Thy-1 Antibody-Triggered Neurite Outgrowth Requires an Influx of Calcium into Neurons via N- and L-type Calcium Channels

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Abstract. We present evidence that the neurite outgrowth stimulated by the binding of Thy-1 antibodies to PC12 cells is mediated by calcium influx through both N- and L-type calcium channels. PC12 cells cultured on a noncellular substratum in the presence of NGF, or on a cellular substratum in the absence of NGF, responded to soluble Thy-1 antibody by extending longer neurites. The response required bivalent antibody and could be blocked by removing Thy-1 from the surface of PC12 cells with phosphatidylinositol specific phospholipase C. The response could also be blocked by reducing extracellular calcium to 0.25 mM, or by antagonists of L- and N-type calcium channels. Additionally, the response could be fully inhibited by preloading PC12 cells with BAPTA/AM which buffers changes in intracellular calcium. A heterotrimeric G-protein is also implicated in the pathway as the response could be fully inhibited by pertussis toxin. These data suggest that antibody-induced clustering of Thy-1 stimulates neurite outgrowth by activating a second messenger pathway that has previously been shown to underlie cell adhesion molecule (NCAM, N-cadherin, and L1), but not integrin or NGF-dependent neurite outgrowth.

Cell-contact phenomenon plays a determining role in many developmental processes. For example, the ability of neurons to migrate and elaborate axons and dendrites (collectively termed neurites for cells in culture), is governed to a large extent by cell–cell interactions that are mediated by cell-membrane glycoproteins (Rathjen and Jessell, 1991; Doherty and Walsh, 1992). A large number of molecules that share sequence homology with the immunoglobulins (the Ig superfamily) have been shown to mediate cell contact–dependent neurite outgrowth. For example, after transfection and expression in non-neuronal monolayer cells, a number of neuronal and/or glial glycoproteins including the cell adhesion molecules (CAMs)[1] NCAM, L1 (a.k.a. NILE/NgCAM), and F3 (a.k.a. F11/contactin), and the myelin associated glycoproteins MAG and Po can all stimulate cell contact–dependent axonal growth from neurons (Doherty and Walsh, 1992; Williams et al., 1992; Gennarini et al., 1991; Johnson et al., 1989; Yazaki et al., 1991). In the case of NCAM and L1, a homophilic binding of NCAM or L1 in neurons to NCAM or L1 in transfected cells stimulates neurite outgrowth by activating a second messenger pathway that culminates in calcium influx into neurons (reviewed in Doherty and Walsh, 1992). Activation of this pathway is both necessary and sufficient to account for cell contact–dependent neurite growth (Saffell et al., 1992; Williams et al., 1992). In the case of F3, Po, or MAG expressed in transfected cells, the nature of the neuronal receptor and the signal transduction pathway underlying neurite outgrowth remains to be determined.

The Thy-1 glycoprotein is the smallest and perhaps the best characterized member of the Ig superfamily (Reif, 1989; Morris, 1985; Williams, 1989; Parekh et al., 1987). Like some other family members, e.g., NCAM, Thy-1 does not show a highly restrictive pattern of expression that might betray an obvious function. It is expressed in several tissues and has recently been shown to contribute to the adhesion of mouse thymocytes to thymic epithelium (He et al., 1991). Thy-1 is probably the most abundant cell surface glycoprotein in the adult brain where it is expressed by several cell types including neurons. Thy-1 expression is upregulated on most neurons towards the end of the period of axonal growth (Xue et al., 1991). After transfection and expression in a neuronal cell line Thy-1 can apparently bind to a heterophilic receptor that is preferentially expressed by mature astrocytes with this interaction inhibiting an otherwise robust neurite outgrowth response (Tiveron et al., 1992). These data have led Morris and his colleagues to suggest that a similar interaction in vivo might stabilize neuronal connections and even suppress regeneration in the damaged CNS.

In an alternative approach to elucidating Thy-1 function some groups have tested the effects of antibodies to Thy-1, either immobilized to a substratum (for example see Lipton et al., 1992) or present in growth media (Mahanthappa and Patterson, 1992) on neurite outgrowth from a variety of primary neurons and/or the PC12 neuronal cell line. In both in-

1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; COLL, collagen; GPI, glycosylphosphatidylinositol; LN, laminin; OPD, *o*-phenylenediamine; PIPLC, phosphatidylinositol-specific phospholipase C; PL, poly-lysine.

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stances the Thy-1 antibodies stimulate neurite outgrowth raising the possibility that the interaction of a natural ligand with neuronal Thy-1 might have positive effects on axonal growth in some circumstances. This could be particularly pertinent for neurons of the peripheral nervous systems such as sympathetic neurons whose axons express Thy-1 early in development and do not encounter astrocytes.

Thy-1 is a glycosylphosphatidylinositol (GPI)-linked molecule and it is not clear if it modulates neurite outgrowth through strictly adhesive interactions or by changing the level of second messengers in neurons. Antibodies to Thy-1 can induce mitosis and increases in intracellular calcium in lymphocytes (Kroczek et al., 1986; Gunter et al., 1987) and can modulate low threshold calcium currents in sensory neurons (Saleh et al., 1988). However this may require the association of Thy-1 with accessory transmembrane spanning molecules such as the CD45 tyrosine phosphatase (Volarevic et al., 1990). In the present study we have investigated the nature of the transmembrane signalling processes that underly Thy-1's ability to stimulate neurite outgrowth.

Materials and Methods

Cell Culture

NIH-3T3 fibroblasts were maintained on 10-cm plastic culture dishes in DME supplemented with 10% FCS. PC12 cells were grown on polylysine-coated 5-cm plastic culture dishes in defined SATO medium containing 2% FCS (Doherty et al., 1991). The PC12 cells were seeded at high density to maximize division rates. All cells were grown at 37°C in 8% CO_2. Monolayers of 3T3 cells were established by seeding 80,000 cells into individual chambers of eight chamber slides (Lab-Tek, Naperville, IL) coated in turn with poly-lysine (PL) and fibronectin. The cells were incubated overnight after which they had formed a confluent monolayer. For the establishment of co-cultures PC12 cells were trypsinized (0.1% wt/vol in PBS/0.02% EDTA) for ~10 min at 37°C. The trypsinized cells were then spun down and a single cell suspension was obtained by resuspending the pellet in SATO containing 2% FCS and by triturating with a 19-gauge needle. These cells were then introduced onto the monolayers at 1.5×10^3 per well of an eight-chamber Lab-Tek slide containing 3T3 monolayers. The co-cultures were maintained for 44–48 h in SATO containing 2% FCS supplemented with the reagents described below, as appropriate. After incubation the co-cultures were fixed for 30 min with 4% paraformaldehyde at room temperature (RT) and immunostained to identify the PC12 cells for quantitation of neurite length.

