

# Annexin II in Exocytosis: Catecholamine Secretion Requires the Translocation of p36 to the Subplasmalemmal Region in Chromaffin Cells

Sylvette Chasserot-Golaz, Nicolas Vitale, Isabelle Sagot,\* Bruno Delouche,\* Sylvie Dirrig,  
Louise-Anne Pradel,\* Jean-Pierre Henry,\* Dominique Aunis, and Marie-France Bader

Institut National de la Santé et de la Recherche Médicale U-338, "Biologie de la Communication Cellulaire," 67084 Strasbourg, France; and \*Centre National de la Recherche Scientifique, URA 1112, Unité de Neurobiologie Physico-chimique, Institut de Biologie Physico-chimique, Paris, France

**Abstract.** Annexin II is a  $\text{Ca}^{2+}$ -dependent membrane-binding protein present in a wide variety of cells and tissues. Within cells, annexin II is found either as a 36-kD monomer (p36) or as a heterotetrameric complex (p90) coupled with the S-100-related protein, p11. Annexin II has been suggested to be involved in exocytosis as it can restore the secretory responsiveness of permeabilized chromaffin cells. By quantitative confocal immunofluorescence, immunoreplica analysis and immunoprecipitation, we show here the translocation of p36 from the cytosol to a subplasmalemmal Triton X-100 insoluble fraction in chromaffin cells following nicotinic stimulation. A synthetic peptide corresponding to the  $\text{NH}_2$ -terminal domain of p36 which contains the phosphorylation sites was microinjected into individual chromaffin cells and catecholamine secretion was monitored by amperometry. This peptide blocked com-

pletely the nicotine-induced recruitment of p36 to the cell periphery and strongly inhibited exocytosis evoked by either nicotine or high  $\text{K}^+$ . The light chain of annexin II, p11, was selectively expressed by adrenergic chromaffin cells, and was only present in the subplasmalemmal Triton X-100 insoluble protein fraction of both resting and stimulated cells. p11 can modify the  $\text{Ca}^{2+}$ - and/or the phospholipid-binding properties of p36. We found that less  $\text{Ca}^{2+}$  was required to stimulate the translocation of p36 and to trigger exocytosis in adrenergic chromaffin cells. Our findings suggest that the translocation of p36 to the subplasmalemmal region is an essential event in regulated exocytosis and support the idea that the presence of p11 in adrenergic cells may confer a higher  $\text{Ca}^{2+}$  affinity to the exocytotic pathway in these cells.

**A**NNEXINS have been isolated from a wide variety of cells and tissues by investigators studying diverse biological processes. The common characteristic of the annexins is that they bind to biological membranes and anionic phospholipids in a  $\text{Ca}^{2+}$ -dependent manner (for reviews see Geisow et al., 1991; Moss, 1992). The function of these proteins within cells remains uncertain (Raynal and Pollard, 1994). Members of the annexin family are substrates for tyrosine kinases, suggesting a function in cell growth (Glenney and Tack, 1985). Several investigators have proposed that annexins may regulate the inflammatory response as they inhibit phospholipase  $\text{A}_2$  and can be induced by steroids (Wallner et al., 1986). Other possible functions of the annexins include roles in the organization of cytoskeletal actin (Gerke and Weber, 1984) or in the organization of lipid domains (Geisow et al., 1991). Furthermore, many proteins of the annexin family have been shown to bind to and promote  $\text{Ca}^{2+}$ -depen-

dent aggregation of secretory granules, indicating that annexins may play a role in promoting membrane interactions during exocytosis (Südhof et al., 1982; Drust and Creutz, 1988). The common sequence principle of the annexin family is an array of 70 residues, which is tandemly repeated four or eight times. Each repeat contains a 17-residue consensus motif, possibly involved in  $\text{Ca}^{2+}$  and/or lipid binding (Geisow et al., 1986; Huber et al., 1992). Diversification of the biological activities of the annexins appears to depend on the  $\text{NH}_2$ -terminal tail which is variable in length and sequence.

Annexin II, one of the best characterized components of the annexin family, is peculiar in that it is present in cells as a 36-kD monomer (p36) and also as a tight heterotetramer complexed with the S-100-related protein p11. The heterotetrameric protein consists of two copies of p36 and two copies of p11 (Erikson et al., 1984; Gerke and Weber, 1984; Glenney and Tack, 1985), and is often found in the subplasmalemmal cytoskeletal network in different cell types (Gerke and Weber, 1984; Thiel et al., 1992). Comparison of the monomeric p36 vs the tetrameric p90 indicates that the formation of the complex results in a mole-

Please address all correspondence to S. Chasserot-Golaz, INSERM Unite 338, Biologie de la Communication Cellulaire, 5 rue Blaise Pascal, 67084 Strasbourg, France. Tel.: 33 88 45 6714. Fax: 33 88 60 0806.

cule with a higher affinity for  $\text{Ca}^{2+}$  and phospholipids (Evans and Nelsestuen, 1994).

Annexin II has been implicated in a number of membrane-related events including the  $\text{Ca}^{2+}$ -dependent regulation of exocytosis in chromaffin cells (Ali et al., 1989; Sarafian et al., 1991; Creutz, 1992) as well as in endocytotic pathways (Emans et al., 1993). Annexin II belongs to the cytosolic proteins described as essential for regulated exocytosis. The addition of annexin II to streptolysin-O (SLO)<sup>1</sup>-permeabilized chromaffin cells with reduced secretory capacity due to the leakage of cytosolic proteins, partially restores the calcium-dependent secretory activity (Ali et al., 1989; Sarafian et al., 1991). This effect is specific for annexin II since other annexins (p32, p37, p67) were unable to stimulate secretion (Sarafian et al., 1991). In addition, the participation of annexin II requires phosphorylation by protein kinase C and this modification promotes the binding of cytosolic p36 to intracellular membranes (Sarafian et al., 1991).

