The EMBO Journal vol.13 no.22 pp.5284–5292, 1994

Activity-dependent mobilization of the adhesion molecule polysialic NCAM to the cell surface of neurons and endocrine cells

Jozsef Z.Kiss[1], Cheng Wang, Sylviane Olive[2], Geneviève Rougon[2], Jochen Lang[3], Danielle Baetens, Daniela Harry[3] and William-F.Pralong[3]

Department of Morphology, University of Geneva Medical School, 1 rue Michel Servet, CH-1211 Geneva 4, [2]Laboratoire de Génétique et Physiologie du Développement, CNRS 9943, Parc Scientifique de Luminy, Case 907, F-13288 Marseille Cedex 9, France and [3]Division of Clinical Biochemistry, 1 rue Michel Servet, CH-1211 Geneva 4, Switzerland

[1]Corresponding author

Communicated by C.Wollheim

The α-2,8-linked sialic acid polymer (PSA) on the neural cell adhesion molecule (NCAM) is an important regulator of cell surface interactions. We have examined the translocation of PSA-NCAM to the surface of cultured cortical neurons and insulin secreting β cells under different conditions of cell activity. Endoneuraminidase N, an enzyme that specifically cleaves PSA chains, was used to remove pre-existing PSA from the plasma membrane and the re-expression of the molecule was monitored by immunocytochemistry. Punctate PSA immunostaining was restored on the surface of 68% of neurons within 1 h. This recovery was almost completely prevented by tetrodotoxin, suggesting that spontaneous electrical activity is required. K^+ depolarization (50 mM) allowed recovery of PSA surface staining in the presence of tetrodotoxin and this effect required the presence of extracellular Ca^{2+}. Rapid redistribution of PSA-NCAM to the surface of β cells was observed under conditions that stimulate insulin secretion. Ca^{2+} channel inhibition decreased both PSA-NCAM expression and insulin secretion to control, non-stimulated levels. Finally, subcellular fractionation of an insulin-secreting cell line showed that the secretory vesicle fraction is highly enriched in PSA-NCAM. These results suggest that PSA-NCAM can be translocated to the cell surface via regulated exocytosis. Taken together, our results provide unprecedented evidence linking cell activity and PSA-NCAM expression, and suggest a mechanism for rapid modulation of cell surface interactions.

Key words: β cell/cortical neuron/NCAM/PSA/regulated exocytosis

Introduction

Morphogenetic transformation of cell shape requires the orchestration of multiple molecular events including cell surface recognition, adhesive interactions and transmembrane signalling.(see Rakic *et al.*, 1994). In the nervous system, NCAM appears to play an important role in these processes (see Edelman, 1986; Rougon *et al.*, 1993). NCAM is a member of the immunoglobulin superfamily that can promote cell adhesion through a homophilic, Ca^{2+}-independent binding mechanism (Edelman, 1986). It is abundant in the nervous system, in skeletal muscle and in certain endocrine organs (Edelman, 1986; Rouiller *et al.*, 1991). NCAM is encoded by a single gene and three major protein isoforms, 180, 140 and 120 kDa, differing mainly by the length of their cytoplasmic domain and their mode of attachment to the membrane, are produced by differential splicing (see Walsh and Dickson, 1989; Goridis and Brunet, 1992). The expression of NCAM is dynamically regulated in different tissues and small changes in the surface density of the molecule are known to produce important alterations in adhesion (Edelman, 1986). An additional regulatory mechanism of NCAM-mediated cell adhesion involves the attachment of large, negatively charged carbohydrate polymers of α-2,8-linked sialic acid polymer (PSA) to the fifth immunoglobulin domain of the molecule (Finne *et al.*, 1983). The modulation of adhesion specifically arises from the length of the PSA; PSA has been shown to decrease NCAM homophilic binding and thereby attenuating cell adhesion (Rutishauser *et al.*, 1988). Recent data also indicate that PSA may act as a global inhibitor of cell adhesion and affect a broad spectrum of cell interactions (Rutishauser *et al.*, 1988; Acheson *et al.*, 1991). In mammalian cells, most if not all PSA is associated with NCAM (Rougon *et al.*, 1986; Cremer *et al.*, 1994). The sialylation state of NCAM is controlled by a developmentally regulated Golgi sialyltransferase activity (Breen and Regan, 1988). The expression of PSA is spatio-temporally regulated and the common denominator between cells expressing PSA-NCAM is that they all display a certain developmental plasticity and potential to change their shape or to move (see Rougon *et al.*, 1993). In the developing brain, NCAM isoforms are highly sialylated; they are then gradually replaced by 'adult' isoforms with lower content of sialic acid (Edelman, 1986). It has been proposed that by attenuating the ability of NCAM to promote cell adhesion, PSA optimizes conditions for normal development of the nervous system (Rutishauser *et al.*, 1988; Rougon *et al.*, 1993). Indeed, PSA on NCAM was shown to play an important role in morphogenetic events such as axonal growth (Doherty *et al.* 1990; Zhang *et al.*, 1992), cell migration (Wang *et al.*, 1994) and muscle innervation (Landmesser *et al.*, 1990). In recent studies, the highly sialylated, 'embryonic' form of NCAM has been found to persist in central structures capable of morphological plasticity (Miragall *et al.*, 1988; Aaron and Chesselet, 1989; Theodosis *et al.*, 1991; Bonfanti *et al.*, 1992; Kiss *et al.*, 1993). Some of these, such as the hypothalamo-neurohypophyseal system, the dentate gyrus of the hippocampal formation and the olfactory bulb, are known

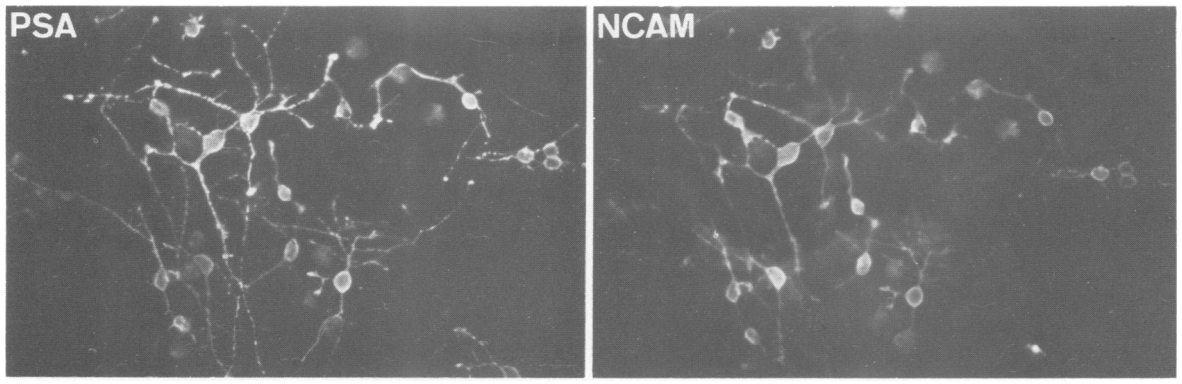

Fig. 1. Double immunofluorescence micrographs (with focus on neurones) showing PSA-NCAM surface staining in primary cultures of cortical neurones. Seven days after plating, neurons were incubated for 1 h in the presence of anti-PSA monoclonal and anti-NCAM polyclonal antibodies at 37°C. Following this incubation cell-bound antibodies were revealed on live cells by FITC and rhodamine-conjugated anti-mouse and anti-rabbit Abs respectively. Note that astrocytes in the background do express NCAM but not PSA. Magnification ×160.

to undergo activity-dependent morphological remodelling in the adult (see Theodosis and Poulain, 1993). A correlation of activity-dependent plasticity and PSA expression was also demonstrated in the neuromuscular junction where the activity-dependent intramuscular nerve branching was blocked by removal of PSA (Landmesser, 1992).

