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Thy-1-mediated regulation of a low-threshold transient calcium current in cultured sensory neurons

(monoclonal antibody/dorsal root ganglia/ligand binding/calcium channel/transmembrane signaling)

MARY SALEH*†, RICHARD J. LANG‡, AND PERRY F. BARTLETT*

*Walter and Eliza Hall Institute of Medical Research, Royal Parade, Parkville, Victoria 3050, Australia; and ‡Department of Physiology, Monash University, Wellington Road, Clayton, Victoria 3168, Australia

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ABSTRACT **The physiological effect of ligand binding to the Thy-1 molecule expressed on the neuronal cell surface was examined. Sensory neurons obtained from dorsal root ganglia of 2-day-old CBA/CAH mice (Thy-1.2) were cultured *in vitro* for 8 days, and then patch-clamp recordings were obtained of calcium currents. It was found that binding of an anti-Thy-1.2 monoclonal antibody to neurons expressing the Thy-1.2 moiety increased the amplitude of a low-threshold transient calcium current. Monoclonal antibodies directed against the Thy-1.1 molecule or a neuronal surface ganglioside (A2B5) had no effect on this calcium current in Thy-1.2-bearing cells, nor did they interfere with subsequent activation by the anti-Thy-1.2 monoclonal antibody. These results demonstrate that ligand binding to the Thy-1 molecule transduces a physiological signal in sensory neurons by increasing a voltage-activated calcium current.**

Thy-1 is a major cell-surface glycoprotein found in the nervous system of all mammalian species studied. Within the nervous system, it is primarily (1), although not exclusively (2), expressed at high levels on the neuronal surface (10^6 molecules per cell; refs. 1 and 3). Expression of the Thy-1 molecule begins at a defined developmental stage (4). The gene has been shown to be under tissue-specific regulation as demonstrated by the DNA-mediated gene transfer studies of the rodent Thy-1 gene into Thy-1-deficient cell lines (5) and shown to be under tight temporal regulation by experiments with Thy-1.1-transgenic Thy-1.2 mice (6) that have shown that Thy-1 is tightly regulated in the nervous system, because the developmental expression of the injected Thy-1 gene in the brain was identical to that of the endogenous Thy-1 gene.

Although the rodent Thy-1 amino acid (7, 8) and cDNA (9) sequences have been determined, the functional role of Thy-1 in the nervous system has yet to be elucidated. The distinctive cellular distribution and developmental regulation of Thy-1 in the nervous system suggest that this molecule may be involved in cell-surface recognition phenomena (10), such as the regeneration of processes of rat retinal ganglion cells (11). The localization of the Thy-1 molecule in synaptosomal fractions of the mouse (12) and chicken (13, 14) brain suggests a role in synaptogenesis. The amino acid homology of the Thy-1 molecule to regions of the immunoglobulin molecule and to other members of the immunoglobulin gene super family further suggests a receptor-like function for Thy-1 (7, 8, 11), which is supported by the report that anti-Thy-1 monoclonal antibodies (mAbs) induce interleukin 2 production (15, 16) and increase intracellular free Ca (17) in mouse T cells when the bound anti-Thy-1 mAb complex is further cross-linked by a second mAb. We have employed whole-cell recordings to demonstrate that anti-Thy-1 mAb binding to sensory neurons increases the amplitude of the voltage-activated Ca current, with or without the binding of a second anti-Thy-1 cross-linking mAb.

MATERIALS AND METHODS

Animals. The CBA/CAH WEHI(H-2K^k, Thy-1.2) and C57BL/Kathy (H-2^b, Thy-1.1) mice used in these experiments were obtained from stocks maintained and bred at the Walter and Eliza Hall Institute of Medical Research.

mAbs. The mAbs used in these experiments were obtained from the following antibody-producing hybridoma cell lines as culture medium supernatants: 30H12 (anti-Thy-1.2; ref. 18), F7D5 (anti-Thy-1.2; ref. 19), and HO22 (anti-Thy-1.1; ref. 20). mAb A2B5 (anti-ganglioside; ref. 21) was used as ascites fluid.

