and (b) phase-contrast photomicrographs of crossed axons with a growth cone, showing uniform surface labeling by mAb 8A2. (c-e) Photographs depicting (c) an axon elongating before exposure to mAb 8A2, (d) evacuated distal strands produced after a period of retraction, and (e) the motile mass (arrow). Scale bar = 10 μ m.

intracellular calcium would induce retraction by utilizing a calcium ionophore and varying extracellular free calcium with an EGTA buffer. In earlier studies (Edmonds and Koenig, 1990a,b), extracellular calcium buffered with EGTA to $\geq 100 \mu$ M in the presence of ionomycin strongly stimulated a calcium-dependent volume re-

duction that culminated in a "syneresis" (i.e., condensation of axoplasmic mass due to volume reduction). The volume reduction, mediated by an apparent potassium efflux, progressively arrested visible particle transport and the syneresis endstage was followed by degeneration. To test whether small elevations in intra-

cellular calcium concentration could induce retraction, a range of submicromolar concentrations of free calcium in the presence of ionomycin were investigated.

RGC axons were treated with 2, 5, or 10 μM ionomycin ($n = 18$) in EGTA-containing Cortland medium, buffered to a range of free calcium concentrations from 0 to 500 μM , and studied with videomicroscopy. Irrespective of the calcium concentration used, including an EGTA-containing calcium-free bathing medium with only the ionophore present, there was a loss of the growth cone lamellipodial veil and formation of an endbulb structure. Occasionally, axoplasmic material was observed to move retrogradely out of the distal axon but this was correlated invariably with the development of a syneresis and was followed by ensuing signs of degeneration (see Edmonds and Koenig, 1990a). A natural, sustained retraction response characteristic of the magnitude and quality induced either by the onset of senescence or by the action of mAb 8A2 (see below) was never observed.

Because the use of an ionophore to elevate intracellular calcium provided no control of intracellular calcium concentration, another approach was explored, using caffeine to grade the release of calcium from intracellular stores (Kuba, 1980; Lipscombe *et al.*, 1988a,b). Treatment of axons with 5 mM ($n = 4$) or 10 mM ($n = 6$) caffeine resulted in a loss of the growth cone lamellipodial veil into an endbulb structure similar to experiments described above. Caffeine treatment also promoted the retrograde movement of axoplasm out of the distal axon for a short distance suggesting that an apparent retraction may have been initiated; however, either the retrograde movement arrested or it progressed to a syneresis.

These experiments, concerned with probing a potential role that calcium may play in inducing retraction, could not possibly simulate the changes in spatiotemporal calcium levels characteristic of normal intracellular regulation (Connor, 1986) and were, therefore, inconclusive in testing the hypothesis. They were instructive, nevertheless, because they strongly argue against the idea that retraction, which entails bulk retrograde translocation of the column of axoplasm, can be produced simply from a nonspecific calcium-mediated perturbation of the growth cone cytoskeletal organization (see Discussion).

Test of 8-bromo-cAMP on inducing retraction. In order to determine if activation of a cAMP-dependent kinase might be involved in retraction, RGC axons were treated with 5 ($n = 1$), 10 ($n = 2$), or 20 ($n = 1$) mM of the membrane permeant cAMP analog 8-bromo-cAMP and monitored by video microscopy. The effect of 8-bromo-cAMP on many axons on the coverslip surface was evaluated for each experiment. Although some growth cone lamellipodial veils were withdrawn either partially or

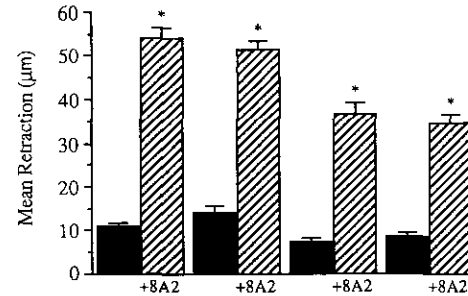


FIG. 3. Determination of whether mAb 8A2 would induce retraction in RGC axons in the absence of extracellular calcium in four separate experiments. Each histogram represents the mean retraction length (μm) for 120 different axons \pm SEM (see Materials and Methods). Preparations were either treated with mAb 8A2 (hatched) or untreated in sister cultures (solid) in a calcium-free, EGTA-containing bathing medium. Mean retraction in untreated controls reflect filopodial length and some axons that retract spontaneously during exchange of bathing medium. Significance level compared to control: * $P < 0.0001$.

into an endbulb/clubbed structure, no significant retrograde movement of axoplasm typical of that produced with mAb 8A2-induced retraction was observed. Quantitative measurements were not made in these experiments because an authentic retraction was never elicited.