Inasmuch as PSA-NCAM regulates cell interactions, the mechanisms that control its level at the cell surface may play a decisive role in modulating morphogenesis and activity-dependent plasticity. To explore the possibility that PSA-NCAM is exposed at the plasma membrane upon cell activation, we have devised a model system in which cell surface expression of PSA can be monitored at the level of individual live cells. To this end, Endoneuraminidase N (Endo N) an enzyme that specifically cleaves PSA chains (Vimr *et al.*, 1984) without altering cell viability, was used to remove all PSA from the cell surface and the re-expression of the molecule was monitored under various experimental conditions. The effect of cell activation on the redistribution of PSA-NCAM was studied in two different cell types: cultured neurons from newborn rat cerebral cortex and insulin-secreting cells.

Results

Primary cultures of cerebral cortex from newborn rats were first assayed under resting or activation conditions with specific antibodies (Ab) directed against PSA or against the NH_2-terminal sequence common to all known NCAM isoforms. Figure 1 illustrates the localization of PSA-NCAM immunoreactivity in cortical cells after 7 days in culture. At this time most neurons were isolated from each other and had developed numerous, moderately branched processes, all of them exhibiting intense PSA-NCAM immunostaining. Double labelling of the same culture with anti-PSA Ab, and Abs directed against specific neuronal or glial markers revealed that only neurons expressed PSA-NCAM surface immunoreactivity (data not shown). To examine whether spontaneous electrical activity is required for cell surface expression of PSA-NCAM immunoreactivity, PSA was first removed by Endo N treatment, then its reapparition on the cell surface was explored in the presence or absence of the Na^+ channel antagonist, tetrodotoxin (TTX, 1 μM). Quantitative analysis of the surface PSA staining is presented in Figure 3. Endo N treatment completely removed PSA immunoreactivity from virtually all neuronal surfaces (Figure 2A and B). As evaluated by confocal microscopy, punctate PSA immunostaining was restored on the surface of 68 ± 4% (mean percentage ± SEM, from 440 cells, $n = 3$ independent experiments) of neurons cultured for 1 h after removal of the enzyme from the medium (Figure 2C and D). Typically, staining was intense and uniformly distributed on processes as well as on the cell body (Figure 2D). However, some cells showed a moderate to low labelling that was randomly distributed on the cell surface. PSA recovery could be largely inhibited by blocking cell metabolism (NaN_3, 10 mM) (Figure 3). Similarly, the recovery was markedly decreased (Figure 3, $P < 0.05$) by the addition of TTX to the culture (Figures 2E and F, and 3); 37 ± 4% (from 650 cells, $n = 4$) of the neurons were only faintly labelled. This suggests that spontaneous electrical activity relying on functional, voltage-sensitive Na^+ channels participates in the recovery process. The inhibitory effect of TTX was completely overcome under depolarizing conditions (in the presence of 50 mM K^+) (Figure 3); 65 ± 5% (from 290 cells, $n = 3$) of neurons exhibited intense punctate immunostaining for PSA. Regulated exo−endocytotic cycles have been demonstrated in cultured developing neurons (Lockerbie *et al.*, 1991; Matteoli *et al.*, 1992); if the PSA moiety of PSA-NCAM is located in the lumen of secretory vesicles, it should be exposed to the extracellular space following Ca^{2+}-dependent exocytosis. We thus explored whether Ca^{2+}-sensitive regulated exocytosis was involved in externalization of PSA-NCAM. Endo N-treated cultures were incubated in Ca^{2+}-free medium both under normal and depolarizing conditions. In all cases, recovery of PSA immunofluorescence was markedly decreased (Figure 3). Taken together, these results strongly suggest that activity-dependent regulated exocytosis is involved in the surface expression of PSA-NCAM in developing neurons.

We next probed for PSA-NCAM expression in another cell system in which secretory vesicles are abundant and regulated secretory pathways are easier to study. We found that the well-differentiated insulin-secreting cell line, ins-1 (Asfari *et al.*, 1992), as well as primary islet cells, exhibited surface labelling for PSA (Figure 4I) and NCAM (not shown). Moreover, both cell types displayed a strong granular staining for PSA and for NCAM in their cyto-