In some experiments PC12 cells were seeded directly onto chambers coated sequentially with PL and then laminin (LN) or collagen (COLL) and these were then maintained as for the co-cultures. The fixing reagent used here was gluteraldehyde and after 30 min of incubation at RT the preparations were washed 3–4 times with PBS, mounted, and analyzed directly under phase contrast microscopy.

Indirect Immunofluorescence

After washing the fixed co-cultures (once with PBS), nonspecific protein binding sites were blocked using 0.5% gelatin. The PC12 cells were visualized by sequential application of the primary antibody (Rabbit anti-mouse L1 IgG fraction at a dilution of 1:50, or polyclonal rabbit anti Thy-1 at 1:500 depending on the reagents used in the co-cultures), the secondary antibody, biotinylated anti-rabbit at a dilution of 1:500, and finally streptavidin-Texas red at 1:500. PBS/BSA was used to dilute all antibodies. Each reagent was incubated for 60 min at RT and the cultures were washed 3–4 times between incubation with PBS/BSA. The cultures were then mounted and analyzed.

For double indirect immunofluorescence staining, a mixture of mouse mAb OX-7 and polyclonal rabbit L1 IgG (both at a dilution of 1:50) directed against the antigens Thy-1 and L1, respectively, were used as the primary antibodies. The secondary antibodies were a mixture of FITC conjugated anti-mouse IgG (1:500) and biotin-conjugated anti-rabbit IgG. Finally streptavidin Texas red (1:500) was applied as normal.

Determination of Cell Surface Levels of the Thy-1 Antigen

PC12 cells were plated at 5,000 cells per well on poly-lysine coated 96-well microtiter plates and incubated at 37°C overnight. The enzyme phosphatidylinositol-specific phospholipase C (PIPLC) was added to each well in threefold tripling dilutions and incubated for up to 48 h at 37°C. The cells were then fixed with 4% paraformaldehyde in DME for 30 min and then washed with PBS and blocked with 0.5% gelatin. The cultures were then incubated with OX-7 (1:50 dilution of tissue culture supernatant) or rabbit anti-L1 IgG for 60 min and washed three times with PBS/BSA. Anti-mouse or anti-rabbit immunoglobulin conjugated to HRP (at a dilution of 1:1,000) was then added for 60 min at RT. Cultures were then washed four times with PBS, once with distilled water, and incubated with 50 μl of 0.2% (wt/vol) *o*-phenylenediamine (OPD) and 0.02% (vol/vol) H_2O_2 in citrate phosphate buffer. After 10–15 min the reaction involving the conversion of OPD to its oxidized product was stopped by the addition of 50 μl of 4.5 M H_2SO_4. The absorbance was read at 492 nm using a Dynatech multiscan apparatus (Dynatech Labs. Ltd., W. Sussex, UK).

For the antibody titration curves PC12 cell cultures were fixed after overnight incubation at 37°C and the antibodies were then added to individual wells in twofold dilutions and incubated for 60 min at RT. This was then followed by incubation with anti-mouse HRP (1:1,000) to detect primary antibody binding. The remaining steps were as described above. The antibodies titrated were OX-7 IgG (tissue culture supernatant); OX-7 bivalent $F(ab)_2$ fragments (2 mg/ml) and OX-7 monovalent Fab fragments (1.7 mg/ml). The $F(ab)_2$ and Fab fragments of OX-7 were prepared by standard methods and were a generous gift from Dr. Roger Morris (Sadie Lee Research Centre, NIMR, Mill Hill, London).

Other Reagents

PIPLC purified from the culture supernatants of *Bacillus thuringiensis* was obtained from ICN Radiochemicals (Irvine, CA) at a stock concentration of 5 U/ml and this was diluted as required for individual experiments. The OX-7 mouse mAb that reacts with rat Thy-1 was used in neurite outgrowth experiments at the given dilutions of the tissue culture supernatant (see Doherty and Walsh, 1987) or given concentrations of purified antibody. Calcium channel antagonists verapamil and diltiazem were purchased from Sigma Immunochemical (St. Louis, MO) and Nifedipine and ώ-conotoxin GVIA from Life Technologies Ltd. (Grand Island, NY). Pertussis toxin was a gift from Dr. J. Kenimer (Bureau of Biologics, FDA, Bethesda, MD). All reagents were used at concentrations previously established as appropriate to block their targets (Doherty et al., 1991*a*,*b*). Secondary antibodies for immunostaining were from Amersham International (Buckinghamshire, England) and those for ELISA assays from Sigma Immunochemicals.

NGF, at a maximally active concentration of 50 ng/ml, was used to induce differentiation of PC12 cells grown on PL/COLL or PL/LN coated plastic as previously described (Doherty and Walsh, 1987).

Image Analysis

The immunostaining was detected using a low light-sensitive video camera and analyzed using a Sight Systems Image Manager (Sight Systems, Newbury, Berkshire, England). The slide was scanned in a systematic manner and the length of the longest neurite or the added length of all the processes per PC12 cell was measured. A minimum of 120 cells per culture were analyzed. For cells with no visible processes a minimum length of 2 μm was assigned to enable their scoring. This did not significantly affect the results.

Measurement of Intracellular Calcium and Calcium Uptake

PC12 cells were loaded with Fura-2 (Grynkiewicz et al., 1985) by incubation at 20°C in 3 μM Fura-2 AM plus 38 mg/liter pluronic F-127 for 30 min. The cells were rinsed with fresh medium and incubated at 36°C for a further 30 min to allow dye deesterification. Intracellular calcium in individual cells was measured from the fluorescence of intracellular dye when illuminated at 350 and then 380 nm as described by Grynkiewicz et al. (1985) using $R_{min} = 0.6$, $R_{max} = 4.93$, $S_{f2}/S_{fb} = 4.27$, Kd = 224 nm.

For calcium uptake studies 5×10^5 PC12 cells were seeded into individual wells of a PL/COLL coated 24-well tissue culture plate 24 h before the experiment. The cultures were washed once with serum free SATO and incubated with serum-free SATO supplemented with 3 μCi/ml $^{45}Ca^{2+}$ at 37°C for the given periods of time. The cultures were then washed 3 times

in ice cold PBS and lysed with 1 ml 0.5M NaOH for 2 h at RT. 0.5 ml of this sample was neutralized with 0.5 ml 0.5 M HCl and counted in a beta counter in 10 ml Aquasol (Dupont de Nemours, Bad Hamburg, Germany). All values were obtained from measurements made on triplicate cultures.