To further elucidate the role(s) of annexin II in regulated exocytosis, we focused here on the intracellular distribution of p36 and its cellular ligand p11 during exocytosis in chromaffin cells. By combining confocal microscopy with a microinjection technique coupled to the amperometric detection of exocytosis in single chromaffin cells, we demonstrate that the translocation of p36 from the cytosol to the subplasmalemmal region is an essential event in catecholamine secretion. Furthermore, our data reveal that p11 is only found in the periphery of adrenergic cells. The presence of p11 may account for the lower  $\text{Ca}^{2+}$  threshold of p36 translocation and exocytosis observed in adrenergic cells.

## Materials and Methods

### Chromaffin Cells

Chromaffin cells were isolated from fresh bovine adrenal glands by retrograde perfusion with collagenase and purified on self-generating Percoll gradients (Bader et al., 1986). Cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% FCS and containing cytosine arabinoside (10  $\mu\text{M}$ ), fluorodeoxyuridine (10  $\mu\text{M}$ ), streptomycin (50  $\mu\text{g}/\text{ml}$ ), and penicillin (50 U/ml). Cells were usually cultured as monolayers on collagen-coated glass coverslips at a density of  $2 \times 10^5$  cells and used within 3–7 d after plating. To trigger exocytosis, chromaffin cells were washed twice with Locke's solution (140 mM NaCl, 4.7 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 11 mM glucose, 0.56 mM ascorbic acid, and 15 mM Hepes, pH 7.2) and then stimulated 5 min with Locke's solution containing either 10  $\mu\text{M}$  nicotine or 59 mM  $\text{K}^+$  (made by decreasing NaCl isosmotically).

Permeabilization of chromaffin cells with streptolysin-O (SLO; Institut Pasteur, Paris, France) was performed as previously described (Sarafian et al., 1991). Briefly, cells were washed with calcium-free Locke's solution (containing 1 mM EGTA) and then permeabilized for 2 min at 37°C with SLO (18 U/ml) in 200  $\mu\text{l}$  calcium-free permeabilizing medium (150 mM glutamate, potassium salt, 10 mM Pipes, 5 mM NTA, 0.5 mM EGTA, 0.2% bovine serum albumin, 5 mM Mg-ATP, 4.5 mM magnesium). Secretion was induced for 5 min with permeabilizing media containing various amounts of  $\text{CaCl}_2$  to yield the indicated free calcium concentration. The exact free  $\text{Ca}^{2+}$  concentration in the medium was calculated as described by Flodgaard and Fleron (1974) using the stability constant given by Sillen and Martell (1971).

1. *Abbreviations used in this paper:* DBH, dopamine  $\beta$ -hydroxylase; NSF, *N*-ethylmaleimide-sensitive fusion protein; PKC, protein kinase C; PNMT, phenylethanolamine *N*-methyl transferase; SLO, streptolysin-O; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor.

### [<sup>3</sup>H]Noradrenaline Release

Catecholamine stores were labeled by incubation of intact cells with [<sup>3</sup>H]noradrenaline (13.3 Ci/mmol; Amersham, Les Ulis, France) for 45 min. Cells were then washed with Locke's solution and subsequently stimulated with 10  $\mu\text{M}$  nicotine in Locke's solution. [<sup>3</sup>H]Noradrenaline release after stimulation was determined by measuring the radioactivity present in the incubation medium and in cells after precipitation with 10% (wt/vol) trichloroacetic acid. The amount of released [<sup>3</sup>H]noradrenaline is expressed as a percentage of total radioactivity present in the cells before stimulation. Data are given as the net secretory values obtained by subtracting the release measured in the presence of nicotine during the stimulation period from the release measured in the absence of nicotine.

### Antibodies

Rabbit polyclonal antibodies raised against annexin II (p36) purified from bovine aorta were used at a 1:200 dilution (generous gift of J.C. Cavadore, INSERM U-249, Montpellier, France). Rat polyclonal antibodies against dopamine- $\beta$ -hydroxylase (EC.1.14.17.1: DBH) were used at a 1:1,200 dilution to specifically label secretory granules in chromaffin cells (Perrin and Aunis, 1985). Mouse monoclonal antibodies against p11 were used at a 1:50 dilution (Chemicon International Inc.). Rabbit polyclonal antibodies against chromogranin A from bovine chromaffin granules (Bader et al., 1981) were used at a 1:2,000 dilution. Sheep polyclonal antibodies against bovine phenyl ethanolamine *N*-transferase (PNMT, Euromedex, France) were used at 1:200 dilution. Donkey anti-rabbit IgG conjugated to either tetramethylrhodamine (TRITC) or fluorescein isothiocyanate (FITC), goat anti-rat IgG conjugated to FITC and goat anti-mouse IgG conjugated to TRITC were from Chemicon International Inc. Donkey anti-sheep IgG conjugated to FITC were from Jackson ImmunoResearch Laboratories (Westgrove, PA).

For immunoprecipitation of annexin II, antibodies were raised in rabbits against bovine lung annexin II heterotetramer (p90) purified as previously described (Regnoui et al., 1995). Affinity-purified antibodies were obtained by passing the serum on annexin II heterotetramer covalently bound to Affigel 10 and eluting the antibodies in glycine-HCl buffer, pH 2.8. The serum was usually tested on immunoblots at a 1:1,000 dilution and the affinity-purified antibodies at 1  $\mu\text{g}/\text{ml}$ . The affinity-purified antibodies did not crossreact with annexin I.

### Immunocytochemistry

The procedure for immunocytochemistry on chromaffin cells has been previously described (Grant et al., 1988). Briefly, cells were fixed for 20 min in 4% paraformaldehyde in 0.12 M Na/K phosphate (pH 7.0) and for an additional 10 min in fixative containing 0.1% Triton X-100. Aldehyde fixation allows a better preservation of the surface features which are of particular interest in the case of p36 staining patterns (Osborn et al., 1988). After several rinses with PBS, cells were pretreated with 3% bovine serum albumin (BSA), 10% normal goat serum in PBS to reduce nonspecific staining. Cells were incubated for 2 h with the primary antibody diluted in PBS containing 3% BSA in a moist chamber, washed, and subsequently incubated for 1 h with the respective secondary antibodies similarly diluted to 1:100. Coverslips were then extensively washed with PBS, rinsed with water and mounted in Mowiol 4-88 (HOECHST).