Preparation of Dorsal Root Ganglion (DRG) Neurons. DRGs from 2-day-old CBA (Thy-1.2) or C57BL/Kathy (Thy-1.1) mice were dissected free of surrounding spinal tissue and placed in Hepes-buffered Eagle's essential medium (HEM). The ganglia were incubated for 30 min at 37°C in HEM containing 0.1% trypsin and 0.01% DNase. After the incubation, fetal calf serum was added and cells were further washed with HEM containing 1% fetal calf serum and 0.001% DNase. The cells were then triturated in this solution with 18–25 gauge needles to attain a single-cell suspension. Approximately 5×10^3 cells were plated onto x-ray irradiated BALB/c 3T3 fibroblast monolayers on glass coverslips, in Dulbecco's modified Eagle's medium with 10% (vol/vol) fetal calf serum and cultured for a further 8 days.

Staining by Immunofluorescence. CBA DRG neurons were incubated with an anti-Thy-1.2 mAb culture supernatant, 30H12, for 20 min (or anti-Thy-1.1 mAb, H022, for C57BL/Kathy neurons), washed in HEM, and then incubated with a fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin mAb (Silenus, Melbourne, Australia, 1:50 dilution). After washing with HEM, the neurons were fixed with acid alcohol [95% (vol/vol) absolute ethanol and 5% (vol/vol) glacial acetic acid] at –20°C, and coverslips were inverted onto glass microscope slides and mounted in 2.6% (vol/vol) 1,4 diazobicyclo[2,2,2]octane (Dabco; Merck, Denmark) dissolved in glycerol/mouse-tonicity phosphate-buffered saline (0.285% $Na_2PO_4 \cdot 2H_2O$/0.0625% $NaH_2PO_4 \cdot 2H_2O$/0.87% NaCl, pH 7.3),, 9:1 (vol/vol) (final pH 8.6), and viewed by fluorescence microscopy.

Whole-Cell Patch-Clamp Recording. Single DRG neurons were selected by bright-field illumination, and low-resistance patch pipettes (2–7 MΩ) were then lowered to gently touch the cell membrane. A high-resistance (>5 GΩ) seal was formed by applying a slight suction. Sharp suction was then applied to rupture the underlying membrane for whole-cell voltage-clamp. After impalement, 15 min were allowed for

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Abbreviations: DRG, dorsal root ganglion(a); mAb, monoclonal antibody.

†To whom reprint requests should be addressed.

complete equilibration of the pipette solution and the internal environment of the cell. Currents through Ca channels were isolated pharmacologically by blocking Na currents with tetrodotoxin (11 μM), and K currents were eliminated by replacing internal K with Cs (22). The charge carrier used in all of these experiments was Ca (3 mM). DRG cells were stimulated at 0.1 Hz and all experiments were carried out at room temperature. The internal pipette solution contained 126 mM CsCl, 10 mM Hepes·NaOH (pH 7.2), 2 mM $MgCl_2$, 3 mM EGTA, 2 mM Na_2ATP, and 11 mM glucose (pH 7.2). The bathing solution contained 15 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 0.15% potassium phosphate buffer (1.68 M KH_2PO_4/1.12 M K_2HPO_4, pH 7.2), and 1% Hepes buffer (1.68 M Hepes/1.68 M NaOH, pH 7.2) with 1 μM tetrodotoxin added. All mAbs were diluted directly into this bathing solution (hybridoma supernatant was diluted 1:10; ascites fluid was diluted 1:100).