Results

Thy-1 Antibody Stimulates Neurite Outgrowth from PC12 Cells

PC12 cells are a line of cells that originate from the adrenal medulla and undergo a switch to a sympathetic neuronal phenotype on treatment with NGF or FGF (Greene, 1984; Togaris et al., 1985). The most overt feature of this change is the extension of neuritic processes that are virtually indistinguishable from those emanating from primary neurons. The OX-7 mAb binds specifically to the Thy-1 antigen on PC12 cells (Doherty and Walsh, 1987) and when presented in solution this and other Thy-1 antibodies have been reported to increase the percentage of process bearing PC12 cells from around 1% to 3–5% of the total population (Mahanthappa and Patterson, 1992). In the present study we added the OX-7 mAb to naive PC12 cells cultured on a PL/COLL or PL/LN substrata in the presence and absence of a maximally active concentration of NGF (50 ng/ml). The effects of OX-7 (1:50 dilution of tissue culture supernatant) on total neurite outgrowth (the sum of all the processes) from PC12 cells is shown in Fig. 1. OX-7 on its own did have a small significant effect on neurite extension increasing total neurite growth per cell on average by 2.2 μm on PL/COLL and on average by 7.4 μm on PL/LN. This compares poorly with NGF which increased process outgrowth per cell by, on average, 35 μm. In the presence of NGF the OX-7 antibody had a much more dramatic effect in increasing total process outgrowth per cell by ~20–30 μm. The effects of OX-7 were confirmed in a further three experiments on PC12 cells grown in the presence of NGF on a PL/COLL substratum. In these experiments OX-7 increased the mean neuritic outgrowth per PC12 cell by 25.1 μm to 163% of the NGF control, by 32.1 μm to 156% of the NGF control, or by 17.2 μm to 153% of the NGF control (values from 100–150 PC12 cells with standard errors of ~5% mean). Reducing the OX-7 antibody concentration to 1:100 did not significantly impair the response, whereas a reduction to 1:200 reduced the response by 10–30% (two experiments). These observations correlate well with the binding of OX-7 to Thy-1 in PC12 cells which for this batch of tissue culture supernatant is highly specific and saturates at a 1:100 dilution (see Doherty and Walsh, 1987).

The effects of OX-7 (1:100 dilution of tissue culture supernatant) were also tested for PC12 cells cultured on confluent monolayers of NIH-3T3 fibroblasts. This model represents a highly permissive environment for basal levels of PC12 cell differentiation as well as differentiation induced by NGF, cholera toxin, K^+ depolarization or by CAMs expressed in 3T3 cells after gene transfer (Doherty et al., 1991*a*; Saffell et al., 1992). In a series of ten consecutive experiments OX-7 induced an approximate doubling (96 ± 14.7% increase) of the mean length of the longest neurite on each PC12 cell from a not inconsiderable basal value of 22.7 ± 2.2 μm (both values mean ± SEM, n = 10). Analysis of neurite length distributions failed to show any evidence for the response being attributable to anything other than an effect on the whole population of PC12 cells. The neurite growth response is comparable in magnitude to that induced by NCAM, N-cadherin and L1 expressed following transfection and expression in the 3T3 cells. (Doherty et al., 1991; Williams et al., 1992).

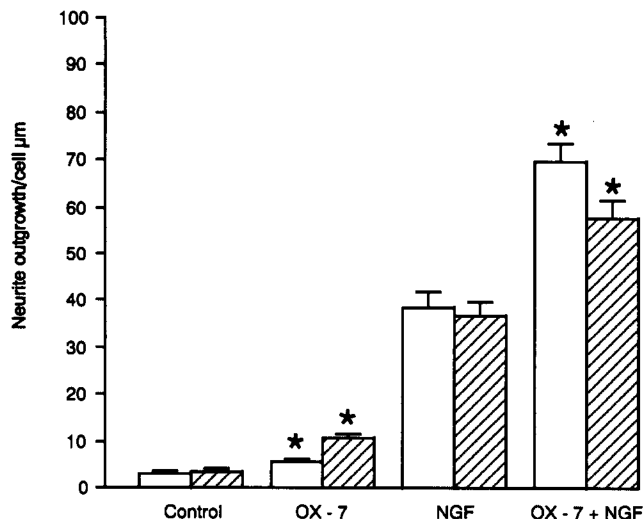

Figure 1. OX-7 antibody induces neurite outgrowth from PC12 cells. PC12 cells were cultured on a PL/COLL (□)- or PL/LN (▨)-coated substratum in control media, media supplemented with the OX-7 mAb (1:50 dilution of tissue culture supernatant), NGF (50 ng/ml), or OX-7 plus NGF. After 48 h the cultures were fixed and the sum of all neuritic processes determined for individual PC12 cells. Each value is the mean ± SEM for 120–150 PC12 cells sampled in replicate cultures. Statistical analysis of the OX-7 response relative to the appropriate control was determined by the single sided t test. * $P < 0.005$.

Mono and bivalent fragments of OX-7 were compared with intact Ig for their ability to promote neurite outgrowth from PC12 cells cultured on 3T3 monolayers with the result shown in Fig. 2. At concentrations that showed saturable binding to PC12 cells (see Fig. 2 legend) the bivalent $F(ab)_2$ was as effective as the whole immunoglobulin in stimulating neurite outgrowth. In contrast the monovalent Fab, which bound to a similar extent to PC12 cells (0.39 ± 0.012 as compared to 0.43 ± 0.0099 OD units for the $F(ab)_2$ as determined by ELISA, both values mean ± SEM, n = 4) had no significant effect on neurite outgrowth. These data are in agreement with Mahanthappa and Patterson (1992) and suggest that clustering of Thy-1 might be required to trigger the response. Examples of PC12 cells grown over monolayers of NIH-3T3 cells in the presence and absence of Thy-1 are shown in Fig 3.