Evaluation of extracellular calcium in mAb 8A2-induced retraction. A Cortland physiological fish saline bathing medium containing EGTA and no added Ca^{2+} was used to determine whether retraction induced by mAb 8A2 required extracellular calcium. In three preliminary experiments, retraction of many individual axons appeared to proceed normally in a Ca^{2+} -free medium. This was confirmed when the response magnitude was quantified (see Materials and Methods) in subsequent experiments where a mean retraction distance was determined for each coverslip, using nontreated sister cultures as controls. The baseline level of retraction, measured in the controls, is a reflection of filopodial length as well as a small number of axons which retract spontaneously, possibly in response to an exchange of the bathing medium. As shown in Fig. 3, significant mAb 8A2 retraction was achieved in the absence of extracellular calcium as compared to nontreated controls ($n = 4$). Although these results indicate that a calcium influx is required neither for inducing retraction nor for sustaining the response, it is possible that there may be a calcium requirement that is satisfied by a release from intracellular stores.

Effect of calmodulin inhibition on mAb 8A2-induced retraction. To determine whether activation of calmodulin played a role that could be measured by an effect on the retraction response magnitude, CGS 9343B (CGS), a new, potent calmodulin antagonist with improved selec-

tivity (Norman *et al.*, 1987) was used. The reported IC_{50} for calmodulin-stimulated cAMP phosphodiesterase activity *in vitro* was $3.3 \mu M$ (Norman *et al.*, 1987), and Polak *et al.* (1991) observed that it reversed the inhibitory effects of 5-HT on neurite outgrowth from *Helisoma* neuron B19.

Axons were preloaded for 1 hr with CGS and then were treated with the antibody in the continued presence of the inhibitor. In the presence of $5 \mu M$ ($n = 1$) or $10 \mu M$ ($n = 1$) CGS, axons retracted in the usual manner as monitored by videomicroscopy. A range of CGS concentrations were tested on axon retraction, and the response magnitudes in treated and controls were quantified in three separate trials. In the range of CGS concentrations tested (i.e., 1, 2, 5, or $10 \mu M$) retraction was not attenuated significantly except in one case at a $2 \mu M$ concentration. These results indicate an apparent lack of a calmodulin dependency for inducing retraction by mAb 8A2 and for maintaining retrograde translocation.

Effects of phorbol esters on retraction. Axons treated with phorbol 12-myristate 13-acetate (TPA) exhibited a retraction response which shared some characteristics with that induced by mAb 8A2. Time-lapse videorecords indicated that elongation stopped when TPA ($80 nM$) was added to the bathing medium, followed by involution of growth cones and retrograde movement of axoplasmic material to varying degrees ($n = 8$). A certain proportion of axons retained a clubbed appearance, with material remaining in the distal axon, while another fraction showed distal evacuation; however, in the latter case, axoplasmic material was distributed along the distal axon in the form of large, cigar-shaped masses, in contrast to the compact and organized nodular motile mass characteristic of mAb 8A2-treated axons. Nonetheless, labeling of F-actin in TPA-treated axons with rhodamine-conjugated phalloidin (not shown) revealed strong labeling having a distribution characteristic of mAb 8A2-treated axons (Finnegan *et al.*, 1992).

Phorbol ester-induced retraction was quantified in terms of mean retraction distance using inactive phorbol esters 4 α -phorbol 12,13-didecanoate (PDC) and 4 α -phorbol (4 α -P) on sister cultures as controls. Treatment with the inactive analogs did not cause a significant response as compared to normal medium (not shown). TPA, at concentrations of $100 nM$ ($n = 5$) and $10 nM$ ($n = 7$), produced significant retraction compared to controls; some typical examples of retraction induced by $10 nM$ TPA are shown in Fig. 4A. The average distances of retraction were less than those typical of the mAb 8A2-induced response. Also, the range of values was large indicating that a subset of axons retracted a distance that was substantially greater than the majority of the population.