RESULTS

Thy-1 Expression on Cultured DRG Neurons. Neurons were obtained from the DRG of 2-day-old CBA (Thy-1.2) and C57BL/Kathy (Thy-1.1) mice and placed into culture. The neurons were then assessed for Thy-1 expression by immunofluorescence staining of bound anti-Thy-1 mAbs (Fig. 1*B*). Significant levels of surface expression of Thy-1 were not detected until after 2–4 days of culture, and the time of appearance of Thy-1 corresponded to that observed in DRG neurons freshly isolated from animals of known postnatal ages (M.S. and P.F.B., unpublished observation). In the cultured DRG neurons, there appear to be several morphologically distinct classes of neurons based on the size of their soma. The neuronal somal diameter varied from 15 to 50 μm (Fig. 1*A*). The larger neurons expressed the Thy-1 molecule on their surface earlier than the smaller neurons (data not shown). To ensure maximal surface expression of Thy-1, whole-cell recordings were carried out on neurons at a time equivalent to postnatal ages of 7–10 days. After recordings, DRG cells were stained by a fluorescein isothiocyanate-labeled anti-mouse inmmunoglobulin mAb to confirm the binding of the anti-Thy-1 mAbs (Fig. 1*B*).

Whole-Cell Patch-Clamp Recordings. The larger sensory neurons were selected by bright-field microscopy (Fig. 1*C*) and voltage-clamped by standard patch-clamp techniques. The large neurons were selected as they were found to have currents that remained consistent throughout the recording period, whereas the smaller neurons gave inconsistent results. A consequence of recording from large neurons was that large currents were difficult to voltage-clamp (see below).

Depolarization from a holding potential of −90 mV to potentials less negative than −70 mV evoked a transient inward current in the large neurons that was maximal at −50 mV. At potentials of more than −50 mV, this Ca current decreased in amplitude and had an accelerated rate of activation and inactivation (Fig. 2*A*). Depolarizations to potentials of more than −30 mV evoked a larger longer-lasting inward current in the sensory neurons that was maximal near −20 mV (Fig. 2 *A* and *B*). At more positive potentials this current decreased in amplitude and showed a slower rate of inactivation. We believe that this current activated at positive potentials is not sufficiently voltage-clamped as evidenced by its steep current–voltage relationship over its activation phase (Fig. 2 *B* and *C*, denoted by stars). However, the lower-threshold transient current showed a gradual increase with potential, indicating that this current was under voltage-clamp. Therefore, the effect of the mAbs binding has been examined only over a limited potential range (−70 to −30 mV), where the transient inward current was recorded. The lower-threshold inward current was abolished when the Ca in the bathing solution was replaced by the nonpenetrating cation Co (3 mM), indicating that this inward current triggered by depolarization was indeed a Ca current (Fig. 2 *A* and *B*).