Effects of Thy-1 Removal on Neurite Outgrowth

Thy-1 (along with some other GPI-anchored molecules) can be removed from PC12 cells by treatment with PIPLC. At a concentration of 0.05 U/ml, PIPLC removed ~95% Thy-1 immunoreactivity from PC12 cells within 2 h, with no recovery of Thy-1 immunoreactivity at up to 48 h as determined by both quantitative ELISA on PC12 cells grown on PL/COLL or by direct immunostaining for PC12 cells cocultured with 3T3 fibroblasts (data not shown). As a control for nonspecific protease activity we have previously shown

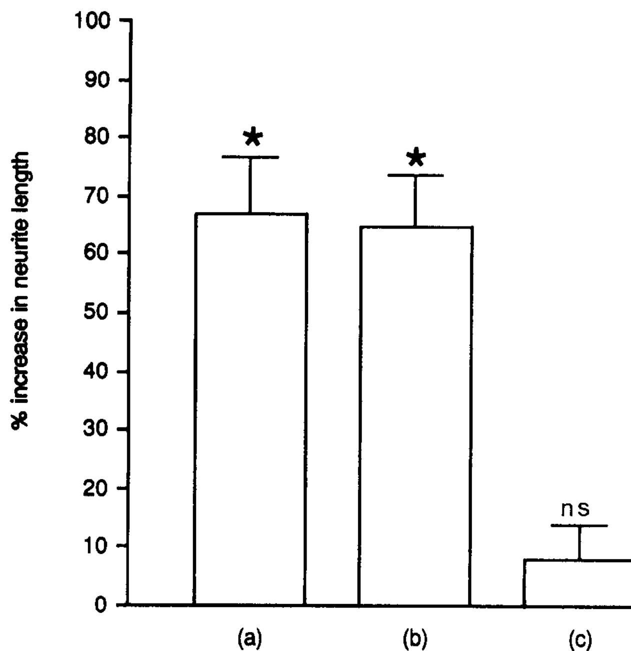

Figure 2. The OX-7 response requires bivalent antibody. PC12 cells were cultured on confluent monolayers of 3T3 fibroblasts in control media or media supplemented with OX-7 IgG (*a*) (1:100 dilution of tissue culture supernatant), OX-7 $F(ab)_2$ (*b*) (1 μg/ml), or OX-7 monovalent Fab (*c*) (1.7 μg/ml). The antibody preparations were titered by ELISA and used at concentrations that showed saturable binding to Thy-1 on PC12 cells. After 48 h the cultures were fixed and the length of the longest neurite on each PC12 cell determined. The results show the percentage increase in neurite length in OX-7-treated cultures relative to control cultures, and each value is the mean +1 SEM for ~120 PC12 cells sampled from replicate cultures. The statistical significance of the three responses was determined as in Fig. 1. * $P < 0.005$; *ns*, not significantly different from growth in control media.

that the enzyme will not remove transmembrane forms of NCAM from neurons or transfected 3T3 cells (Doherty et al., 1990*a*). We have also found that the enzyme has no effect on PC12 cell L1 immunoreactivity (Singh, A., and P. Doherty, unpublished observations).

We tested the effects of removal of Thy-1 (and other GPI-anchored molecules) on the ability of PC12 cells to extend neurites under control conditions as well as in response to a large number of external stimuli. PIPLC tested over the full concentration range determined to affect Thy-1 levels had no significant effect on neurite outgrowth from PC12 cells cultured in the presence and absence of NGF on a PL/COLL or PL/LN substratum. It is unlikely that effects on a small subpopulation of PC12 cells were masked as none of the cells in the treated cultures extended a process longer than 20 μm. Likewise in a number of tests PIPLC did not affect neurite outgrowth from PC12 cells cultured over confluent monolayers of parental 3T3 or NCAM expressing 3T3 fibroblasts (data not shown, however see Fig. 4, *a* and *b*).

We next determined the ability of PIPLC to inhibit the Thy-1 antibody-triggered response. As a control we first determined that the OX-7 mAb did not compromise the ability of PIPLC to remove Thy-1 from the PC12 cell membrane. For example, after a 60-min treatment with 0.025 U/ml PIPLC, Thy-1 immunoreactivity was reduced by 77.2% in control cultures and by 80.5% in cultures simultaneously treated with PIPLC and OX-7 (Thy-1 immunoreactivity was determined by the binding of rabbit anti-Thy-1 and each value is the mean for six replicate cultures). Treatment with PIPLC completely inhibited the OX-7 induced increase in neurite outgrowth from PC12 cells cultured on monolayers of parental 3T3 cells (Fig. 4 *a*), or from PC12 cells cultured on PL/COLL in the presence of NGF (Fig. 4 *b*). Thus the binding of OX-7 to Thy-1 in the PC12 cells membrane triggers the response. In contrast, in two independent experiments the addition of an anti-ganglioside mAb (A2B5), at up to 1% of ascites fluid, did not affect neurite outgrowth from PC12 cells cultured on a 3T3 cell monolayer (values in presence of antibody being 111 ± 5.5% and 94 ± 4.8% of the respective control; both values mean ± SEM of approximately 150 individual determinations of the length of the longest neurite).

Thy 1 Antibody-Triggered Neurite Outgrowth Requires Calcium Influx into PC12 Cells

At least three CAMs (NCAM, N-cadherin, and L1) two of which are Ig superfamily members, trigger neurite outgrowth by stimulating calcium influx into neurons (reviewed in Doherty and Walsh, 1992). Their responses can be blocked by reducing extracellular calcium to 0.25 mM, or by antagonists of N- and/or L-type calcium channels or by treatment (including preloading of neurons) with a chelator of intracellular calcium (BAPTA-AM). The effects of these agents on OX-7 antibody-triggered neurite outgrowth were determined for PC12 cells grown on 3T3 monolayers. OX-7 failed to trigger neurite outgrowth when BAPTA-AM was present in the culture media (two experiments) or when PC12 cells were pre-loaded with BAPTA-AM for 2 h before co-culture (two experiments). Overall, in the presence of OX-7 and BAPTA-AM the longest neurite was 105 ± 6.9% (mean ± SEM of four independent experiments) of the untreated control. BAPTA-AM did not significantly affect basal neurite outgrowth over 3T3 cells (Fig. 5 *a*) nor neurite outgrowth stimulated by NGF or cholera toxin (not shown, but see Williams et al., 1992). Similarly, in two independent experiments reducing extracellular calcium to 0.25 mM completely inhibited the OX-7 response without affecting basal neurite outgrowth (Fig. 5 *b*) or neurite outgrowth induced by NGF or cholera toxin (not shown). In five independent tests OX-7 failed to trigger significant neurite outgrowth when L-type calcium channels were blocked by diltiazem (three experiments), nifedipine (one experiment), or verapamil (one experiment) (overall the length of the longest neurite in OX-7 plus L-type blocker was 99.5 ± 3.2% ($n = 5$) of the untreated control; see Fig. 5 *c*). The OX-7 response was also absent in cultures treated with the N-type calcium channel blocker ω-conotoxin (overall the length of the longest neurite in OX-7 plus ω-conotoxin being 98.3 ± 5.4% ($n = 3$) of the untreated control). None of the calcium blockers affected basal neurite outgrowth from PC12 cells or neurite outgrowth stimulated by NGF or cholera toxin (for example see Saffell et al., 1992).