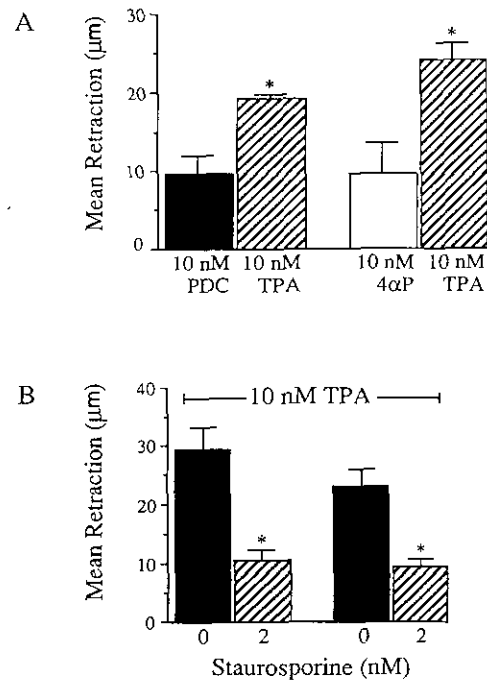


FIG. 4. Comparison in the efficacy of inducing retraction in RGC axons (A) by phorbol esters that are either active (phorbol 12-myristate 13-acetate (TPA)) or inactive (4 α -phorbol (4 α -P) and 4 α -phorbol 12,13-didecanoate (PDC)) in activating PKC (examples are representative of seven experiments), and (B) by TPA in the absence and presence of the kinase inhibitor staurosporine. In (A) histograms represent mean retraction lengths of axons treated with either $10 nM$ TPA (hatched), $10 nM$ PDC (solid), or $10 nM$ 4 α -P (open). In (B) histograms represent mean retraction lengths of axons treated with $10 nM$ TPA in the presence (hatched) or absence (solid) of $2 nM$ staurosporine. Each histogram represents the mean retraction length \pm SEM for 120 different axons. Significance level compared to control: * $P < 0.0001$.

While activation of PKC by a phorbol ester appeared to mimic the antibody retraction response, it was not as global, robust, or reliable. It is possible, therefore, that while activation of PKC may represent an integral component of the signal transduction cascade underlying the retraction response, additional pathways are likely to be involved. To investigate other potential pathways, use was made of kinase and phosphatase inhibitors.

Effects of staurosporine on the retraction response. Staurosporine, a microbial alkaloid, is a general kinase inhibitor having an IC_{50} of $2.7 nM$ for rat brain PKC (Tamaoki *et al.*, 1986). Staurosporine has been reported to inhibit PKA from bovine heart with an IC_{50} value of $8.2 nM$ as well as protein tyrosine kinase activity of p60^{src} *in vitro* with an IC_{50} value of $6.4 nM$ (Nakano *et al.*, 1987). In addition, staurosporine has been found to inhibit CaM kinase II from rat brain with an IC_{50} value of $20 nM$ (Yanagihara *et al.*, 1991) and myosin light chain kinase (MLCK) in platelets (Watson *et al.*, 1988). Despite the lack of specificity, this agent is a potent inhibitor of kinase activity.

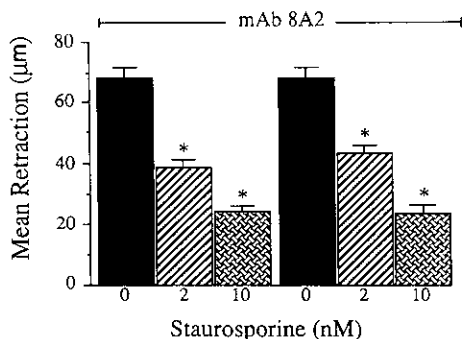


FIG. 5. Dose-dependent inhibition of the mAb 8A2-induced retraction response in RGC axons by staurosporine (examples are representative of three experiments where both 2 and 10 nM staurosporine were tested). Magnitude of the retraction response induced by mAb 8A2 in the presence of 0 (solid), 2 (hatched), or 10 (weave) nM staurosporine. Each bar represents the mean retraction length \pm SEM for 120 different axons. Significance level compared to control: * $P < 0.0001$.