The transient accessibility of dopamine- $\beta$ -hydroxylase (DBH) on the plasma membrane of stimulated chromaffin cells (Perrin and Aunis, 1985) was tested by incubating SLO-permeabilized cells for 10 min in permeabilizing medium containing 10 or 100  $\mu\text{M}$  free calcium in the presence of anti-DBH antibodies diluted to 1:50. Cells were then fixed, washed, and processed for immunofluorescence labeling.

Immunofluorescence labeling was evaluated on samples observed with a planapo oil (63 $\times$ ) immersion objective by counting the cells present in a randomly selected transect covering the entire width of the coverslip. Counts were performed in duplicate on samples taken from 3 to 6 different experiments.

### Confocal Laser Scanning Microscopy and Image Analysis

To acquire sequential through-focus images of labeled cells, we have used a Zeiss laser scanning microscope (LSM 410 invert) equipped with a planapo oil (63 $\times$ ) immersion lens ( $n_a = 1.4$ ). FITC emission was excited using the argon laser 488-nm line whereas TRITC was excited using the He/Ne laser line 543-nm line. The emission signals were filtered with a Zeiss

515–565-nm filter (fluorescein emission) or with a long pass 595-nm filter (rhodamine signal).

The rate of photobleaching during image acquisition was determined by measuring the rate of decrease in emitted light intensity from a labeled cell over a period to collect the three dimensional (3D) data sets. Using this procedure we found no significant decrease in the rhodamine fluorescence whereas the photobleaching of fluorescein was important. For this reason, p36 was always labeled with rhodamine for quantitative confocal microscopy.

Nonspecific fluorescence was assessed by incubating cells with the secondary fluorescent-labeled antibodies and measuring the average intensity value for each fluorochrome. This value was then subtracted from all p36-specific images.

Cells were subjected to optical serial sectioning to produce images in the X-Y plane. Each optical section was scanned eight times to obtain an averaged image which was used for analysis. 3D imaging was constructed from 50 serial optical sections of 0.2  $\mu\text{m}$  depth. The images were recorded digitally in a 768  $\times$  576 pixel format and saved on an erasable magneto optical disk. Digital data were later down loaded to a digital image recorder FOCUS.

For quantification, three optical sections from the same cell were recorded at 1- $\mu\text{m}$  intervals in the Z-direction and stored on optical disc for subsequent display. Quantification of the p36 labeling was performed as previously described (Verschuer et al., 1994) by determining the intensity of fluorescence associated with the cell periphery. This area was manually selected. This area and the average intensity ( $\text{x}/\mu\text{m}^2$ ) were calculated using ZEISS CLSM instrument software. To compare the relative quantities of p36 labeling associated with the plasma membrane and the cytoplasm, the labeled area was then multiplied by the associated fluorescence intensity (100% corresponded to the total labeling of each cell). The use of the mean gray level from a nuclear region as a correction factor significantly decreased the SEM, indicating the usefulness of this measure for correcting cell to cell variations.

### **Incubation with $^{32}\text{P}$ and Stimulation of Chromaffin Cells**

For phosphorylation studies, cultured chromaffin cells were incubated for 4 h at 37°C in buffer A (154 mM NaCl, 5.6 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 3.6 mM  $\text{HNaCO}_3$ , 1.2 mM  $\text{MgSO}_4$ , 5.6 mM glucose, 0.56 mM ascorbic acid, 10 mM Hepes, pH 7.5) containing 5% FCS and 300  $\mu\text{Ci}$  carrier free [ $^{32}\text{P}$ ]-orthophosphate (Amersham). Cells were then rinsed and stimulated with buffer A containing 20  $\mu\text{M}$  nicotine. EGTA was subsequently added to the incubation medium in order to reduce the external free  $\text{Ca}^{2+}$  concentration to either  $10^{-8}$  M for resting cells or  $10^{-5}$  M for stimulated cells. These solutions were then removed and cells were rinsed once with buffer A containing EGTA to yield either  $10^{-8}$  M or  $10^{-5}$  M free  $\text{Ca}^{2+}$ . Cells were subsequently placed in liquid nitrogen for rapid freezing.

### **Immunoprecipitation of Unphosphorylated and Phosphorylated Annexin II from Chromaffin Cell Subcellular Fractions**

To prepare total cell homogenates, frozen chromaffin cells were rapidly thawed by addition of 250  $\mu\text{l}$  extraction buffer (10 mM imidazole, pH 7.4, 10 mM EGTA, 0.1 M NaCl, 0.5 mM dithiothreitol, 0.02%  $\text{NaN}_3$ , 0.75% Triton X-100) containing 100  $\mu\text{M}$  sodium orthovanadate, 0.1 mM PMSF, 20  $\mu\text{g}/\text{ml}$  leupeptin, 50  $\mu\text{g}/\text{ml}$  pepstatin, aprotinin, trypsin inhibitor, 20  $\mu\text{g}/\text{ml}$  benzonuclease, and 0.1  $\mu\text{M}$  okadaic acid. The culture dishes were scraped with a plastic blade to release adhering cell membranes and the total lysate was centrifuged at 50,000 rpm for 45 min in a Ti-70 rotor (Beckman Instrs., Fullerton, CA) (200,000 g). The supernatant was saved and the protein content determined.