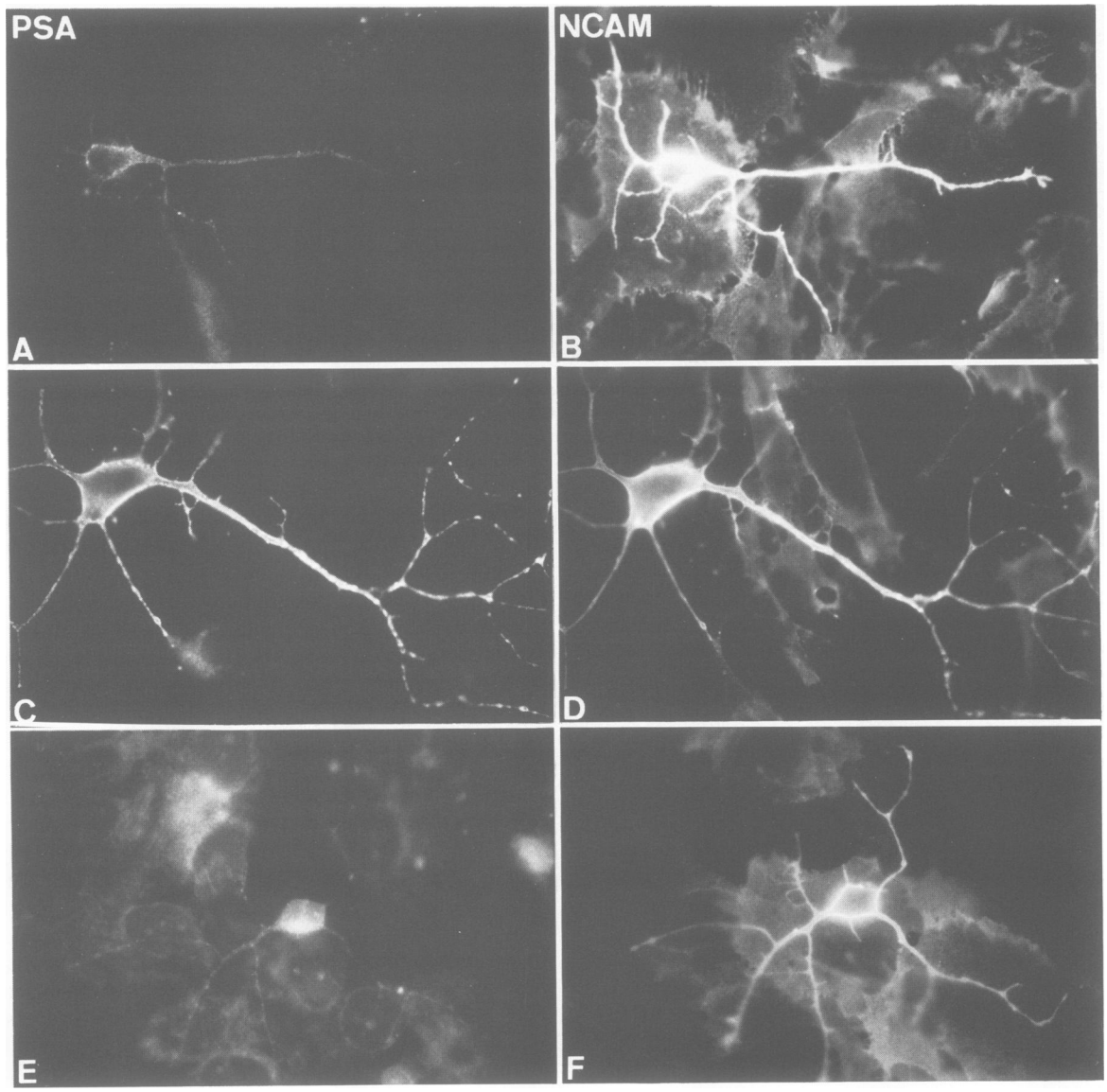

Fig. 2. PSA-NCAM surface immunoreactivity of cortical neurones after Endo N treatment. (**A** and **B**) Neurons were fixed and double-immunostained for PSA and for NCAM immediately after Endo N treatment. Cell-bound Abs were revealed by anti-mouse IgG conjugated to fluorescein (FITC) (A, C and E); or anti-rabbit IgG conjugated to rhodamine (TRITC) (B, D and F). Notice that PSA immunoreactivity has virtually disappeared, whereas NCAM immunostaining is not affected by the treatment. (**C** and **D**) Endo N-treated live neurons were allowed to recover for 1 h in normal medium in the presence of anti-PSA and anti-NCAM antibodies. Labelling on this neurone is intense and uniformly distributed on the surface. However, it should be noted that some other cells exhibited moderate to low degree of patchy immunoreactivity. (**E** and **F**) TTX (1 μM) was included in the medium during the 1 h recovery period. Notice that the neuron shown in E and F is devoid of PSA immunoreactivity, but strongly labelled for NCAM. However, some neurones displayed weak immunostaining even in the presence of TTX (not shown). Magnification ×420.

plasm that was comparable to that obtained for insulin (Figure 4I). These observations complement previous reports showing that NCAMs are expressed in pancreatic islet cells (Rouiller *et al.*, 1991). PSA-NCAM expression on the β cell surface was then probed under conditions known to stimulate or inhibit regulated insulin secretion (Figure 4II) (Wollheim *et al.* 1981). Endo N-pre-treated islet cells in primary culture were incubated for 30 min at a non-stimulating glucose concentration (3 mM) in the presence of PSA Ab. Under these conditions, 27 ± 3 % (from 250 cells, $n = 4$) of all islet cells exhibited a faint surface immunostaining for PSA (Figure 4II, panel A). Incubation in the presence of a stimulating glucose concentration (20 mM for 15 min) in combination with the cAMP raising agent, forskolin (10 μM), a condition known to induce maximal insulin secretion in cultured β cells (Pipeleers *et al.*, 1985), doubled the number of positive cells (72 ± 4 %, from 240 cells, $n = 4$) and enhanced PSA labelling intensity at the cell surface (Figure 4II, panel C). Addition of the Ca^{2+} channel blocker, SR7037 (Li *et al.*, 1991), to these cells maintained PSA expression at control levels (30 ± 2%, from 170 cells, $n = 3$), (Figure 4II, panel E), indicating the Ca^{2+} dependence of the phenomenon. These results were confirmed by quantitative analysis of PSA surface staining (Figure 5): a shift towards higher fluorescence values was obtained after glucose stimulation; this was prevented by the calcium channel blocker (Figure 5). Correlative evidence between exocytosis and enhanced surface exposure of PSA proceeded from the parallel assessment of insulin

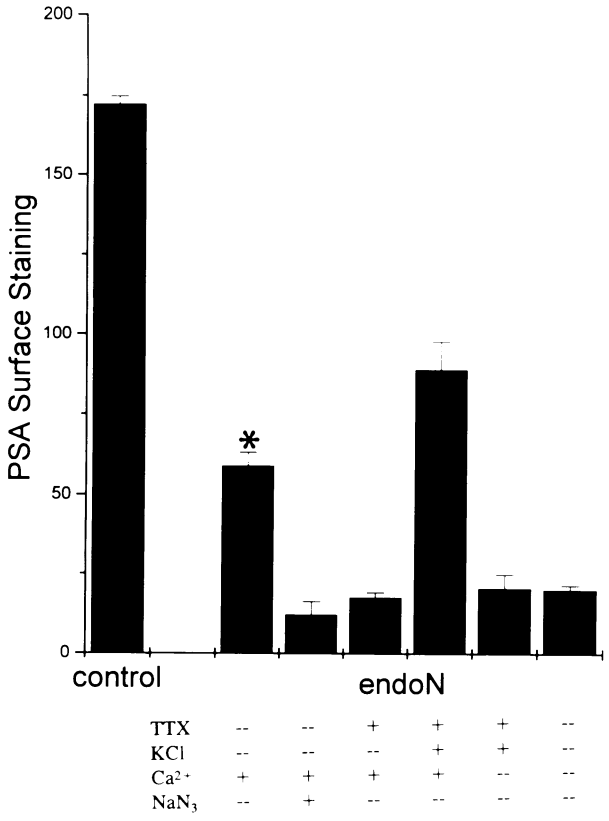

Fig. 3. Recovery of PSA surface staining in cortical neurones after Endo N treatment as evaluated by quantitative confocal microscopy. Cultures were treated with Endo N for 4 h, rinsed and allowed to recover in normal medium with the indicated compounds for 1 h (see Materials and methods). Control groups have not been treated with Endo N. Values are means ± SEM (n = 2–4 independent experiments) from 300 cells, normalized to that obtained at the end of Endo N treatment (see in Material and methods: experimental group 1). *, significantly different from all others (P <0.05).

secretion in the culture medium. When basal level was taken as control (1 ± 0.2) insulin secretion was 10-fold increased in stimulated cultures, whereas the presence of the Ca^{2+} channel blocker markedly inhibited the hormone secretion (Figure 5).

Further evidence for the presence of PSA-NCAM in the regulated secretory pathway of insulin secreting cells was obtained from immunoblots performed after subcellular fractionation of INS-1 cells (Asfari *et al.*, 1992; Figure 6). Proteins from fractions highly (lane 1) or partially (lane 2) enriched in secretory granules and from whole cell homogenates (lane 3) were analysed by Western blotting with both anti-PSA (Figure 6A) and anti-NCAM Abs (Figure 6B). A broad band of ~ 200 kDa was identified as PSA-NCAM according to its labelling by both anti-PSA and anti-NCAM Abs, and to its apparent molecular weight (Rougon *et al.*, 1986). The relative abundance of PSA-NCAM directly correlated with the enrichment of secretory granules as assayed by the insulin content of the fractions (see legend to Figure 6); when estimating the relative enrichment (per mg protein) of PSA-NCAM in the secretory granule fraction (lane 1), it should be taken into account that more than 80% of the proteins of that fraction represent insulin peptides. A faint band of non-polysialylated NCAM (116 kDa) was only detected in the homogenate. These results indicate that PSA-NCAM is specifically and highly enriched in insulin-containing secretory granules.

Discussion

Past work has shown that PSA-NCAM is associated with brain structures that undergo activity-induced morphological remodelling during development and in the adult (Miragall *et al.*, 1988; Aaron and Chesselet, 1989; Theodosis *et al.*, 1991; Bonfanti *et al.*, 1992; Kiss *et al.*, 1993). The question we thus raised is whether PSA-NCAM can be mobilized to the cell surface as a consequence of functional cell activity. To approach this problem we have developed an *in vitro* experimental system in which Endo N was used to remove pre-existing PSA from the cell surface; the subsequent re-expression of new PSA on the cell surface was monitored by an immunocytochemical assay. Our experiments indicate that PSA immunoreactivity is rapidly re-expressed at the cell surface upon enhancement of cellular activity. This was observed in two different cell types: developing cortical neurons and insulin-secreting β cells. In neurons, the recovery of PSA staining was blocked by TTX, suggesting that spontaneous electrical activity relying on functional, voltage-sensitive Na^+ channels is required for this process. This conclusion is consistent with previous studies demonstrating that neurons in primary cultures express mature Na^+ channels after 4 days in culture (Berwald-Netter *et al.*, 1981). Membrane depolarization with high concentration of potassium further increased PSA surface staining. Similarly, the stimulation of β cells by secretagogues such as high glucose and forskolin also enhanced the recovery of PSA staining following removal by Endo N. Since in both cases the activity-dependent redistribution of PSA-NCAM was calcium-dependent, our data suggest that the regulated exocytotic pathway is involved in the cell surface re-expression of PSA.