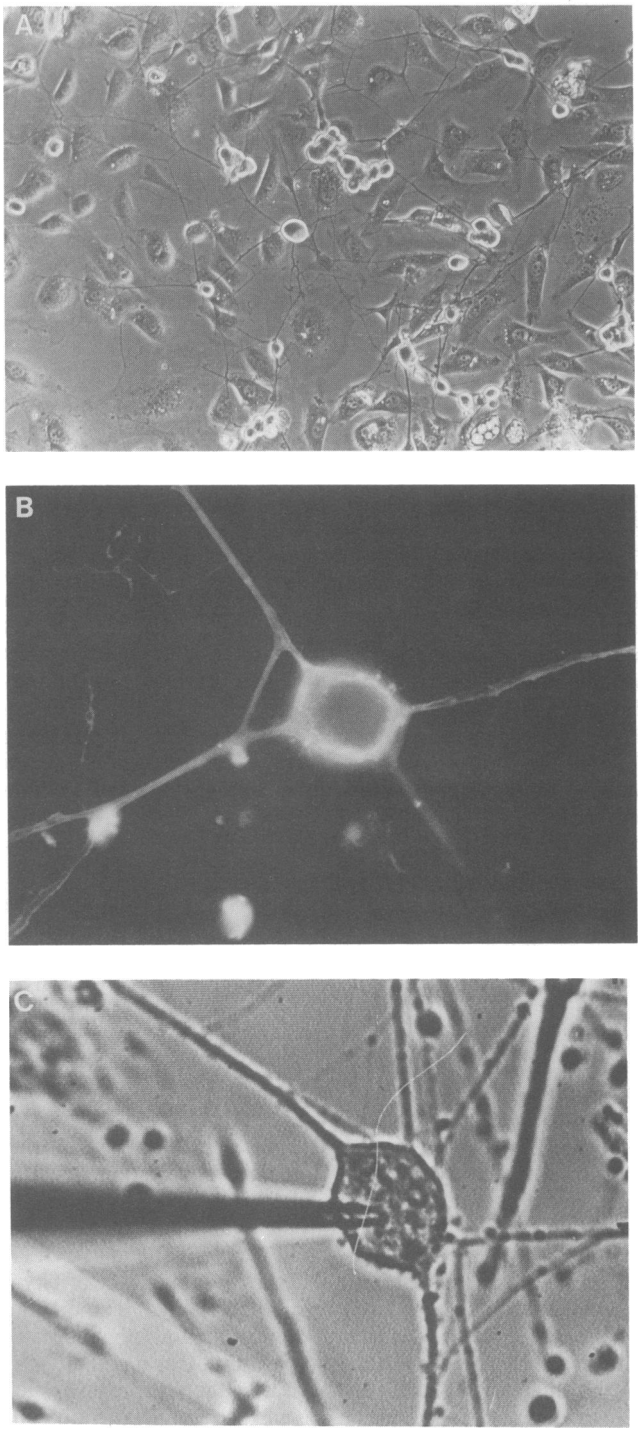

FIG. 1. DRG neurons with various morphologies are shown; the larger neurons were selected for patch-clamp recording. (*A*) A phase-contrast photomicrograph of a population of DRG neurons cultured on an x-irradiated monolayer of BALB/c 3T3 cells on glass coverslips, illustrating the variation in the neuronal soma diameter. (×75.) (*B*) A fluorescence photomicrograph showing the expression of the Thy-1.2 molecule on a cultured CBA DRG neuron stained with the mAb 30H12 and visualized with a fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin mAb. (×300.) (*C*) Bright-field photomicrograph of a single voltage-clamped DRG neuron. (×290.)

Effects of mAb Binding to Neurons on the Transient Inward Ca Current. To investigate the possible effect(s) of ligand

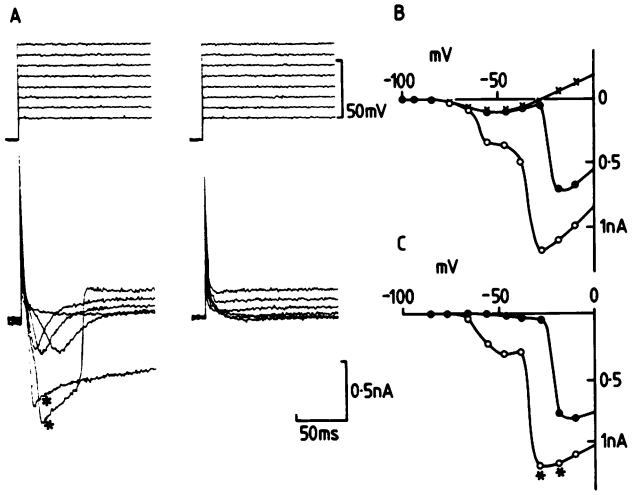

FIG. 2. Two types of voltage-sensitive Ca currents recorded during whole-cell recordings of cultured voltage-clamped DRG neurons. (*A*) Superimposed current traces evoked from a holding potential of −90 mV when 3 mM Ca (on the left) was in the bathing solution and when Ca was replaced by 3 mM Co (on the right). (*B*) Peak current (○) and current at the end of test (140 ms) depolarization (●) are plotted against the potential. The current (X) at the end of each depolarization when Ca was replaced by Co is shown. Leak conductance was estimated from the linear instantaneous current–voltage relationship at potentials more negative than −90 mV and has been subtracted. (*C*) Co-sensitive Ca current is plotted against the potential. ○, Peak current; ●, current at the end of each depolarization; *, activation phase.