For subcellular fractionation, frozen chromaffin cells were rapidly thawed in 35  $\mu\text{l}$  of 10 mM imidazole, pH 7.3, 0.1 mM sodium orthovanadate, 0.1 mM PMSF, 20  $\mu\text{g}/\text{ml}$  leupeptin, 50  $\mu\text{g}/\text{ml}$  pepstatin, aprotinin, trypsin inhibitor, 20  $\mu\text{g}/\text{ml}$  benzonuclease, and 0.1  $\mu\text{M}$  okadaic acid, incubated 10 min on ice and then centrifuged for 45 min at 200,000 g. The supernatant was saved (cytosol) and the pellet was homogenized with a Hamilton microsyringe in 150  $\mu\text{l}$  of 10 mM imidazole, pH 7.3, 75 mM KCl, 2 mM  $\text{MgCl}_2$ , 1 mM  $\text{NaN}_3$ , 0.5% Triton X-100 containing the inhibitors mentioned above. The suspension was then incubated for 10 min at 0°C and centrifuged for 45 min at 200,000 g. The supernatant was saved (Triton X-100 soluble fraction) and the pellet was suspended in 150  $\mu\text{l}$  of 10 mM imidazole, pH 7.3, 150 mM NaCl, 10 mM EGTA, 1 mM dithiothreitol

and protease inhibitors, sonicated, incubated for 5 min at 0°C and then centrifuged for 45 min at 200,000 g. The supernatant was saved (Triton X-100 insoluble fraction).

Immunoprecipitation of annexin II was performed with affinity-purified antibodies raised against the annexin II heterotetramer. Cell fractions diluted to 1:10 with 50 mM Tris buffer pH 7.4 were incubated for 1 h at 4°C with anti-annexin II antibodies (5–10% per total protein, wt/wt). 200  $\mu\text{l}$  of protein A-Sepharose CL-4B beads (50 mg in 1.5 ml 50 mM Tris pH 7.4) were then added. After a 1-h incubation at 4°C, the protein A-Sepharose-annexin II-anti-annexin II complexes were collected by centrifugation (microfuge, 20,000 rpm for 5 min), extensively washed at 4°C with (1) 50 mM Tris buffer, pH 7.4, 0.05% Triton X-100, (2) 50 mM Tris, pH 7.4, (3) 0.5 M LiCl, (4) 50 mM LiCl, (5) water and solubilized in 40  $\mu\text{l}$  SDS buffer for polyacrylamide gel electrophoresis. The amount of annexin II (p36) on Coomassie blue-stained gels was quantified by densitometry directly on gel slabs using a scanning densitometer (model Shimadzu C-R3A). Standard curves were obtained with known quantities of purified p36 loaded on the same gel.

### **Electrophoretic Blotting and Immunological Detection**

Proteins resolved on SDS polyacrylamide gels were transferred electrophoretically to nitrocellulose sheets. Blots were incubated with rabbit anti-p90 antiserum diluted 1:500 and monoclonal anti-p11 antibodies diluted 1:500. After washing, the blots were incubated with anti-rabbit IgG (1:10,000) and anti-mouse IgG (1:7,500) conjugated to alkaline phosphatase. After visualization of the immunoreactive bands, the sheets were incubated overnight with  $^{125}\text{I}$ -labeled protein A (1  $\mu\text{Ci}/\text{ml}$ ) and quantification was performed with a phosphorimager.

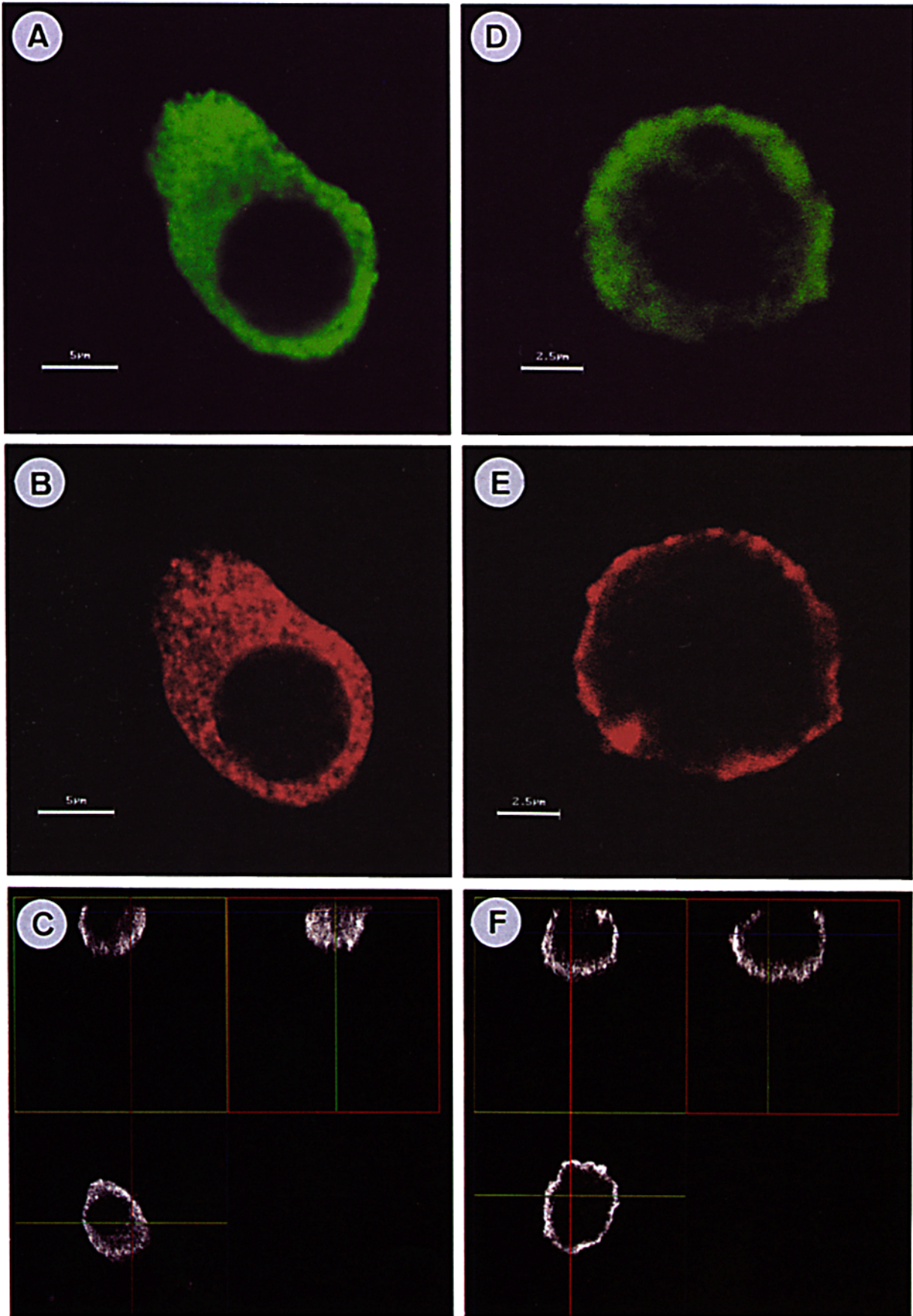
### **Microinjection in Single Chromaffin Cells**