Previous studies had indicated that β cells resemble neurons in a number of ways including, the expression of NCAMs (Rouiller *et al.*, 1991; Moller *et al.*, 1992) and neuronal markers such as neurofilament proteins (Teitelman, 1990) and biosynthetic enzymes for neurotransmitters (Okada and Taniguchi Shimada, 1976; Teitelman and Lee, 1987; Teitelman and Evinger, 1988). Just as neurons, β cells make use of at least two pathways for the regulated exocytosis: one which involves large, dense core-containing storage vesicles (DV) and the other which involves small 'synaptic' vesicles (SV) (Cameron *et al.*, 1993). DVs are involved in the storage and secretion of neuropeptides and hormones, while SVs are the vesicle carriers of fast non-peptide neurotransmitters (Hökfelt *et al.* 1986; Cameron *et al.* 1993). Although we have not definitely identified the intracellular compartment of β cells and neurons that contain PSA-NCAM, our experiments suggest that PSA-NCAM could be sorted and stored, at least partly, in the DV compartment. This conclusion is based on the following observations: (i) PSA-NCAM immunoreactivity is abundant in the cytoplasm of β cells, where it has a granular appearance and extensively co-localizes with insulin immunostaining; (ii) increased surface staining and decreased cytoplasmic immunoreactivity for PSA-NCAM (unpublished results) after stimulation was paralleled by a decrease in insulin staining

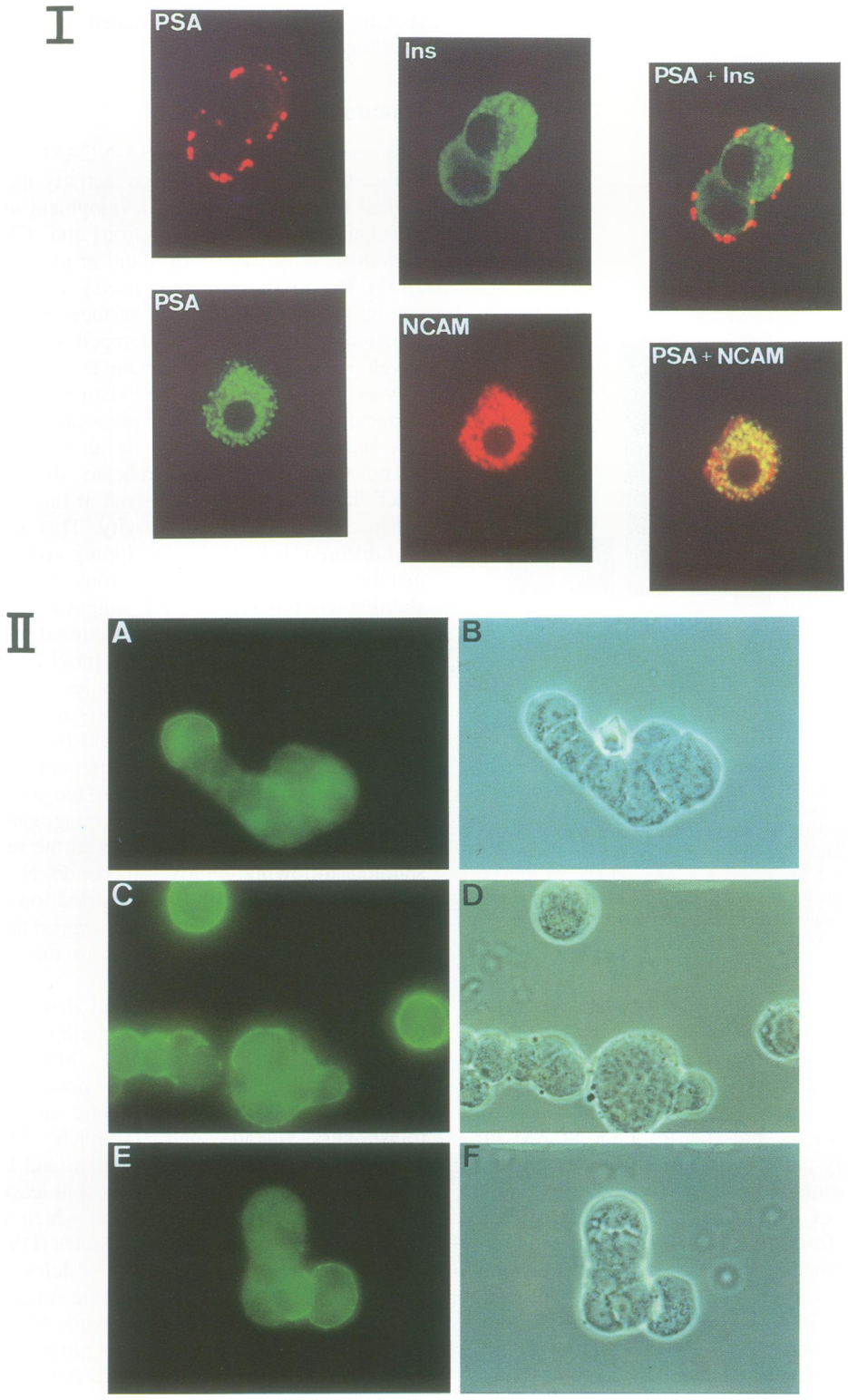

Fig. 4. PSA-NCAM in primary islet cells. (**I**) First row: confocal fluorescence microscopy showing an insulin (ins) positive primary islet cell exhibiting PSA surface labelling. PSA staining was performed on live cells, cultures were then fixed, detergent permeabilized and immunostained for insulin. Below: the co-localization of PSA and NCAM immunoreactivity in the cytoplasm of a second islet cell is shown. In these series, both stainings were performed on permeabilized cells. PSA+Ins and PSA+NCAM show superimposed digitized images where the yellow pseudo colour indicates the co-localization of PSA and NCAM staining. Magnification ×570. (**II**) Immunofluorescence and phase−contrast micrographs showing PSA surface re-distribution on live islet cells pre-treated with Endo N (see Materials and methods) and incubated thereafter for 30 min with 3 mM glucose (A and B) (basal, non-stimulating condition), 20 mM glucose + forskolin (10 μM, Sigma; stimulating condition) in the absence (C and D) and in the presence (E and F) of the Ca^{2+} channel blocker SR7037 (5 mM, Symphar, Geneva). Anti-PSA Ab was added to the medium during stimulation and cell bound Ab was revealed on live cells with the FITC-conjugated anti-mouse IgG at 37°C for 20 min. Magnification ×435.