binding to the Thy-1 membrane glycoprotein on the transient Ca current in sensory neurons, a panel of anti-Thy-1 mAbs was employed (see Table 1 for the list of mAbs and a summary of our results). The mAb concentration employed in these experiments gave maximal binding as assessed by indirect immunofluorescence assay. The addition of the anti-Thy-1.2 mAb 30H12 to cultures of CBA (Thy-1.2) DRG neurons evoked an increase in this Ca current within 10–15 min, as observed in recordings from eight individual neurons (Fig. 3). The holding current and resting membranes conductance, however, remained unaffected. The addition of the

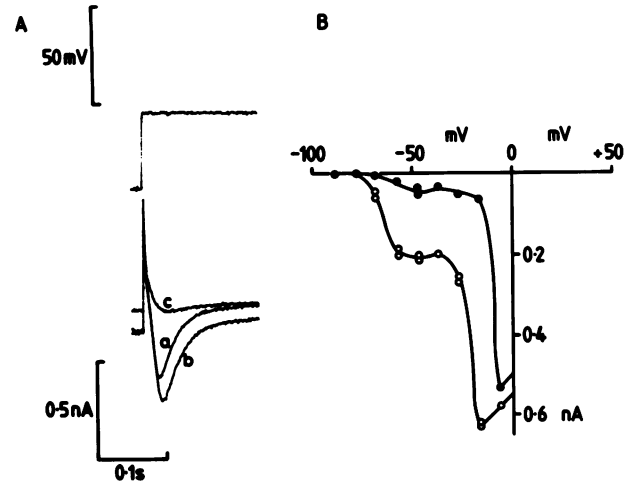

FIG. 3. Anti-Thy-1.2 mAb, 30H12, increases the transient inward Ca current recorded in cultured DRG neurons. (*A*) Superimposed current traces evoked at −45 mV, from a holding potential of −90 mV, are shown in the presence of 3 mM Ca and anti-Thy-1.1 mAb H022 (curve a), 10 min after 30H12 was added (curve b), and when the extracellular Ca was replaced by Co (curve c). (*B*) Plot of Co-sensitive peak Ca current in the absence (●) or the presence (○) of 30H12 is shown.

Table 1. Changes in the transient Ca current activity induced by mAb binding on mouse DRG neurons

mAb	Antibody specificity	Immunoglobulin isotype	Neuronal Thy-1 genotype	Effect on Ca current
30H12	Anti-Thy-1.2	IgG2b	Thy-1.2*	Increased
			Thy-1.1†	NE
F7D5	Anti-Thy-1.2	IgM	Thy-1.2*	Increased
			Thy-1.1†	NE
H022	Anti-Thy-1.1	IgM	Thy-1.2*	NE
A2B5	Anti-ganglioside	IgG2a	Thy-1.2*	NE
			Thy-1.1†	NE

NE, no significant effect.
*DRG neurons were from CBA mice.
†DRG neurons were from C57BL/Kathy mice.

anti-Thy-1.1 mAb H022 to the Thy-1.2-bearing neurons had no effect on the transient Ca current, whereas the subsequent addition of the 30H12 mAb to the same cell elicited an increase in the Ca current similar to that observed in cells without antibody (anti-Thy-1.1) preincubation (Fig. 3*B*). Furthermore, 30H12 had no effect when added to cultures of C57BL/Kathy (Thy-1.1) sensory neurons (data not shown). These experiments were repeated with another anti-Thy-1.2 mAb, F7D5, of a different immunoglobulin isotype (IgM instead of IgG2b, Table 1). This mAb produced an increase in the transient Ca current in a manner similar to that produced with 30H12 (Fig. 4*A*).