A synthetic peptide corresponding to the p36  $\text{NH}_2$ -terminal domain (p36<sub>[15-26]</sub> peptide; DHSTPPSA YGSV), was obtained from Neosystem (Strasbourg, France), purified by HPLC and dissolved in water at 1 mM. Control experiments were performed with a synthetic peptide containing the same amino acid sequence but with the serine residues replaced by alanine residues ([Ala<sup>17</sup>, Ala<sup>21</sup>, Ala<sup>25</sup>]p36<sub>[15-26]</sub> peptide, DHATPPAAY-GAV). This peptide was synthesized on a multiple peptide synthesis instrument using Fmoc chemistry (432A Peptide Synthesizer SYNERGY, Applied Biosystems, Warrington, UK). Purity was checked on HPLC and sequence analysis was performed by Edman degradation on an automated gas phase protein sequencer (Applied Biosystems).

Microinjection experiments were performed on 3–5-d-old cultures. In each experiment, 50–150 cells were selected and substances were microinjected into their cytosol. Microinjection was performed with an injection system (Eppendorf, Hamburg, Germany) using commercial glass microcapillaries (Femtotips, Eppendorf) with an outlet diameter of  $0.5 \pm 0.2$   $\mu\text{m}$ . Injection time was 0.5 s and the pressure was 40–50 hPa. From these parameters, the calculated injected volume represented 50–100 fl (Graessmann and Graessmann, 1983). Substances to be microinjected were dissolved in water. Before injection samples were mixed with FITC-conjugated dextran 10,000 (5 mg/ml) to visualize microinjected cells. Electrochemical measurements of catecholamine secretion or fixation for immunocytochemistry were performed 30 min after microinjection.

### **Electrochemical Measurement of Catecholamine Secretion from Single Chromaffin Cells**

Culture dishes were washed with Locke's solution in the absence of ascorbic acid (140 mM NaCl, 4.7 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 0.01 mM EDTA, 11 mM glucose and 15 mM Hepes, pH 7.2) and placed on the stage of an inverted microscope. Working electrodes (Soficar, Paris, France) were prepared from 8- $\mu\text{m}$ -diam carbon fibers sealed in glass capillaries as previously described (Gonon et al., 1984). The sensing tip of the electrode was 30- $\mu\text{m}$  long. The electrode was positioned in tangent contact with a single chromaffin cell using a three-dimensional micromanipulator (Narishige, Tokyo, Japan). The reference electrode was a silver wire coated with AgCl immersed in the medium bathing the cells. A two electrode potentiostat (AMU 130, Radiometer Analytical, Villeurbanne, France) was used to apply +0.6 V to the carbon fibre electrode vs the reference electrode and to record the current passing through them (response time of the amplifier: 1 ms). The amplified signal was digitized at 100 Hz by a MacLab/2e system (AD Instruments, Castle Hill, Australia) coupled to a Macintosh computer. Catecholamine secretion was





evoked by applying  $K^+$  (100 mM) or nicotine (100  $\mu$ M) in Locke's solution without ascorbic acid for 5 s to single cells by means of a glass micropipette (Femtotips, Eppendorf) positioned at a distance of 100  $\mu$ m from the cell using a hydraulic micromanipulator. The amplitude of secretion was quantified by measuring the area below the current curve using the MacLab system.

## Results

### Subcellular Localization of p36 in Resting and Stimulated Chromaffin Cells

The intracellular localization of the 36-kD monomer of annexin II (p36) was analyzed in cultured chromaffin cells by confocal laser scanning microscopy using rabbit anti-p36 antibodies and rhodamine-conjugated anti-rabbit antibodies. Chromaffin cells were identified with rat anti-dopamine- $\beta$ -hydroxylase (DBH) antibodies, which specifically label chromaffin secretory granules, and fluorescein-conjugated secondary antibodies. Double immunofluorescence experiments indicated that p36 was uniformly distributed in the cytosol of resting chromaffin cells (Fig. 1, A and B). This pattern did not allow a precise distinction between a cytosolic and an eventual membrane-associated pool of p36 as shown by three-dimensional (3D)-imaging of serial section series (Fig. 1 C). However, previous biochemical studies performed by cell-fractionation techniques indicated that p36 is found in both a soluble and membrane-bound form in resting chromaffin cells (Sarafian et al., 1991).

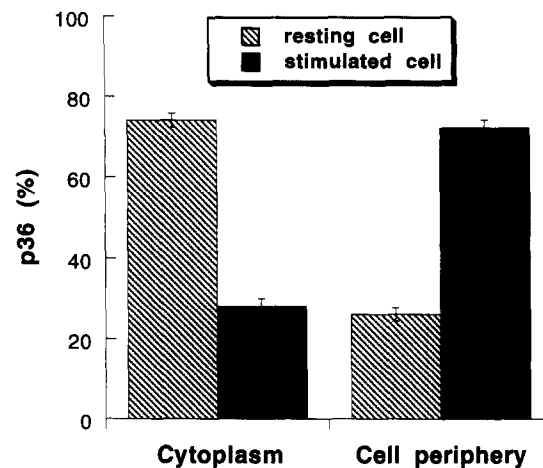
Upon nicotine stimulation, the distribution of p36 was modified in more than 90% of the chromaffin cells, as shown by the appearance in the rhodamine channel of a strong immunofluorescent ring in the cell periphery (Fig. 1, D and E). This observation suggested that nicotine was able to trigger the translocation of p36 from the cytosol to the subplasmalemmal region. 3D-imaging of serial sections obtained by confocal microscopy confirmed the preferential association of p36 with the submembranous network in stimulated chromaffin cells (Fig. 1 F).