The effect of staurosporine was tested and analyzed on the TPA-induced retraction. The response induced by 100 nM TPA was blocked by 107 nM staurosporine ($n = 3$) (not shown), and the response produced by 10 nM TPA was inhibited significantly by 2 nM staurosporine ($n = 2$) (Fig. 4B). Treatment of axons with 107 nM staurosporine alone caused a slight withdrawal of the growth cone lamellipodial veil without otherwise producing significant morphological changes as monitored by videomicroscopy and no significant retraction compared to control when quantified. These results indicate that the retraction induced by the active phorbol ester was probably due to kinase activity such as PKC.

Staurosporine was tested on mAb 8A2-induced retraction by comparing the response quantitatively to nontreated sister cultures as controls. Staurosporine completely blocked mAb 8A2 retraction to control levels at a concentration of 107 nM ($n = 4$) (not shown). On the other hand, Hedberg *et al.* (1990) have reported that concentrations in excess of 20 nM staurosporine can induce a disruption of microfilament bundles in cultured fibroblasts and epithelial cells. The integrity of the actin cytoskeleton is vital for normal function of the growing axon, particularly the growth cone; however, any major perturbation of the actin filament network in the growth cone would have produced visible morphological changes. Despite the fact that no significant changes were noted, we chose to examine lower concentrations to minimize any potential nonspecific effects of the kinase inhibitor.

Staurosporine inhibited mAb 8A2 retraction in a dose-dependent manner at lower concentrations of 0.5 nM ($n = 1$), 2 nM ($n = 8$), and 10 nM ($n = 4$) (typical examples are shown in Fig. 5). The results of these ex-

periments indicate that at least one kinase plays a role in the signal transduction cascade mediating mAb 8A2 retraction response. While experiments using phorbol esters (see above) are consistent with the possibility that PKC may lie in the latter pathway, the relative lack of specificity of staurosporine militates against drawing this conclusion.

Effects of genistein on the retraction response. Genistein is a fairly specific inhibitor of tyrosine kinase, with an IC_{50} of about 20–30 μ M for receptor tyrosine kinases, while requiring in excess of 370 μ M as an IC_{50} for serine- and threonine-specific kinases, such as PKA and PKC (Akiyama *et al.*, 1987). Genistein treatment alone did not perturb the normal axon morphology as viewed by videomicroscopy.

Axons treated with mAb 8A2, with or without genistein, were evaluated quantitatively in terms of mean retraction distances. Genistein completely blocked retraction at 100 μ M ($n = 3$) (not shown) and inhibited retraction in a dose-dependent manner at 2 ($n = 5$) and 20 μ M ($n = 6$); typical examples are shown in Fig. 6. These results indicate that a tyrosine kinase is likely to be involved in the signal transduction cascade underlying the retraction response induced by mAb 8A2.

Effect of okadaic acid on the retraction response. Okadaic acid is a powerful inhibitor of protein phosphatases-1 and -2A (PP1 and PP2A) and rapidly promotes increased levels of phosphorylated proteins in intact cells (Haystead *et al.*, 1989). Treatment of axons with 10 ($n = 4$) or 25 ($n = 5$) nM okadaic acid resulted in a robust retraction response (typical examples shown in Fig. 7). When monitored by videomicroscopy, the response resembles the phorbol ester response in terms of the quality of the response, but it is more robust, global and sustained. Material moves out of the distal axon as a column of axoplasm, leaving strands behind. The results

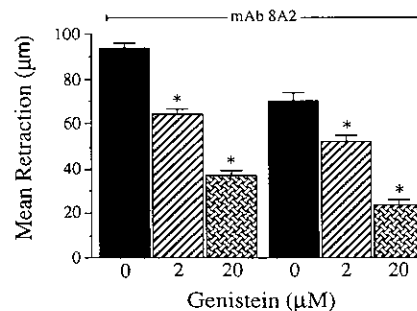


FIG. 6. Dose-dependent inhibition of mAb 8A2-induced retraction response in RGC axons by kinase inhibitor genistein (examples are representative of five experiments where both 2 and 20 μ M genistein were tested). Magnitude of mAb 8A2-induced axon retraction in the presence of 0 (solid), 2 (hatched), or 20 (weave) μ M genistein. Each bar represents the mean retraction length \pm SEM for 120 different axons. Significance level compared to control: * $P < 0.001$.

are consistent with the conclusion that an augmentation of protein phosphorylation plays an important role in promoting retraction.