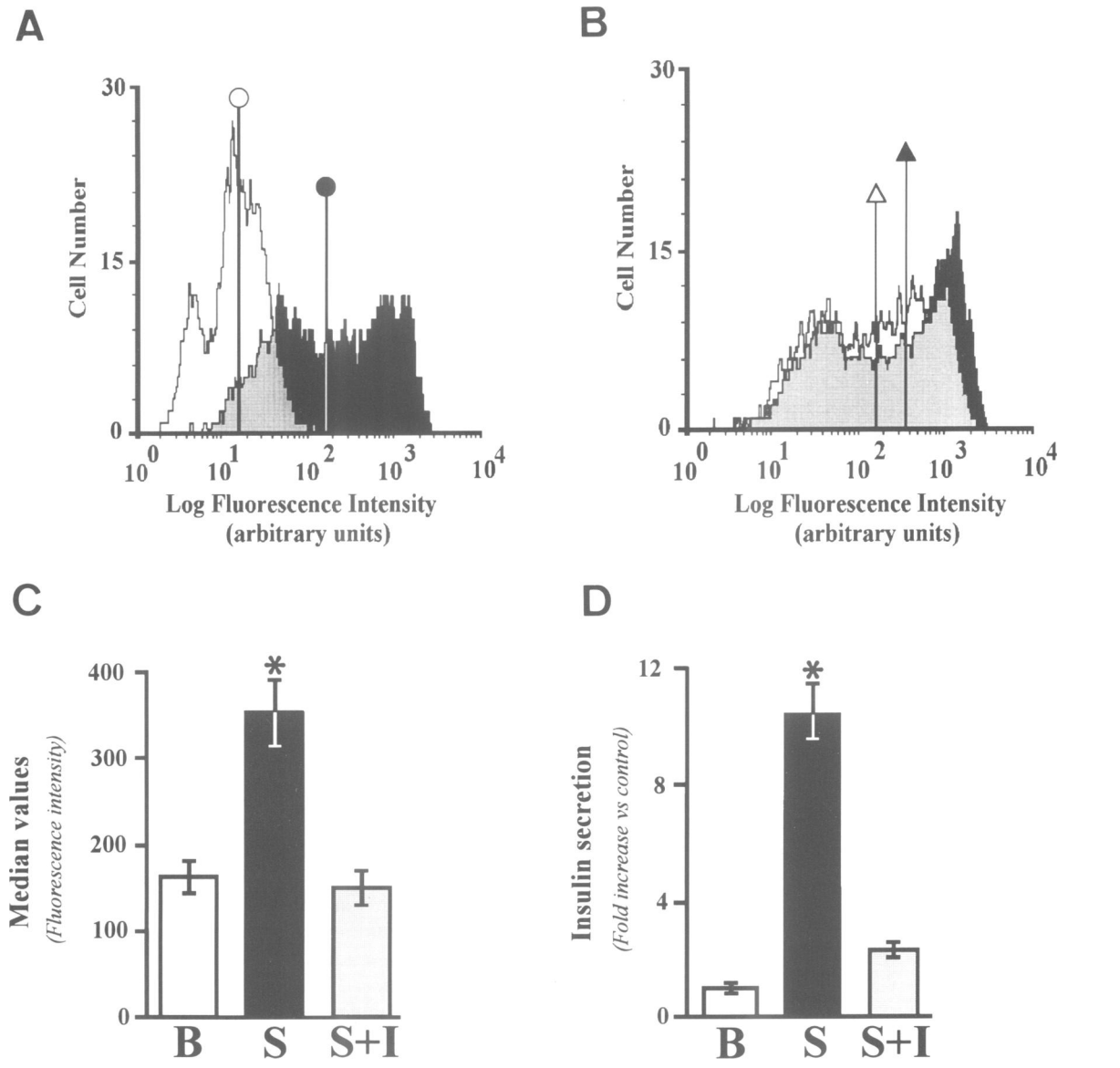

Fig. 5. Recovery of PSA surface staining in Endo N non-treated primary islet cells as evaluated by the fluorescence-activated cell sorting technique (FACS). In (**A**) cells underwent FACS following incubation under non-stimulating secretory conditions only with the anti-PSA secondary Ab (white control) and with the anti-PSA Ab followed by second antibodies (black histogram). In (**B**) cells were exposed to stimulating secretory conditions (see Materials and Methods) in the absence (white histogram) or the presence (black) of the Ca^{2+} channel blocker SR 7037; the grey area corresponds to the intersection of histograms. Vertical lines represent the position of median values indicating the respective distribution of the sorted populations (in log scale). (**C**) is a summary diagram of the median values (means ± SEM of four independent experiments) of the fluorescence sorted populations under basal B, high glucose + forskolin S or high glucose + forskolin + SR 7037 S+I. In (**D**), values (means ± SEM) of the immunoreactive insulin measured in the cell supernatants of the same experiments are shown. Notice that a shift to the right of the median values coincides with the stimulation of insulin secretion. *, significantly different from all others ($P < 0.05$).

and an increase in insulin secretion; (iii) cell fractionation and Western blot analysis showed that the secretory vesicle fraction of an insulin-secreting cell line was highly enriched in PSA-NCAM. In developing neurons, the plasmalemmal expansion at the growth cone is a calcium-mediated regulated process (Lockerbie *et al.*, 1991) that involves characteristic large clear vesicles different from neurotransmitter containing vesicles (Pfenninger and Bunge, 1974). It is possible that in addition to secretory vesicles, this type of growth cone vesicle is a carrier for PSA-NCAM, because growth cones are strongly labelled for PSA-NCAM; however, these vesicles are most likely not the only such carriers, since PSA immunoreactivity was not preferentially concentrated in the growth cone and the redistribution of PSA-NCAM on the surface of neurons after depolarization was virtually uniform along the cell surface. This distribution pattern is not surprising if one considers recent results obtained with cultured hippocampal neurons (Matteoli *et al.*, 1992). At earlier developmental stages that precede synaptic contacts, even the exo−endocytosis of synaptic vesicles appears in all cellular compartments, including dendrites, though in a lesser degree than in axons. In mature cultures rich in synaptic contacts, exocytosis of synaptic vesicles is restricted to axon terminals (Matteoli *et al.*, 1992). Other examples of molecules that exhibit a polarized distribution in mature but not in developing cells include Thy1, transferrin receptor, cytoskeletal protein MAP2 and tau

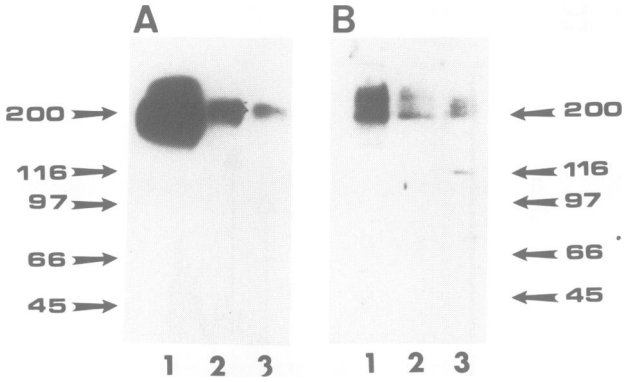

Fig. 6. Immunoblots with anti-PSA (**A**) and anti-NCAM Abs (**B**) of subcellular fractions from the insulin secreting cell line, INS1 cells. Fractions highly (lane 1, 11 μg protein) or partially (lane 2, 7.5 μg protein) enriched in secretory granules or homogenates (lane 3, 33 μg protein) were separated by SDS–PAGE (7% gels) followed by electrotransfer onto nitro-cellulose sheets and immunoblotted. The three fractions contained 0.86, 0.25 and 0.04 μg of insulin/mg of protein, respectively.

(for a review see de Hoop and Potti, 1993). It remains to be determined whether PSA surface expression becomes polarized by the maturation of neurons, for example after establishing synaptic contacts. However, it should be noted that PSA expression is known to be lost in most neurones after that synaptogenesis is completed.