To examine whether the effects produced on the transient Ca current were merely due to nonspecific perturbation of the neuronal membrane by antibody binding, the mAb A2B5 was bound to the voltage-clamped neurons. A2B5 is directed against a surface ganglioside and was chosen because it was shown by immunofluorescence to be present on both CBA and C57BL/Kathy DRG neurons (data not shown). The binding of the A2B5 mAb to neurons of either Thy-1 genotype failed to elicit a change in the transient Ca current (Table 1 and Fig. 4*B*). Furthermore, voltage-clamp recordings taken from the same A2B5-bound cell showed an increase in the Ca

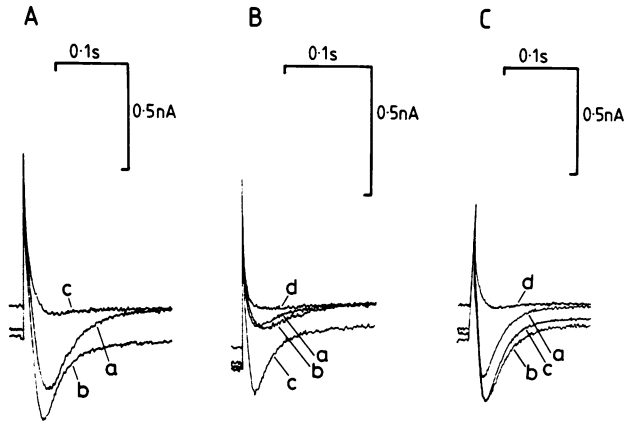

FIG. 4. Superimposed current traces evoked at −45 mV from a holding potential of −90 mV in the presence of various mAbs bound to the DRG neurons. (*A*) The anti-Thy-1.2 mAb F7D5 (curve b) increased the transient inward Ca current in the presence of 3 mM Ca and anti-Thy-1.1 mAb H022 (curve a) but not when the extracellular Ca was replaced by Co (curve c). (*B*) The A2B5 mAb (curve b) did not increase the transient Ca current nor prevent induction by the anti-Thy-1.2 mAb 30H12 (curve c) in the presence of 3 mM Ca and anti-Thy-1.1 mAb (curve a), or when the extracellular Ca was replaced by Co (curve d). (*C*) Cross-linking the bound anti-Thy-1.2 mAb with a sheep anti-mouse immunoglobulin mAb (curve c) failed to further increase the transient Ca current obtained with the anti-Thy-1.2 mAb 30H12 (curve b), in the presence of 3 mM Ca and anti-Thy-1.1 (curve a). Extracellular Ca was replaced by Co (curve d).

current upon the subsequent addition of the anti-Thy-1.2 mAb 30H12 (Fig. 4*B*). The magnitude and time course of the increase of this Ca current was identical to that observed with the anti-Thy-1.2 mAb alone.

Cross-Linked mAbs. In peripheral T cells (16), T-cell hybridomas (16), and various cell types transfected with a murine Thy-1.2 gene (17), the Thy-1–anti-Thy-1 complex must be further cross-linked with an anti-immunoglobulin mAb to obtain an increase in the intracellular free Ca concentration. We therefore cross-linked the anti-Thy-1.2 or A2B5 mAbs with sheep anti-mouse immunoglobulin mAb to test for an amplification of Ca influx. However, it was found that this did not increase the transient Ca current beyond the effects produced by anti-Thy-1.2 or A2B5 mAb binding alone (Fig. 4*C*).

DISCUSSION

Thy-1 expression on the cultured DRG neurons was assessed by immunofluorescence staining with the anti-Thy-1 mAbs and the developmental time of its surface expression was determined prior to the recording experiments. Within the DRG neuronal population, the cell bodies of neurons appear to have various sizes. The cultured DRG neurons varied in the size of their cell bodies, and the larger neurons were observed to have different characteristics than the smaller neurons. The large neurons express Thy-1 at an earlier stage and under voltage-clamp conditions, they retained their voltage-activated membrane current throughout the experiment, whereas the smaller neurons were unstable and often deteriorated after impalement. The necessity to record from larger neurons has meant that the effects of the mAbs on the membrane currents could only be studied over a limited potential range. In the larger neurons, a transient inward current was recorded. This Ca current shares a number of properties with the transient (T) or low-voltage activated (LVA) Ca current described in a number of cardiac and neuronal preparations (23–25). It is not clear whether the variable electrical properties observed between the different neurons correlate with the staggered surface appearance of the Thy-1 molecule on the neurons of various soma diameters.