3D-microscopy coupled with an appropriate image processing allows a quantitative estimation of the dynamic association of proteins with subcellular compartments (Lynch et al., 1991). To determine the relative amount of cytosolic and peripheral p36 in resting and stimulated chromaffin cells, images representing a single confocal microscope section of p36 labeling were acquired from both control and nicotine-stimulated cells. For each cell, three optical slices taken at 1- $\mu$ m intervals in the Z direction were analyzed. In resting chromaffin cells, 75% of the total p36 labeling was found in the cytoplasm (Fig. 2). By contrast, stimulation with nicotine reduced the cytosolic form of p36 to  $\sim$ 25% and greatly enhanced the amount of p36 associated with the subplasmalemmal region (Fig. 2).

These results obtained by immunofluorescence staining

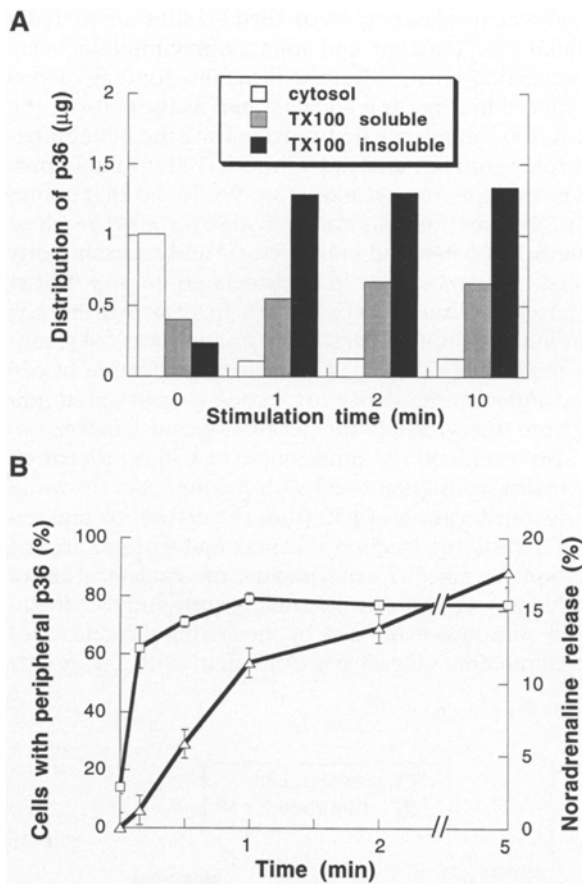
and confocal microscopy were further substantiated by subcellular fractionation and immunoprecipitation using affinity-purified anti-p90 antibodies. The content of p36 was analyzed in three fractions defined as the cytosol, the Triton X-100 soluble fraction representing the membrane-bound compartment, and the Triton X-100 insoluble proteins representing the cytoskeleton. We found that stimulation of chromaffin cells with nicotine strongly reduced the quantity of p36 found in the cytosol and concomitantly enhanced the amount of p36 associated to the Triton X-100 insoluble fraction (Fig. 3 A). In contrast, the p36 content in the Triton X-100 soluble fraction was not significantly modified (Fig. 3 A). Thus, the translocation of p36 that accompanies the exocytotic process occurs predominantly from the cytosol to the peripheral cytoskeleton.

We also examined the time course of p36 translocation in chromaffin cells stimulated with nicotine. As shown in Fig. 3 A, translocation of p36 from the cytosol to the Triton X-100 insoluble fraction was maximal within 1 min of stimulation. In parallel experiments, the translocation of p36 was monitored by confocal microscopy and catecholamine secretion was estimated by measuring the release of [ $^3$ H]noradrenaline (Fig. 3 B). Consistent with the results



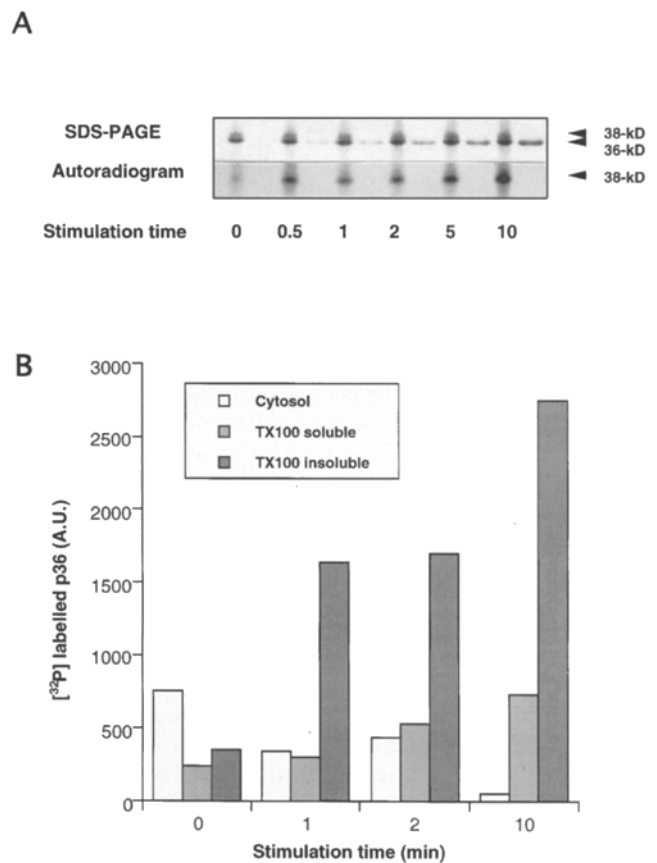
**Figure 2.** Quantitative analysis of the distribution of p36 in resting and nicotine-stimulated chromaffin cells. Images representing a single confocal microscope section of p36 labeling were acquired from 20 resting and 20 nicotine-stimulated cells. Correction for nonspecific labeling by the fluorophore-conjugated secondary antibodies was introduced. The fluorescence was quantified by multiplying the labeled area with its intensity in gray level (data given by the "area measure function" of the CLSM instrument software). Images were then analyzed to estimate the ratio of p36 present in the cytoplasm or associated with the plasma membrane. Results are expressed relative to the labeling obtained in the whole cell (100%).