Calcium channel blockers do not inhibit NGF stimulated neurite outgrowth from PC12 cells cultured on a collagen coated substratum (Doherty et al., 1991). In a representative experiment on collagen in the presence of NGF (see Fig. 1)

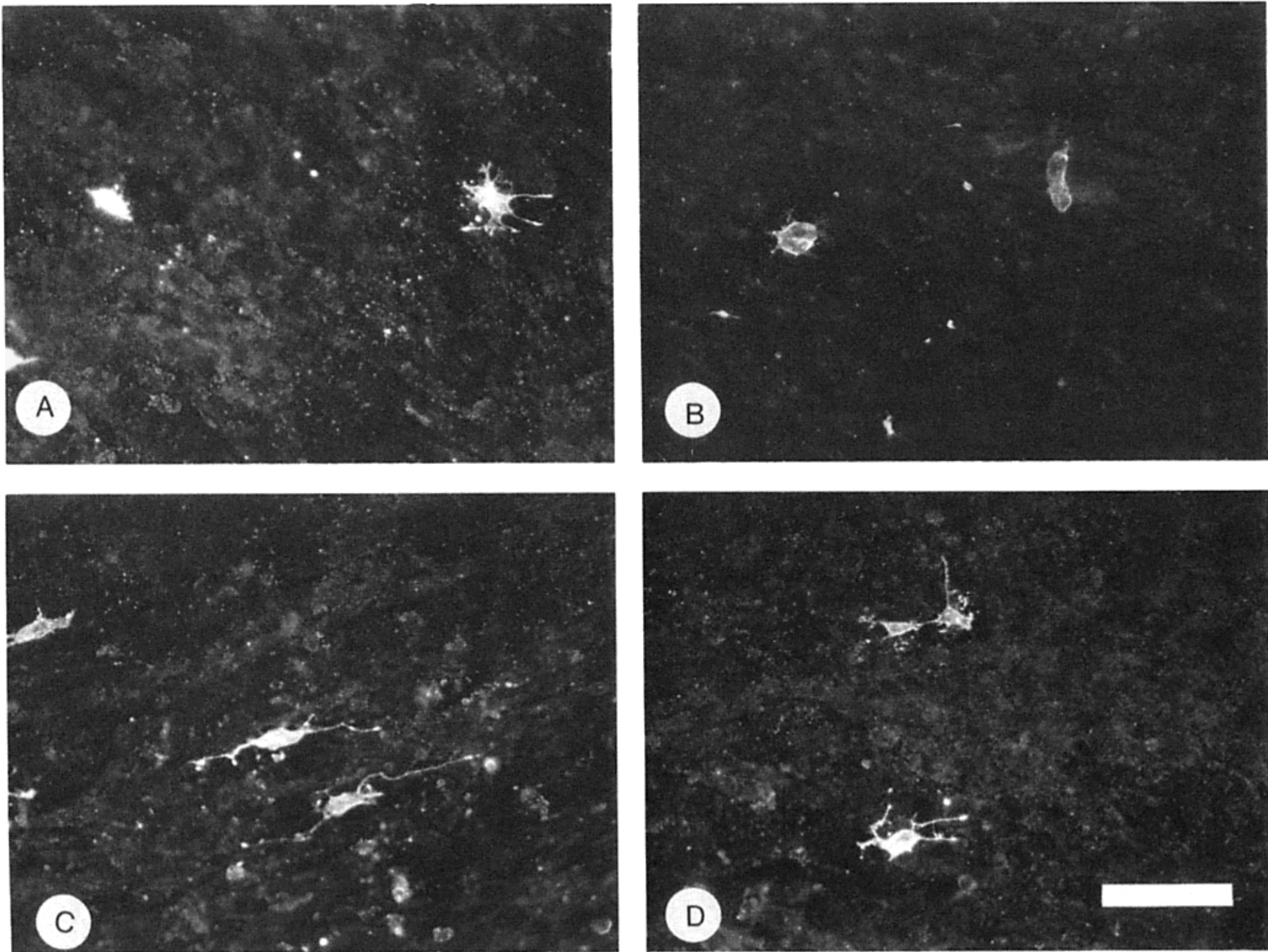

Figure 3. PC12 cell morphology. PC12 cells were cultured on confluent monolayers of 3T3 fibroblasts in the absence (*A* and *B*) and presence (*C* and *D*) of OX-7 (1:100 dilution of tissue culture supernatant). After 48 h the cultures were fixed and PC12 cells identified by immunostaining with a polyclonal antiserum raised against Thy-1. Unlike the OX-7 mAb which reacts only with rat Thy-1 in PC12 cells, this rabbit antiserum also bound to Thy-1 in the 3T3 monolayer. Bar, 50 μm.

OX-7 stimulated neurite outgrowth to 159 $\pm$ 11.5% ($P <$ 0.005) of control in the absence of calcium channel blockers, but had no effect in media further supplemented with 10 μM diltiazem or 0.25 μM ω-conotoxin (measured value being 95.7 $\pm$ 6.4% and 104 $\pm$ 4.3% of the respective control; all values mean $\pm$ SEM from 130–170 PC12 cells). Thus the effects of calcium channel blockers cannot be mediated via indirect effects on the confluent monolayers of 3T3 fibroblasts.

The above data point to an influx of calcium through N- and L-type calcium channels being required for Thy-1 antibody-triggered neurite outgrowth. We have previously shown that direct activation of this particular pathway either by K^+ depolarization or by treatment with a calcium channel agonist is sufficient to stimulate a similar response (Saffell et al., 1992). It was of interest to determine to what extent the OX-7 antibody could actually change the level of free intracellular calcium in PC12 cells and this was measured by $^{45}Ca^{2+}$ accumulation, and by loading neurons with Fura-2 (see Materials and Methods) and acquiring fluorescence images at 505 nm while exciting first at 350 and then 380 nm. Transformation of fluorescence ratios to absolute calcium values was done as previously described (see Materials and Methods).

In the first series of experiments the effects of K^+ (80 mM) and OX-7 on $^{45}Ca^{2+}$ accumulation into PC12 cells cultured on PL/COLL was determined. After 5 min $^{45}Ca^{2+}$ accumulation in cells treated with K^+ was 286 $\pm$ 51% (n = 5) greater than in untreated controls. In contrast $^{45}Ca^{2+}$ accumulation was not significantly affected by a 5 min treatment with OX-7 (93 $\pm$ 7%, control n = 3, all values mean $\pm$ SEM for the given number of independent experiments). Likewise, a 2-h treatment with OX-7 did not significantly affect $^{45}Ca^{2+}$ accumulation (not shown). Thus the OX-7 antibody does not trigger substantial accumulation of calcium in PC12 cells over short periods of time.