The binding of two anti-Thy-1.2 mAbs to sensory neurons bearing the Thy-1.2 moiety increased the amplitude of the transient inward Ca current. The two mAbs were of different immunoglobulin isotypes, indicating that the activation of the Ca current is independent of the isotype class of the mAb. The activation of the Ca current appears to be specifically due to the binding of the anti-Thy-1.2 mAbs and not to a factor present in the supernatant medium, as evidenced by the addition of an anti-Thy-1.1 mAb to Thy-1.2-bearing neurons. Furthermore, mAb binding to neuronal molecules does not of itself result in a discernible change in the Ca current as shown by A2B5 binding nor does it interfere with the specific induction by the anti-Thy-1.2 mAbs. Binding of mAbs directed against other cell-surface antigens (such as the histocompatibility class I and class II antigens, the common leukocyte antigen, and the common lymphocyte function antigen) to murine T-cell hybridomas and human T-cell tumors failed to increase the intracellular Ca level (17). This is further evidence that signaling by way of the Thy-1 molecule may be a selectively specific phenomenon that is not found with all cell-surface molecules. The Thy-1 mode of transmembrane signaling in sensory neurons also appears to differ from that in T cells, because the anti-Thy-1 mAbs did not require cross-linking to a second mAb to alter the intracellular Ca concentration.

Although the precise mechanism by which mAb binding to the Thy-1.2 glycoprotein increases the Ca current in sensory neurons is not known, the activation of a protein kinase is likely to be involved. It has been shown that phospholipase C hydrolysis of phosphatidylinositol (26), the proposed Thy-1 membrane anchorage domain (26, 27), produces various inositol phosphates and diacylglycerol (28). In concert with phospholipids, 1,2-diacylglycerol specifically activates protein kinase C, which in turn affects membrane ion channels in a number of systems (28–31). In *Hermissenda* B photoreceptor cells, the activation of protein kinase C by phorbol esters results in an enhancement of the recorded Ca currents (31), and similar observations have been described with *Aplysia* bag-cell neurons (30–32). Another possible mode of activation may involve the direct association of the Thy-1 molecule with the Ca channels. This type of direct association occurs between γ-aminobutyric acid receptors and Ca channels in rat DRG neurons (33) and is independent of the protein kinase C pathway. Further work needs to be done to determine by which of these pathways Thy-1 binding modifies the membrane currents in sensory ganglia.

Whereas the importance of internal Ca concentration to neuronal function is well documented and Ca as a second messenger has been shown to mediate many vital processes, such as neurotransmitter release (25, 34), the major question arising from this study is the relative importance of the Thy-1-mediated pathway in regulating these processes. It has been speculated (17) that Thy-1 mediates an alternative activation pathway in T cells that has been largely superceded by the antigen-specific T-cell receptor and that this explains the lack of conservation of Thy-1 expression on T cells in some species. In contrast to T cells, the expression of Thy-1 like molecules has been retained on neurons of all species studied, including invertebrates (7). This conservation suggests that Thy-1-mediated activation of the transient Ca current may not only represent a primordial function of this molecule. Furthermore, the retention of Thy-1 at high levels on neurons indicates that this pathway may still be a major mode of regulating intracellular Ca concentration. The possible effects of Thy-1 binding on other membrane currents, such as Na or K currents, in sensory ganglia must also be established. These results stress the importance of identifying the putative natural Thy-1-ligand(s) that regulate this pathway within the nervous system.

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