To our knowledge, the only adhesion molecule that has so far been identified in the regulated exocytotic pathway is P-selectin (Sternberg *et al.*, 1985; Berman *et al.*, 1986), a member of a family of lectin-like adhesion molecules, that has been localized in storage granules of platelets and endothelial cells (McEver, 1991). Stimulation of these cells by agonists, such as thrombin, produces a rapid mobilization of P-selectin to the cell surface, where it mediates adhesion of leukocytes (McEver, 1991). P-selectin is subsequently re-internalized from the surface of activated endothelial cells (Hattori *et al.*, 1989). A signal directing P-selectin to secretory vesicles has been identified in the cytoplasmic domain of the molecule (Disdier *et al.*, 1992). It will be important to determine whether similar to P-selectin, sequence domains of the NCAM protein core are required for its sorting to storage vesicles and/or whether PSA residues have a determining role in this process. NCAM comprises several isoforms (Goridis and Brunet, 1992) which might be delivered by distinct mechanisms to the membrane. It also remains to be seen if the activity-dependent increase in PSA at the cell surface is transient and whether the molecule undergoes consecutive endo- and exocytosis cycles (Thomas *et al.*, 1994) through which it may be targeted to specific membrane domains.

Accumulating data suggest that interactions between adhesion molecules and transmembrane signalling play a crucial role in neuritogenesis (Doherty *et al.*, 1990; Arcangeli *et al.*, 1993; Fredette *et al.*, 1993; Teng and Green, 1993). Doherty *et al.* (1990; 1991) provided evidence that axonal outgrowth of cultured hippocampal neurones and PC12 cells can be induced by NCAM-mediated cell surface interactions and that this could be blocked by Ca^{2+} channel inhibitors. NCAM-promoted increase of axonal outgrowth could be mimicked by membrane depolarization, which again could be inhibited by blocking calcium influx (Doherty *et al.*, 1990, 1991). Based on these data an 'outside-to-in' signalling pathway was outlined as a transducing sequence from NCAM to activation of calcium channels, via a pertussis sensitive G protein (Doherty *et al.*, 1990, 1991). This explanation was consistent with the hypothesis that adhesion molecules, including CAMs and integrins, may promote axonal outgrowth through altered membrane potential and calcium influx. Our studies raise the possibility that this interacting pathway may also work in the opposite direction; depolarization and calcium ion influx could modulate adhesion molecules by influencing their expression at the plasma membrane. This may represent one mechanism by which membrane depolarization (Doherty *et al.*, 1990, 1991; Teng and Green, 1993) or alternatively hyperpolarization (Arcangeli *et al.*, 1993) affects neurite outgrowth and maintenance.

More generally, our observations have implications for the cellular mechanisms that relate environmental signals and cell activation to structural remodelling and morphogenesis. Neuronal activity is known to have a pronounced effect on the development of neuronal circuits in the neuromuscular (Landmesser *et al.*, 1988) as well as in the visual (Goodman and Schatz, 1993) systems during the early post-natal period. Moreover, activity-related synaptic rearrangement in hippocampal and neocortical structures has been implicated in learning and memory processes (Bailey and Kandel, 1993). Similarly, reorganization of the islet micro-organ (Orci, 1982) and the recruitment of secretory cells is believed to permit the endocrine pancreas to adapt to changes in hormone demand (Bonner-Weir *et al.*, 1989; Pipeleers, 1992). This structural remodelling may require the orchestration of multiple molecular events including the rapid and transient expression of cell surface receptors that mediate cellular recognition, cell adhesion and the activation of specific ion channels. The activity-triggered and targeted surface expression of PSA-NCAM (present study) or conversely its potential removal by endocytosis (Bailey *et al.*, 1992), may represent essential steps in these events.

Materials and methods

Antibodies and Endo N

To identify astrocytes, a rabbit polyclonal (Dakopatts, Denmark) and a mouse monoclonal Ab (mAb; Boehringer Mannheim) to glial fibrillary acidic protein (GFAP) were used at a dilution of 1:200 and 1:15, respectively. Anti-Men B (Meningococcus group B) Ab (clone 2-1 B) is a mouse IgM monoclonal Ab that recognises specifically α-2,8-linked PSA with chain length superior to 12 residues (Rougon *et al.*, 1986; Rougon, 1993). Ascites fluid was used at a dilution of 1:400. The rabbit antiserum directed against NCAM protein core was a site-directed Ab recognizing the seven NH_2-terminal residues of NCAM whose sequence is shared by every isoform (Rougon and Marshak, 1986). It was used at a dilution of 1:1000. The guinea-pig anti-porcine insulin Ab (Lot 573) was a kind gift from Dr P.Wright (University of Indianapolis). Neurones were specifically labelled with a polyclonal Ab directed against neurofilament, a kind gift of Dr V.Y.Lee (University of Pennsylvania). To remove pre-existing PSA from cell surfaces, the enzyme Endo N purified from phage K_1 (see Wang *et al.*, 1994) was used. Endo N was shown to degrade rapidly and specifically linear polymers of sialic acid with 2,8-linkage, with a minimum length of 7–9 residues (Vimr *et al.*, 1984). α-2,8 Polysialic acid glycans with chain length in excess of 10 sialosyl residues have so far been described in association with NCAM (Finne and Mäkelä, 1985).

Culture of cortical neurones

Primary mixed cultures of neuronal and glial cells were prepared from new-born rat cerebral cortex as described (Lyles *et al.*, 1984), except that cells were obtained by mechanical dissociation without trypsin digestion. Cultures were grown on polylysine-coated coverslips in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (BSA). Neurones remained viable up to 2 weeks in culture and were usually used 7 days after plating. Cells were treated with Endo N (0.5 U/ml) at 37°C for 4 h to remove pre-existing PSA from the cell surface. After the treatment, cultures were rinsed in serum-free medium (see below) containing 0.5% BSA and five experimental groups were processed in parallel. (i) To confirm the removal of PSA by Endo N, a group of cultures (group 1) were fixed and immunostained immediately after the enzymatic treatment. The serum-free medium was prepared by adding a supplement mixture (15 μg/ml insulin, 20 μg/ml transferrin, 20 nM progesterone, 100 mM putrescine and 30 nM sodium selenite) to the medium (DMEM, Gibco; Bottenstein, 1984). (ii) Group 2 included cultures that were allowed to recover in normal, serum-free medium in the presence of anti-PSA (1:400) and anti-NCAM (1:1000) Abs at 37°C for 1 h. (iii) Group 3 was the same as group 2, but sodium azide (10 mM) was added to the medium to block cellular metabolism. (iv) Group 4 was the same as group 2, but tetrodotoxin TTX (1 μM) (Sigma) was added to the medium. (v) Group 5 was the same as group 3 but depolarized with KCl (50 mM); (vi) Group 6 was the same as group 4 but depolarized in stimulation buffer devoid of $CaCl_2$, and containing 2mM EGTA; (vii) group 7 was the same as group 6, but without depolarizing concentration of KCl.

Immunocytochemistry

After 1 h of stimulation, neurones from groups 2–5 were fixed in 4% paraformaldehyde, incubated with the secondary FITC or TRITC conjugated anti-mouse or anti-rabbit Abs (Boehringer) at room temperature for 1 h as described (Wang *et al.*, 1994). Cells were then examined with either an Axiophot microscope (Zeiss, Germany) equipped with epifluorescence device or a Bio-Rad MCR 600 confocal laser scanning microscope (CSLM) system (Bio-Rad Micro Science Ltd, UK) attached to an Axiophot microscope. The laser source was a krypton/argon mixed gas laser. This laser emits three strong lines in exact coalignment at 488, 568 and 647 nm (Ion Laser Technologies, Model 5425, Salt Lake City, Utah). This makes possible simultaneous double fluorescence using 488 and 568 lines together. The following filters were applied: (i) selection filter 488 and 568 nm in the filter wheel; (ii) K1 dual excitation block: dual excitation filter 488 and 568 nm, K1 double dichroic mirror; (iii) K2 block: LP dichroic mirror, 522 DF 32 green emission filter (FITC), 585 EFLP red emission filter (TRITC). To avoid any interference in fluorescence emission, FITC and TRITC images of each double cells were taken consecutively.