**Figure 1.** Intracellular distribution of p36 in resting (A, B, and C) and nicotine-stimulated chromaffin cells (D, E, and F). Confocal immunofluorescent images obtained by labeling with anti-DBH antibodies (diluted 1:1,200) visualized with fluorescein-conjugated secondary antibodies are shown in A and D. Images obtained with anti-p36 polyclonal antibodies (diluted 1:200) and rhodamine-conjugated secondary antibodies are shown in B and E. Sections were taken with minimum pinhole size in the plane of the nuclei using excitation and emission filtering as described in Materials and Methods. C and F show orthogonal sections of the p36 labeling obtained from analysis of the three dimensional imaging of 50 serial optical sections (0.2  $\mu$ m). Note that p36 displays a cytoplasmic distribution in resting cells but is mainly concentrated in the subplasmalemmal region in stimulated cells.



**Figure 3.** (A) Distribution of p36 in subcellular fractions from resting and stimulated chromaffin cells. Chromaffin cells were stimulated for the indicated periods of time with 20  $\mu$ M nicotine in Locke's solution. Cells were then processed to separate the cytosol and the Triton X-100 soluble and insoluble fractions. The content of p36 in each fraction was estimated by immunoprecipitation and quantification on SDS polyacrylamide gels (see Materials and Methods). Stimulation of chromaffin cells triggers a rapid translocation of p36 from the cytosol to the Triton X-100 insoluble fraction. (B) Time course of catecholamine secretion (triangles) and p36 translocation (squares) in stimulated chromaffin cells. Cells labeled with [ $^3$ H]noradrenaline were stimulated for the indicated periods of time with nicotine. Extracellular fluids were then collected and the radioactivity present in solutions and in cells was assayed. Data are given as the mean of triplicate determinations on the same cell preparation. Similar results were obtained on ten different cell preparations. In parallel experiments, chromaffin cells were stimulated with nicotine, fixed, and labeled with rabbit anti-p36 antibodies. Immunoreactivity was detected with rhodamine-conjugated anti-rabbit immunoglobulins. The percentage of chromaffin cells with a subplasmalemmal labeling of p36 was determined in randomly selected areas covering the entire width of the coverslip. Counts were performed in duplicate on cells taken from three different preparations. The translocation of p36 is maximal within 1 min of nicotine stimulation and seems to precede the exocytotic release of catecholamines.

obtained by subcellular fractionation, the translocation of p36 visualized by immunocytochemistry was rapid and almost complete within 30 s of nicotine stimulation (Fig. 3 B). By comparison, several minutes of stimulation were required to reach the maximal level of [ $^3$ H]noradrenaline



**Figure 4.** (A) Kinetics of  $^{32}$ P incorporation into p36 after stimulation of chromaffin cells with nicotine. Cells labeled with  $^{32}$ P were stimulated with 20  $\mu$ M nicotine for the indicated periods of time. Annexin II was immunoprecipitated from the total cell lysate and the associated radioactivity was determined by SDS gel electrophoresis and autoradiography. Purified p36 (0.25–1  $\mu$ g) was run in the lanes between the samples. Nicotine triggers within seconds the phosphorylation of p36 in chromaffin cells as seen by the appearance of a radioactive 38-kD band. Similar results were obtained in five separate experiments. (B) Distribution of phosphorylated p36 in subcellular fractions from resting and stimulated chromaffin cells.  $^{32}$ P-labeled cells were stimulated with nicotine for various periods of time. Cells were then processed to separate the cytosol and the Triton X-100 soluble and insoluble fractions. p36 was immunoprecipitated and the radioactivity incorporated into the 38-kD band after electrophoretic separation was estimated by scanning densitometry on the corresponding autoradiogram. Values are expressed as arbitrary units (A.U.) from one representative experiment. Similar results were obtained in three separate experiments.

release (Fig. 3 B). Thus, it seems unlikely that p36 translocates to the cell periphery in stimulated cells due to its association with secretory granules which move to the plasma membrane and fuse with it during exocytosis. Our results rather suggest that the translocation of p36 may be an early event in the pathway of regulated exocytosis in chromaffin cells.

#### **Effect of the Synthetic $^{15-26}$ $\text{NH}_2$ -Terminal Peptide of p36 on the Translocation of p36 and on Exocytosis of Catecholamines**