The absolute level of intracellular free Ca^{2+} in control, K^+ (40–80 mM) and OX-7–treated cultures was determined by taking measurements from large numbers of PC12 cells at 30 min (Fig. 6) or by following individual cells for 30 min after addition of test agents (Fig. 7). K^+ is able to induce a very rapid increase in Ca^{2+} that peaks within a few minutes and is still detectable after 30 min (see also Reber and Reuter, 1991). In contrast the OX-7 antibody had no obvious

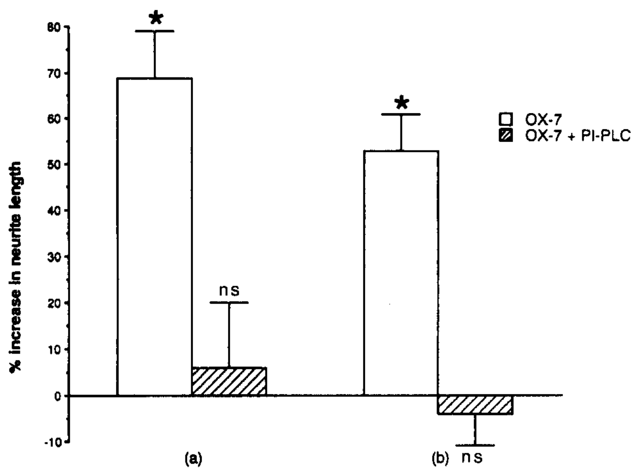

Figure 4. Cleavage of Thy-1 from PC12 cells inhibits the OX-7 response. PC12 cells were cultured on confluent monolayers of 3T3 fibroblasts in the absence of NGF (*a*) or on a PL/COLL–coated substratum in the presence of 50 ng/ml NGF (*b*) in media further supplemented with OX-7 plus PIPLC (0.05 U/ml) as indicated. After 48 h the cultures were fixed and the mean length of the longest neurite per PC12 cell (*a*) or the mean sum of all neurites per cell (*b*) determined as in Fig. 2 and 1, respectively. The results show the percentage increase in these values in cultures treated with OX-7 relative to untreated controls and each value is the mean + 1 SEM for ~120 PC12 cells sampled in replicate cultures. The statistical significance of the response was determined as in Fig. 1. * $P < 0.005$; *ns*, not significantly different from growth in control media.

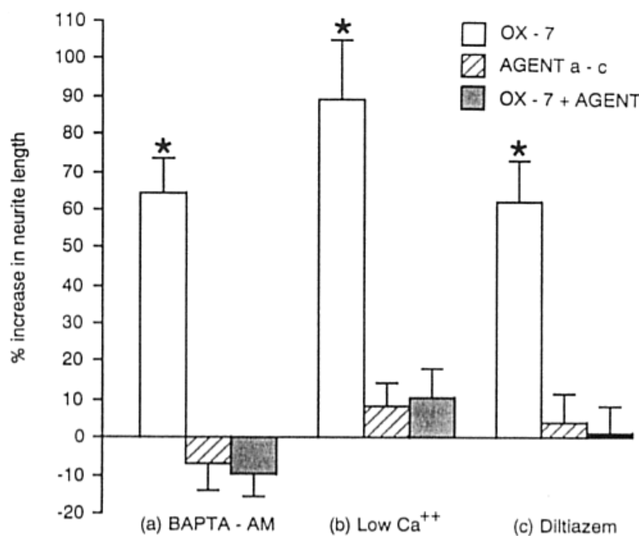

Figure 5. Calcium influx into PC12 cells is required for the OX-7 response. PC12 cells were cultured on confluent monolayers of 3T3 fibroblasts in the presence and absence of OX-7 (1:100 dilution of tissue culture supernatant) in media further supplemented with 20 μM BAPTA-AM (*a*), in media containing reduced Ca^{2+} at 0.25 mM (*b*), or media containing diltiazem at 10 μM (*c*). After 48 h the cultures were fixed and the percentage increase in the mean neurite length determined relative to PC12 cells grown in control media as described in Fig. 2. The results show the percentage increase in neurite length induced by OX-7, by the test agent or by OX-7 in the presence of the test agent for the three independent experiments as indicated. Each value is the mean +1 SEM for ~120 PC12 cells sampled in replicate cultures. The only significant responses, determined as in Fig. 1, were obtained in the presence of the OX-7 antibody on its own. All other values were not significantly different from growth in control media. * $P < 0.005$.

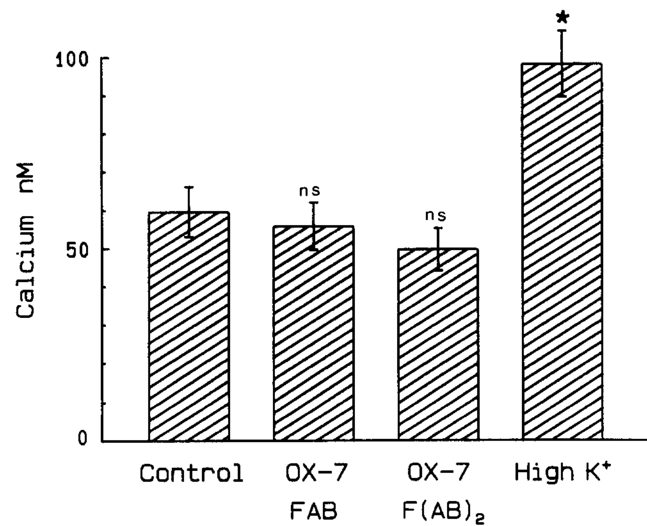

Figure 6. Cytosolic calcium in OX-7 and K^+ treated PC12 cells. Cultures of PC12 cells were established on a PL/COLL–coated substratum and loaded with Fura-2 as described in Materials and Methods. Cytosolic calcium levels were determined 30 min after the addition of fresh control media or media supplemented with OX-7 Fab (10 μg/ml), OX-7 $F(ab)_2$ (10 μg/ml), or KCl (40 mM). Cytosolic calcium in cells treated with OX-7 was not significantly different from the control, but cytosolic calcium in cells treated with 40 mM K^+ was significantly higher. Bars represent mean +1 SEM of 29, 40, 40, and 39 cells, respectively. * $P < 0.005$.

effect on calcium levels at any time point within the 30-min period (Fig. 6 and 7). Thus the OX-7 induced increase in neurite outgrowth most probably requires very subtle or spatially restricted changes in calcium influx into PC12 cells.