For intracellular staining, cultures were fixed in 4% paraformaldehyde, detergent-permeabilized and incubated with the primary Abs (anti-GFAP or anti neurofilament) at 4°C for 12 h. Bound antibodies were revealed with FITC- or rhodamine-conjugated sheep anti-rabbit IgG. Cell counts presented in the text were obtained by random sampling of 2–8 coverslips for each group in at least three independent experiments.

Immunostaining was not seen in controls using non-specific mouse IgM, pre-immune sera or secondary antibodies alone. In double immunolabelling experiments the use of single primary antibodies with both anti-mouse FITC and anti-rabbit TRITC-conjugated secondary antibodies resulted in only single labelling. Endo-N pretreatment completely eliminated PSA immunostaining (see Figure 2) without affecting NCAM, GFAP or insulin staining.

Quantitation of PSA surface fluorescence was accomplished using the confocal microscopy and the Comos software package, version 4.62 (Bio-Rad Microscience). Cells double-labelled for NCAM and PSA were randomly taken on the basis of NCAM staining (TRITC) using a ×63 oil immersion Plan-I objective. We collected images of PSA (FITC) of each selected cell and stored them on Plasmon Optical Disc. Thus each captured image contained an individual cell profile to be evaluated. Images of 10 to 20 cells were generated for each culture. The intensity of fluorescent excitation of cells, gain and black levels were kept constant for each session of measurement. The pixel intensity threshold was adjusted such that the background corresponded to level 0 (maximum in arbitrary units was 255). The peak intensity measured over individual neurones were averaged for each culture and then normalized to that obtained from cultures of group 1 (fixed immediately after the treatment of Endo N) which had been processed in the same experiment. Data were evaluated by one way analysis of variance, followed by Duncan and Bonferroni's tests for multiple comparisons. The limit of significance was $P < 0.05$.

Culture of pancreatic islet cells

Primary cultures of adult rat islet cells were obtained and cultured for 3 days on coverslips as described (Pralong *et al.*, 1990); 12 h before the experiment, the glucose concentration in the medium was lowered to 3 mM. Pre-existing PSA immunoreactivity was removed by Endo N treatment (see above), then preparations were rinsed and incubated thereafter for 30 min with either 3 mM glucose (basal, non-stimulating condition) or 20 mM glucose + forskolin (10 μM, Sigma) (stimulating condition) in the absence and in the presence of the Ca^{2+} channel blocker SR7037 (5 μM, Symphar, Geneva, Switzerland). Anti-PSA Ab was added directly into the test media, cells were then fixed in 4% paraformaldehyde and further incubated with FITC- and TRITC-conjugated secondary antibodies. Using this protocol, no intracellular staining was detected, even when permeabilization of cells by Triton X preceded the incubation with the secondary antibodies. In order to rule out 'antigen capping', β cells were stimulated as described above, fixed and thereafter immunolabelled for PSA. The surface labelling was superimposable to that obtained with immunostaining of living cells. Insulin secretion was measured from the culture medium as described (Asfari *et al.*, 1992).

Fluorescence-activated cell analysis

Primary islet cells were cultured for 3 days in 8 cm^2 surface area Petri dishes as described above (Pralong *et al.*, 1990). Before incubation, all cells were rinsed with KRBH buffer and pre-incubated for 30 min at 37°C in the presence of 2.8 mM glucose. They were then rinsed and incubated for 30 min according to the basal and stimulating conditions described above. At the end of the incubation periods, the supernatants were collected for immunoreactive insulin assay and the cells still attached to the Petri dishes were rinsed with cold PBS containing 1% BSA. Immediately after, the anti-PSA Ab (1:4000) was applied for 20 min in PBS−BSA, then rinsed with PBS−BSA and re-incubated for 20 min in the presence of the second Ab, an anti-mouse IgM coupled to phycoerithrin (Caltag, San Francisco, CA). The cells were then washed three times for 5 min at room temperature and left for 10 min on ice under minimized light exposure in the presence of PBS−BSA; thereafter cells were gently detached by a flow of ice-cold PBS buffer with a Pasteur pipette, spun down and resuspended at a density of 1×10^6 cells/ml in PBS. They were kept on ice in the dark until fluorescence-activated cell analysis. Control cells were incubated only with the second Ab.

The cell-associated PSA immunoreactivity was determined with a FAC scan (Becton Dickinson) fluorescence cell analyser. The excitation was 488 nm and the emission light was selected with an interference filter at 575 nm. The data were arranged in histograms, the medians of which were given by the attached (Becton Dickinson) histogram statistical analysis software. The level of immunofluorescence light is expressed on the x axis in log scale; the y axis represents the distribution of the frequency (Figure 5). Histograms were compared using the Mann−Whitney U-test. Significance level of $p < 0.05$ was chosen.

Subcellular fractionation assay

INS-1 cells were prepared as already described (Asfari *et al.*, 1992), homogenized in HB (0.25 M sucrose, 5 mM HEPES and 0.5 mM EDTA, pH 7.4) by the use of a Parr bomb (20 bar for 20 min) and the post-nuclear supernatant was loaded on a discontinuous Nycodenz (Fluka, Buchs, Switzerland) gradient (16%/23%). After centrifugation at 100 000 *g* for 45 min, the 16%/23% interface was collected, washed and the sample was either used as partially purified fraction or further processed by resuspension in 40% metrizamide. The suspension was loaded under a discontinuous gradient consisting of 19%/25% metrizamide (Fluka, Buchs, Switzerland) and centrifuged at 100 000 *g* for 2 h. The 19%/25% interface, containing secretory granules was collected, washed and resuspended in HB. Incubations for detection of PSA were performed with anti-PSA Ab (1:500 dilution of ascites fluid); NCAM was detected on the same blot after desaturation with 2% SDS−100 mM mercaptoethanol in 50 mM Tris−HCl, pH 6.8 for 30 min at 70°C, followed by extensive rinsing in PBS and incubation with polyclonal anti-NCAM Ab (1:1000). Blots were revealed either by the direct use of ^{125}I-labelled protein A (anti-NCAM) or after incubation with anti-mouse IgM Ab (anti-PSA) and autoradiographed.

Acknowledgements

We wish to thank C.Saadi for technical assistance, D.Wohlwend for technical assistance in cell sorting and J.-P.Gerber for photographic work. This work was supported by the Swiss National Science Foundation grants 31-32745.91 to J.Z.K, 32-32376.97 to W.-F.P. and by grants from the Association Française contre la Myopathie and Association de Recherche contre le Cancer to G.R and from EEC Biotechnology Concerted Action (920326) to G.R and J.Z.K.