Effect of subthreshold doses of okadaic acid on mAb 8A2-induced retraction. Subthreshold doses of okadaic acid, in combination with mAb 8A2, caused a retraction response that was augmented in a dose-dependent manner compared to the response induced by the antibody alone. The enhancement exceeded the addition of the two independent responses, indicating that the retraction induced by the two modes of induction may have operated via a common pathway. Treatment with 0.5 nM okadaic acid enhanced mAb 8A2 retraction in a significant manner five out of seven times; addition of 2 nM okadaic acid enhanced mAb 8A2 retraction in every case ($n = 5$). Typical examples of potentiation of mAb induced retraction by subthreshold doses of okadaic acid are shown in Fig. 8.

DISCUSSION

During development and regeneration of the nervous system, growth cones respond to a variety of extrinsic cues in the extracellular environment presumably by way of transmembrane signaling pathways. It is reasonable to assume that extrinsic attractive and repulsive cues are transduced into changes in cytoskeletal dynamics by second messenger systems in the growing axon which ultimately governs its behavior. A number of second messenger systems have been implicated in the control of axon outgrowth, growth cone motility, and cytoskeletal organization (Bixby and Harris, 1991; Strittmatter and Fishman, 1991). One behavior which is now recognized as playing an integral part in the forma-

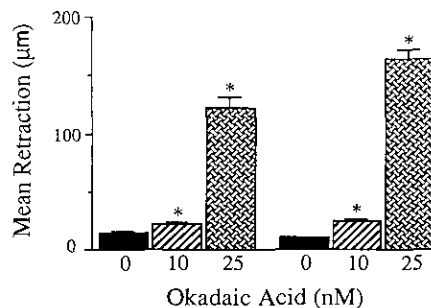


FIG. 7. Robust retraction response in RGC axons induced by phosphatase inhibitor, okadaic acid (examples are representative of 4 experiments where both 10 and 25 nM okadaic acid were tested). Magnitude of axon retraction in response to 0 (solid), 10 (hatched), or 25 (weave) nM okadaic acid. Each bar represents the mean retraction length \pm SEM for 120 different axons. Note the dramatic change in response magnitude over a narrow dose range. Significance level compared to control: $*P < 0.0001$.

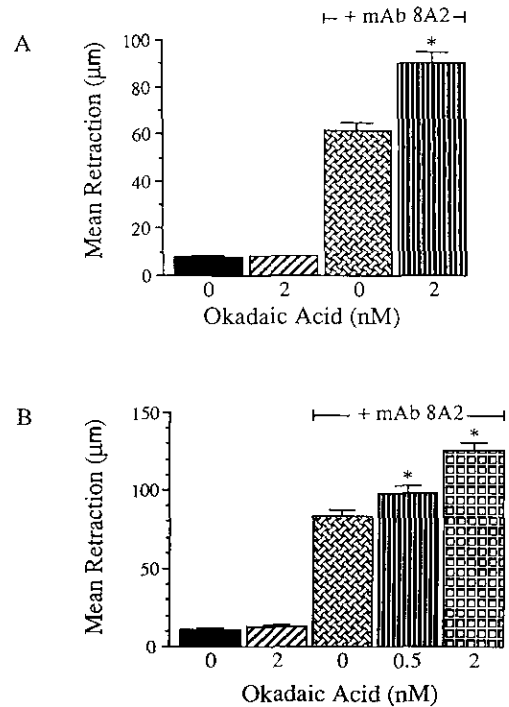


FIG. 8. Potentiation of the mAb 8A2-induced retraction response by subthreshold concentrations of okadaic acid (examples are representative of five experiments where 2 nM okadaic acid was tested in A; and three experiments where both 0.5 and 2 nM okadaic acid were tested in B). (A) Comparisons of retraction in untreated RGC axons with 0 (solid) or 2 (hatched) nM okadaic acid, and in mAb 8A2-treated axons with 0 (weave) or 2 (stripes) nM okadaic acid. (B) Comparisons of retraction in untreated RGC axons with 0 (solid) or 2 (hatched) nM okadaic acid, and in mAb 8A2-treated axons with 0 (weave), 0.5 (stripes), or 2 (grid) nM okadaic acid. Each bar represents the mean retraction length \pm SEM for 120 different axons. Treatment with 0.5 or 2 nM okadaic acid was insufficient to trigger retraction, but in combination with mAb 8A2, the retraction response was significantly augmented over that triggered by the antibody alone. Significance level compared to control: $*P < 0.0001$.

tion of appropriate neuronal connections is that of axon retraction.