Pertussis toxin ribosylates some members of the heterotrimeric family of G-proteins and thereby inhibits their function. Pertussis toxin blocks CAM (NCAM, N-cadherin, and L1)-dependent neurite outgrowth from PC12 cells but does not inhibit neurite outgrowth stimulated by NGF, cholera toxin or K^+ depolarization. This suggests the highly specific involvement of Go and/or Gi in the CAM pathway upstream of calcium channel opening (reviewed in Doherty and Walsh, 1992). The effects of pertussis toxin on OX-7–triggered neurite outgrowth were determined in two independent experiments for PC12 cells cultured on 3T3 monolayers, with the results of one experiment shown in Fig. 8. In both cases pertussis toxin completely abolished the OX-7 response, implicating a G-protein in the second messenger pathway.

Discussion

Despite a relatively complete knowledge of the structure and distribution of the Thy-1 glycoprotein the function of this molecule in the nervous system remains elusive. A strong case has been made that Thy-1 functions to stabilize synaptic structures. Interaction with a heterophilic receptor, which based on functional studies appears to be expressed by mature astrocytes but not other cells, inhibits neurite outgrowth from a neuronal cell line expressing transfected Thy-1 (Tiveron et al., 1992). The same study showed that the expression of Thy-1 by the neuronal cell line did not affect neurite outgrowth over several other cellular substrata. Also, neurite outgrowth from primary neurons such as retinal ganglion

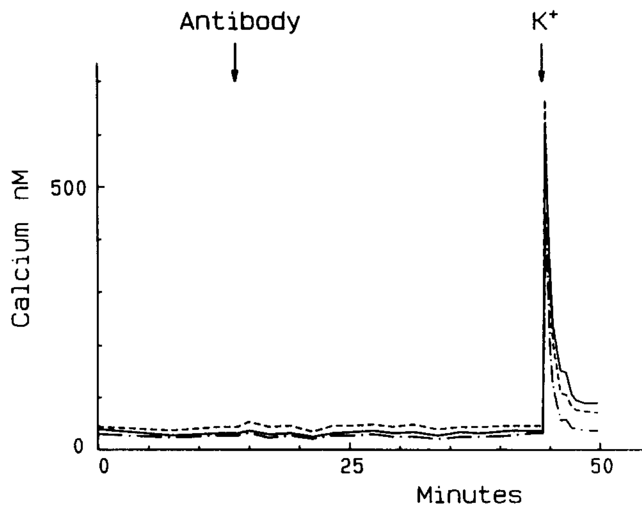

Figure 7. Cytosolic calcium in OX-7 and K^+ treated PC12 cells. The cytosolic calcium levels in three PC12 cells was monitored before and after the addition of OX-7 antibody (1:25 dilution of tissue culture supernatant) and the subsequent addition of KCl to 80 mM. On average measurements were made at 160-s intervals before the addition of KCl and 40-s intervals after KCl addition. The cells were incubated at 36°C in an atmosphere of 5% CO_2, 95% air on the stage of a fluorescence microscope throughout the period of acquiring images for calcium determination.

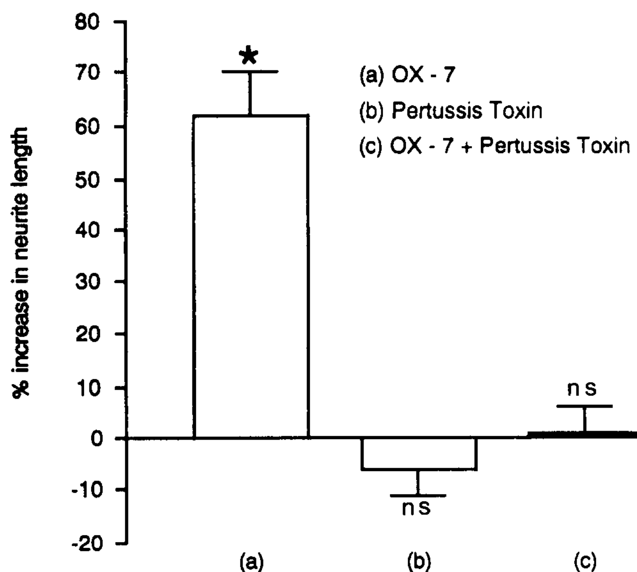

Figure 8. Pertussis toxin inhibits the OX-7 response. PC12 cells were cultured for 48 h on confluent monolayers of 3T3 fibroblasts in control media or media supplemented with OX-7 (1:100 dilution of tissue culture supernatant), pertussis toxin at 800 ng/ml, or OX-7 and pertussis toxin. The percentage increase in the mean length of the longest neurite per cell relative to control media was determined. Each value is the mean + 1 SEM for ~120 PC12 cells sampled from replicate cultures. The statistical significance of the response was determined as in Fig. 1. * $P < 0.005$; *ns*, not significantly different from growth in control media.

cells, even over astrocytes, is not obviously compromised by their expression of Thy-1 (e.g., see Doherty et al., 1990*b*). Thus the expression of Thy-1 per se is not inhibitory for neurite growth.

A case for a positive role of Thy-1 in axonal growth can also be made. For example Thy-1 is a member of the Ig superfamily and has recently been shown to be an adhesion molecule (He et al., 1991). Almost every other molecule that shows these characteristics has been shown to be capable of supporting neurite outgrowth (for example see Doherty and Walsh, 1992). The fact that immobilized Thy-1 antibodies can support neurite outgrowth from primary neurons also suggests a positive role (Lipton et al., 1992); however the possibility that the antibodies simply provide an adhesive substratum for the Thy-1 positive neurons must be borne in mind. The fact that Thy-1 is expressed at relatively high levels by sympathetic neurons early in development (Birren and Anderson, 1990) and the observation that NGF induces increased expression of Thy-1 on PC12 cells over a similar time course to neurite outgrowth (Doherty and Walsh, 1987) would also be consistent with a positive role. A dual role for Thy-1, inhibitory in some and positive in other neurons, cannot be discounted.

Over a 4-d culture period soluble antibodies to Thy-1 can stimulate neurite outgrowth and this has been interpreted as being consistent with an inhibitory role for Thy-1 in neurite outgrowth (Mahanthappa and Patterson, 1992). The authors provided evidence that the antibodies induced shedding of Thy-1 from membranes and also showed that removal of Thy-1 by PIPLC had similar effects as the antibodies. However removal of Thy-1 from the total PC12 cell population was associated with neurite outgrowth from only a few percent of the cells over a 4-d culture period.