References

Aaron,L.I. and Chesselet,M.F. (1989) *Neuroscience*, **28**, 701–710.
Acheson,A., Sunshine,J.L. and Rutishauser,U. (1991) *J. Cell Biol.*, **114**, 143–153.
Arcangeli,A. *et al.* (1993) *J. Cell Biol.*, **122**, 1131–1143.
Asfari,M., Janjic,D., Meda,P., Li,G., Halban,P.A. and Wollheim,C.B. (1992) *Endocrinology*, **130**, 167–178.
Bailey,C.H. and Kandel,E.R. (1993) *Annu. Rev. Physiol.*, **55**, 397–426.
Bailey,C.H., Montarolo,P., Chen,M., Kandel,E.R. and Schacher,S. (1992) *Neuron*, **9**, 749–758.
Berman,C.L., Yeo,E.L., Wencel-Drake,J.D., Furie,B.C., Ginsberg,M.H. and Furie,B. (1986) *J. Clin. Invest.*, **78**, 130–137.
Berwald-Netter,Y., Martin Moutot,N., Koulakoff,A. and Couraud,F. (1981) *Proc. Natl Acad. Sci. USA*, **78**, 1245–1249.
Bonfanti,L., Olive,S., Poulain,D.A. and Theodosis,D.T. (1992) *Neuroscience*, **49**, 419–436.
Bonner-Weir,S., Deery,D., Leahy,J.L. and Weir,G.C. (1989) *Diabetes*, **38**, 49–53.
Bottenstein,J.E. (1984) In Barnes,D.W., Sirbasku,D.H. and Sato,G.H (eds), *Methods for Serum-free Culture of Neuronal and Lymphoid Cells*. Alan R.Liss, New York pp. 3–13.
Breen,K.C. and Regan,C.M. (1988) *Development*, **104**, 147–154.
Cameron,P., Mundigl,O. and De Camilly,P. (1993) *J. Cell Sci.*, **17**, 93–100.
Cremer,H. *et al.* (1994) *Nature*, **367**, 455–459.
de Hoop,M.J. and Dotti,C.G. (1993) *J. Cell Sci.*, **17** (suppl.), 85–92.
Disdier,M., Morrissey,J.H., Fugate,R.D., Bainton,D.F. and McEver,R.P. (1992) *Mol. Biol. Cell*, **3**, 309–321.
Doherty,P., Cohen,J. and Walsh,F.S. (1990) *Neuron*, **5**, 209–219.
Doherty,P., Rowett,L.H., Moore,S.E., Mann,D.A. and Walsh,F.S. (1991) *Neuron*, **6**, 247–258.
Edelman,G.M. (1986) *Annu. Rev. Cell Biol.*, **2**, 81–116.
Finne,J. and Mäkelä,P.H. (1985) *J. Biol. Chem.*, **260**, 1265–1270.
Finne,J., Finne,U., Deagostini Bazin,H. and Goridis,C. (1983) *Biochem. Biophys. Res. Commun.*, **112**, 482–487.
Fredette,B., Rutishauser,U. and Landmesser,L. (1993) *J. Cell Biol.*, **123**, 1867–1888.
Goodman,C.S. and Schatz,C. (1993) *Cell*, **72** (suppl), 77–98.
Goridis,C. and Brunet,J.F. (1992) *Semin. Cell Biol.*, **3**, 189–197.
Hattori,R., Hamilton,K.K., Fugate,R.D., McEver,R.P. and Sims,P.J. (1989) *J. Biol. Chem.*, **264**, 7768–7771.
Hökfelt,T., Fuxe,K. and Pernow,P. (1986) *Progr. Brain Res.*, **68**, 1–411.
Kiss,J.Z., Wang,C. and Rougon,G. (1993) *Neuroscience*, **53**, 213–221.
Landmesser,L. (1992) *J. Neurobiol.*, **23**, 1131–1139.
Landmesser,L., Dahm,L., Schultz,K. and Rutishauser,U. (1988) *Dev. Biol.*, **130**, 645–670.
Landmesser,L., Dahm,L., Tang,J.C. and Rutishauser,U. (1990) *Neuron*, **4**, 655–667.
Li,G.D., Milani,D., Dunne,M.J., Pralong,W.F., Theler,J.M., Petersen,O.H. and Wollheim,C.B. (1991) *J. Biol. Chem.*, **266**, 3449–3457.
Lockerbie,R.O., Miller,V.E. and Pfenninger,K.H. (1991) *J. Cell Biol.*, **112**, 1215–1227.
Lyles,J.M., Norrild,B. and Block,E. (1984) *J. Cell Biol.*, **98**, 2077–2081.
McEver,R.P. (1991) *Thromb. Haemostasis*, **66**, 80–87.
Matteoli,M., Takei,K., Perin,M.S., Sudhof,T.C. and De Camilli,P. (1992) *J. Cell Biol.*, **117**, 849–861.
Miragall,F., Kadmon,G., Husmann,M. and Schachner,M. (1988) *Dev. Biol.*, **129**, 516–531.
Moller,C.J., Christgau,S., Williamson,M.R., Madsen,O.D., Zhan-Po,N., Bock,E. and Baekkeskov,S. (1992) *Mol. Endocrinol.*, **6**, 1332–1342.
Okada,Y. and Taniguchi Shimada,C. (1976) *Science*, **194**, 620–622.
Orci,L. (1982) *Diabetes*, **31**, 538–565.
Pfenninger,K.H. and Bunge,R.P. (1974) *J. Cell Biol.*, **63**, 180–196.
Pipeleers,D.G. (1992) *Diabetes*, **41**, 777–781.
Pipeleers,D.G., Schuit,F.C., in'T Veld,P.A., Maes,E., Hooghe-Peters,E.L. and Van de Winkel,M. (1985) *Endocrinology*, **117**, 824–833.
Pralong,W.-F., Bartley,C. and Wollheim,C.B. (1990) *EMBO J.*, **9**, 53–60.
Rakic,P., Cameron,R. and Kumoro,H. (1994) *Curr. Opin. Neurobiol.*, **4**, 63–69.
Rougon,G. (1993) *Eur. J. Cell Biol.*, **61**, 197–207.
Rougon,G. and Marshak,D. (1986) *J. Biol. Chem.*, **261**, 3396–3401.
Rougon,G., Dubois,C., Buckley,N., Magnani,J.L. and Zollinger,W. (1986) *J. Cell Biol.*, **103**, 2429–2437.
Rougon,G., Olive,S. and Figarella-Branger,D. (1993) In Roth,J., Rutihauser,U. and Troy,F. (eds), *Polysialic acid*. Birkhäuser Verlag, Basel, pp. 323–333.
Rouiller,D.G., Cirulli,V. and Halban,P.A. (1991) *Dev. Biol.*, **148**, 233–242.
Rutishauser,U., Acheson,A., Hall,A.K., Mann,D.M. and Sunshine,J. (1988) *Science*, **240**, 53–57.
Sternberg,P.E., McEver,R.P., Shuman,M.A., Jacques,Y.V. and Bainton,D.F. (1985) *J. Cell Biol.*, **101**, 880–886.
Teitelman,G. (1990) *Dev. Biol.*, **142**, 368–379.
Teitelman,G. and Evinger,M.J. (1989) In Hanahan,D., McDevitt,H.O. and Cahill,G.S. (eds), *Perspectives on the Molecular Biology and Immunology of the Pancreatic β Cell*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 37–42.
Teitelman,G. and Lee,J.K. (1987) *Dev. Biol.*, **121**, 454–466.
Teng,K.K. and Green,L.A. (1994) *J. Neurosci.*, **13**, 3124–3135.
Theodosis,D.T. and Poulain,D.A. (1993) *Neuroscience*, **57**, 501–535.
Theodosis,D.T., Rougon,G. and Poulain,D.A. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 5494–5498.
Thomas,P., Lee,A.K., Wong,J.G. and Almers,W. (1994) *J. Cell Biol.*, **124**, 667–675.
Vimr,E.R., McCoy,R.D., Vollger,H.F., Wilkison,N.C. and Troy,F.A. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 1971–1975.
Walsh,F.S. and Dickson,G. (1989) *BioEssays*, **11**, 83–88.
Wang,C., Rougon,G. and Kiss,J.Z. (1994) *J. Neurosci*, in press.
Wollheim,C.B. and Sharp,G.W. (1981) *Physiol. Rev.*, **61**, 914–973.
Zhang,H., Miller,R.H. and Rutishauser,U. (1992) *J. Neurosci.*, **12**, 3107–3114.

Received on April 11, 1994; revised on June 27, 1994