We have recently analyzed and described the induction and sustained retraction of RGC axons in response to binding by mAb 8A2 (Finnegan *et al.*, 1992). The response is reliably induced in vigorously growing axons by surface binding of the antibody such that focal application to the distal axon is sufficient to trigger it. It is similar in most respects to the spontaneous retraction that occurs in these same axons with the onset of senescence in explant culture. We believe that it represents a suitable model for studying physiological retraction in RGC axons regenerating *in vitro*. The antibody binds to the surface membrane and by way of an unknown transmembrane signaling mechanism induces a complex sequence of apparent actomyosin-based cytoskeletal activities which mediate a dramatic bulk redistribution of

axoplasm, leaving the distal axon evacuated of its contents. It is worth emphasizing that in the case of the retraction induced by mAb 8A2, F-actin in the growth cone is preserved during the process in which a motile growth cone, active in anterograde elongation, is transformed into a compact motile mass that retains an intrinsic capacity to translocate as a discrete entity, albeit, in a retrograde direction (Finnegan *et al.*, 1992).

Here, we report findings which indicate that the retraction response is correlated with an augmented level of phosphorylation of one or more proteins and provide evidence that implicates both a tyrosine kinase and one or more serine/threonine kinases. Monoclonal Ab 8A2 has been demonstrated to recognize a novel set of *O*-acetylated gangliosides in embryonic chick retina (Drazba *et al.*, 1991) and may bind to similar ganglioside antigens on goldfish retinal axons. Gangliosides have been shown to modulate the activity of kinases such as phosphotyrosine kinase (Chan, 1989a), a ganglioside-stimulated kinase (Chan, 1989b), PKC (Magal *et al.*, 1990), and a tyrosine kinase (Bremer *et al.*, 1986), suggesting they play an important role in signal transduction in cells.

In view of the evidence that calcium may play a significant role in modulating growth cone motility (Kater and Mills, 1991), there have been reports that elevated intracellular calcium concentration may also mediate retraction. For example, two studies by Lankford and Letourneau (1989, 1991) showed that elevation of intracellular calcium causes loss of the lamellipodium, which was correlated with disruption of the underlying actin filament meshwork. In the literature, loss of the lamellipodium is frequently referred to as "growth cone collapse" and is regarded as an incipient sign of retraction, or, indeed, may even be referred to as retraction. In addition to the question of whether or not calcium plays a key role in retraction, however, there is also the issue of what constitutes an authentic retraction response.

In the present ionomycin experiments, small nominal elevations in intracellular calcium concentrations did not induce a response that in any way resembled an authentic retraction. Elevating intracellular calcium in these axons, using an ionophore technique, has been shown to stimulate a calcium-regulated volume reduction, which culminates in an endstage of syneresis (Edmonds and Koenig, 1990a,b). Even the use of caffeine to stimulate release of calcium from internal stores in the present study caused the development of a condensed state characteristic of syneresis. Occasionally, there were signs of a distoproximal bulk movement of axoplasm in the distal axon, which, along with particle transport, arrested as shrinkage supervened. Elevation of $[Ca^{2+}]_i$ disrupts actin filaments in the growth cone and destabilizes microtubules, leading to what has been characterized as form of "neurite retraction" (Lankford

and Letourneau, 1989). However, any shortening that may result from perturbation of the cytoskeleton in distal axons is clearly unphysiological and should not be classified as retraction, notwithstanding a collapse of the growth cone lamellipodium. As previously noted, the induction of the retraction response by mAb 8A2, involving the retrograde translocation of axoplasm and formation of evacuated distal strands, was absolutely dependent on preserving organized F-actin in the growth cone (Finnegan *et al.*, 1992).