In the present study we have confirmed that antibodies to Thy-1 can trigger neurite outgrowth from PC12 cells and identified two culture conditions whereby a robust response from a large percentage of the population can be observed after a 48-h period of culture. These robust responses were observed when PC12 cells were cultured on PL/COLL or PL/LN in the presence of NGF, or alternatively over confluent monolayers of 3T3 cells in the absence of NGF. The observation that bivalent antibody is required for the response suggests that clustering of Thy-1 may be important. We found no evidence to support the idea that the removal of Thy-1 by antibody might cause the response; e.g., there was no obvious decrease in Thy-1 immunoreactivity on PC12 cells even after 48 h in the presence of the OX-7 antibody (see Fig. 3). Also complete removal of Thy-1 by PIPLC did not mimic to any extent the robust response induced by the OX-7 antibody. In this context removal of Thy-1 and presumably other GPI-anchored molecules from PC12 cells had no effect on basal neurite outgrowth or neurite outgrowth stimulated by NGF or CAMs (Singh, A., and P. Doherty, unpublished observations). These data are in accord with those of Tiveron et al. (1991) in that they show that the expression of Thy-1 per se is not inhibitory for neurite outgrowth over a variety of cellular substrata. This function has only been observed after Thy-1's interaction with a ligand expressed primarily by mature astrocytes. Treatment of rat cerebellar neurons and chick retinal ganglion cells with PIPLC also does not alter their ability to extend neurites in response to CAMs (Doherty et al., 1990*a*,*b*) again suggesting that the removal of GPI-anchored molecules from neurons has no effects on general neurite outgrowth.

The fact that PIPLC inhibits the effects of OX-7 suggests that OX-7 binding to PC12 cells is required for the biological response. The relative specificity of the response was shown by the fact that the binding of antibodies to other surface anti-

gens (in this case gangliosides) did not trigger a neurite growth response. Also, the binding of polyclonal antibodies to L1 or to NCAM in PC12 cells does not trigger neurite outgrowth (Williams et al., 1992; Saffell, J. L., F. S. Walsh, and P. Doherty manuscript in preparation).

How might the binding of antibodies trigger a complex response like neurite outgrowth? One obvious possibility is that this interaction might mimic the effects of a natural ligand binding to Thy-1 with consequent activation of neuronal second messenger pathways. In this context antibodies to Thy-1 can trigger changes in calcium in lymphocytes (Kroczek et al., 1986) and also increase the amplitude of the low threshold voltage-activated calcium current in sensory neurons (Saleh et al., 1988). In some cells Thy-1 coprecipitates with a tyrosine phosphatase (Volarevic et al., 1990) or a tyrosine kinase (Stefanova et al., 1991) activity. These data suggest that antibodies to Thy-1 might trigger neurite outgrowth by activating second messenger pathways in neurons.

There are at least four independent pathways that can lead to neurite outgrowth in PC12 cells and/or primary neurons. Providing an appropriate substratum is available soluble neurotrophic agents can stimulate neurite outgrowth as can agents that elevate cyclic AMP levels in neurons. Also, the interaction of integrin receptors or CAMs (NCAM, N-cadherin, and L1) with their appropriate ligands can stimulate "cell contact"-dependent neurite outgrowth. Calcium influx is both necessary and sufficient to account for CAM-dependent neurite outgrowth (Doherty and Walsh, 1992) but is not required for neurite outgrowth stimulated by NGF, increased cAMP, or by integrin receptors. In the present study three experiments suggest that Thy-1 antibodies can activate the same pathway as the CAMs. Reducing extracellular calcium to 0.25 mM, preloading neurons with a chelator of intracellular calcium to buffer any changes in intracellular calcium and blocking neuronal calcium channels all specifically inhibited OX-7-stimulated neurite outgrowth without affecting basal neurite outgrowth or neurite outgrowth stimulated by other agents. Unlike K^+ depolarization, the Thy-1 response was not associated with substantial changes in calcium influx into neurons or significant changes in the level of intracellular calcium in the PC12 cell bodies. However, this is not inconsistent with calcium influx mediating the Thy-1 response as measurements were made only in cell bodies over relatively short periods after antibody addition (up to 2 h) whereas the neurite outgrowth response takes up to 48 h to develop. Thus much more subtle and/or spatially restricted movements of calcium into neurons, including increased cycling of calcium rather than changes in level (for example see Rasmussen, 1989) may underlie both the antibody triggered and CAM dependent neurite outgrowth.

Calcium influx into PC12 cells and primary neurons can mimic CAM-dependent neurite outgrowth (Saffell et al., 1992; Williams et al., 1992). We have reported that a pertussis toxin-sensitive heterotrimeric G-protein is a key component of the CAM pathway that acts upstream of calcium channel activation. In the present study pertussis toxin has been shown to inhibit the Thy-1 antibody-triggered neurite outgrowth suggesting that Thy-1 acts upstream of a G-protein in the activation of the pathway. We have previously suggested that the above CAMs might activate the second messenger pathway by co-clustering a common adaptor or effector molecule and this is partly supported by the observation that NCAM and L1 (Kadmon et al., 1990), and L1 and axonin 1 (Kuhn et al., 1991) can be colocalized to the same punctate patches in membranes. In preliminary studies we have found that NCAM will cocluster with Thy-1 in PC12 cells and this might suggest that both can cluster a common adaptor or effector molecule. However we do not observe this phenomenon in 3T3-fibroblasts that express both Thy-1 and transfected NCAM (Rimon, G., and P. Doherty, unpublished observation). Thus the coclustering in PC12 cells may be indirect and/or mediated by carbohydrate groups present only on neuronal NCAM or Thy-1.

An important question concerns the physiological relevance of our observations. At the present moment it can only be stated that Thy-1 has the potential to trigger neurite outgrowth via activation of the CAM pathway in neurons. Whether the interaction of Thy-1 with a natural ligand would also result in neurite outgrowth via activation of this pathway can only be determined after identification of the Thy-1 binding molecule(s). At present it is clear that the binding of Thy-1 to an as yet to be identified ligand on astrocytes actually inhibits neurite outgrowth (presumably stimulated by CAMs) on astrocytes. This observation is not completely irreconcilable with the results of this study. For example, calcium influx into neurons can also be inhibitory for neurite outgrowth (Kater and Mills, 1991). A number of agents that inhibit neurite outgrowth by causing the collapse of the growth cone do so via a mechanism that can be inhibited by pertussis toxin (Igarashi et al., 1993) with, in at least one instance, a very large increase in calcium concentration preceding the collapse (Bandtlow et al., 1993). Alternatively, the interactions of Thy-1 with the astrocyte ligand may actually restrict the mobility of Thy-1 and indirectly affect the ability of CAMs and/or effector molecules to cluster and thereby activate the pathway.

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