While the question of what role calcium may play in either the induction or the maintenance of retraction remains unanswered by these and similar types of experiments, a recent study by Ivins *et al.* (1991), in which intracellular calcium concentration was monitored by Fura-2, revealed that intracellular calcium levels did not change during retraction of dorsal root ganglion cell axons when they encountered retinal ganglion cell axons. Furthermore, it is clear from present results that extracellular calcium is not required for mAb 8A2-induced retraction. Finally, the use of the highly specific calmodulin inhibitor CGS (Norman *et al.*, 1987) also failed to block the retraction response induced by mAb 8A2, which would appear to indicate that calcium/calmodulin-dependent kinase activity is probably not necessary to support the response.

Among the kinase-activating probes tested independently of mAb 8A2, 8-bromo-cAMP was ineffective, while TPA induced a retraction response. Nevertheless, the latter retraction response was weaker, more variable in magnitude (i.e., distance retracted) and qualitatively different with respect to the gross morphology of the distal axon, compared to the retraction response induced by mAb 8A2. Recently, Lankford and Letourneau (1991) also found that TPA caused retraction in chick dorsal root ganglion cell axons. It is interesting to note in the latter study that Fura-2 measurements during TPA-induced retraction yielded no evidence of a change in calcium concentration; furthermore, unlike the effects produced by artificially elevating $[Ca^{2+}]_i$ (Lankford and Letourneau, 1989), the retraction was not associated with a loss of actin filaments. If mAb 8A2 binding activates PKC via the usual pathway, involving phospholipase C, resulting in the formation of diacylglycerol and IP_3 , then the expectation is that there should be a release of calcium from intracellular stores. Without directly measuring changes in calcium during the mAb 8A2 retraction response, however, we are unable to firmly assign a role for intracellular calcium release in the evolution of the response. While staurosporine blocked TPA-induced retraction and the retraction induced by mAb 8A2, the lack of specificity of the inhibitor and the qualitative differences between the two modes of retraction makes the question of PKC involve-

ment in physiological retraction moot at the present time.

Okadaic acid proved to be a potent agent for inducing retraction. Indeed, retraction appeared to be even more robust than that induced by mAb 8A2 alone. Okadaic acid is an inhibitor of serine/threonine phosphatases. At doses, which by themselves did not produce a retraction, okadaic acid significantly potentiated the retraction induced by mAb 8A2, indicating that the action of the serine/threonine phosphatase inhibitor must have involved one or more phosphoproteins operating in the mAb 8A2-mediated signal transduction pathway. As the evidence available does not implicate a calcium/calmodulin-dependent kinase, nor is it conclusive in implicating PKC, the identification of the serine/threonine protein kinase(s) remains an open question.

Given the specificity of genistein as a tyrosine protein kinase inhibitor (Akiyama *et al.*, 1987), the fact that it inhibited the mAb 8A2-induced retraction response strongly implicates a tyrosine kinase in the signal transduction pathway. Tyrosine kinases and serine/threonine kinases, such as PKC, have been shown to act in concert in signal transduction cascades. For example, T cell antigen receptor-mediated activation of phospholipase C, and subsequent cell proliferation require tyrosine phosphorylation of one of the receptor subunits (Mustelin *et al.*, 1990).

In the context of actin-based motility, which appears to mediate the mAb 8A2-induced retraction response (Finnegan *et al.*, in press), some recent studies from Pollard's laboratory are especially relevant. Profilin, a G-actin-sequestering protein, also binds phosphatidylinositol 4,5-bisphosphate (PIP₂) (Goldschmidt-Clermont *et al.*, 1990). The latter complex not only inhibits binding of soluble actin by profilin, but it also inhibits the hydrolysis of PIP₂ by PLC- γ 1; however, phosphorylation of PLC- γ 1 by the epidermal growth factor receptor tyrosine kinase overcomes the inhibitory effect of profilin and allows PLC- γ 1 to become activated (Goldschmidt-Clermont *et al.*, 1991). The latter study provides evidence of a potential linkage between signal transduction and regulation of the actin cytoskeleton. It also provides a model whereby both a tyrosine kinase and a serine/threonine kinase, such as PKC, could be involved in the membrane-mediated signal transduction cascade causing retraction.

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