Next, we examined whether the translocation of p36 is an

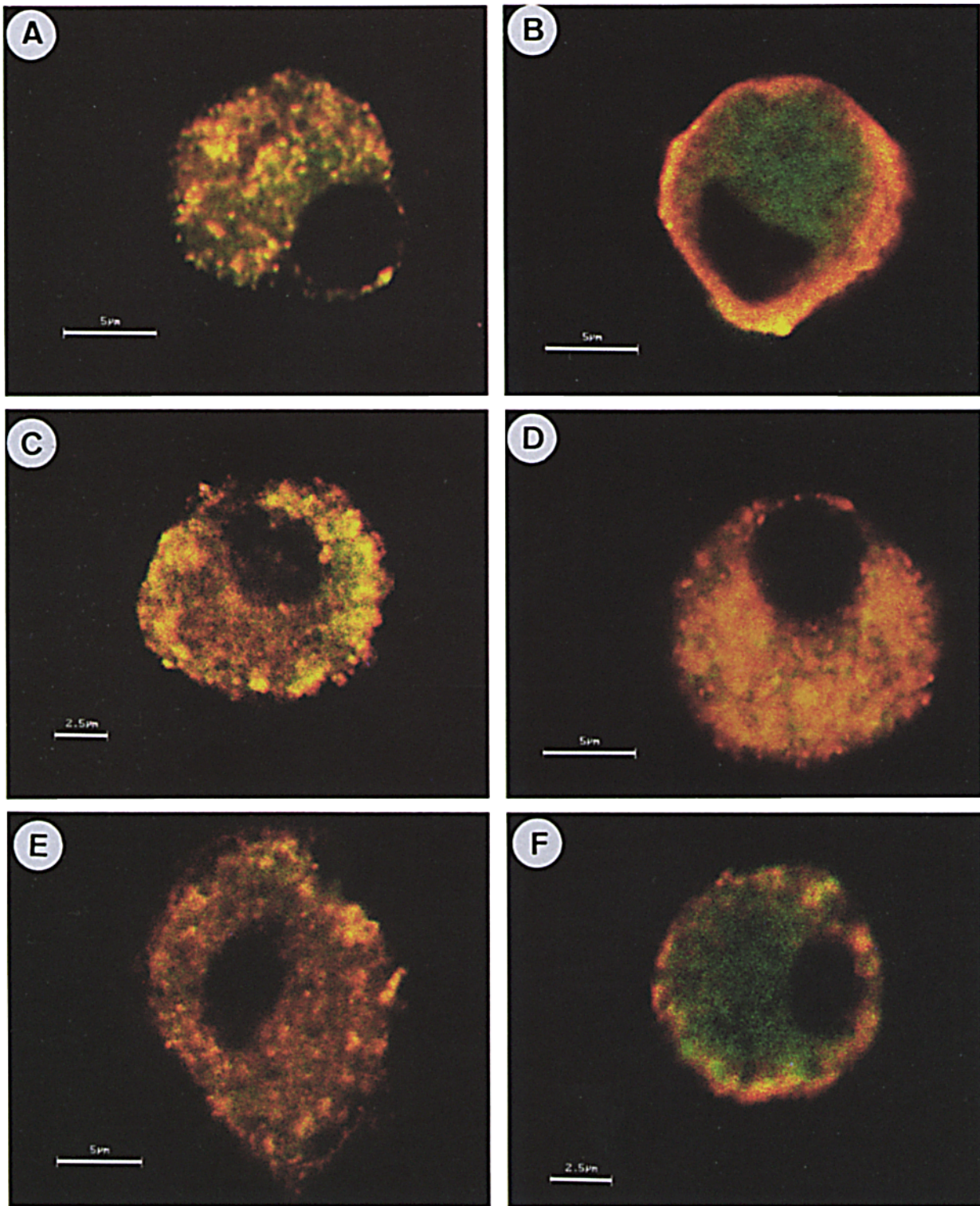
essential event of the exocytotic machinery. The NH<sub>2</sub>-terminal "tail" region of p36 contains the binding site for p11 (Powell and Glenney, 1987) and the serine and tyrosine residues phosphorylated by protein kinase C (PKC) and tyrosine kinase pp60<sup>src</sup> (Glenney and Tack, 1985). We previously observed that phosphorylation of p36 by PKC promotes the association of p36 with chromaffin cell membranes (Sarafian et al., 1991). Furthermore, the phosphorylation of p36 by PKC seems to be required for the reconstitution of exocytosis in permeabilized chromaffin cells with reduced secretory activity (Sarafian et al., 1991). The phosphorylation of p36 in stimulated chromaffin cells is illustrated in Fig. 4. Cells loaded with [<sup>32</sup>P]orthophosphate were stimulated with nicotine for various periods of time and the radioactivity specifically incorporated into p36 was estimated by immunoprecipitation. Nicotine triggered a rapid phosphorylation of p36 which reached a threefold increase of the initial radioactivity after 10 min of stimulation (Fig. 4 A). Coomassie blue staining of the immunoprecipitated p36 indicated the presence of a doublet of apparent molecular mass 36 kD and 38 kD. The radioactivity was only associated with the 38-kD component, indicating that phosphorylation changed the protein mobility. Accordingly, previous *in vitro* observations showed that purified p36 phosphorylated by purified brain PKC migrates with a reduced mobility on SDS gel electrophoresis (Regnoui et al., 1995). Upon nicotinic stimulation, the incorporation of <sup>32</sup>P was primarily observed in p36 associated to the Triton X-100 insoluble fraction (Fig. 4 B). Thus, the translocation of p36 to the cell periphery may precede the phosphorylation process in stimulated chromaffin cells.

The PKC phosphorylation site of p36 is a serine residue in the NH<sub>2</sub>-terminal domain in position 25 (Gould et al., 1986). Thus, the effect of a synthetic peptide corresponding to residues 15–26 of p36 on the translocation of p36 in stimulated chromaffin cells was tested. Preliminary *in vitro* experiments using purified PKC indicated that the synthetic p36<sub>[15–26]</sub> peptide did not significantly interfere with the phosphorylation of the total cytosolic protein fraction, slightly reduced the phosphorylation of purified p36 (≈6%) and inhibited the phosphorylation of purified p90 by 22% (data not shown). Chromaffin cells were individually microinjected with 100 μM of p36<sub>[15–26]</sub> peptide in a water solution containing fluorescein (FITC)-conjugated dextran to visualize microinjected cells. After a 30-min interval, cells were incubated 5 min in Locke's solution or stimulated with 10 μM nicotine. Cells were then fixed and immunostained with anti-p36 antibodies revealed with rhodamine-conjugated secondary antibodies. Control experiments were performed with a p36<sub>[15–26]</sub> analogue peptide in which the three serine residues in positions 17, 21, and 25 were replaced by alanine ([Ala<sup>17</sup>, Ala<sup>21</sup>, Ala<sup>25</sup>] p36<sub>[15–26]</sub> peptide). Microinjected chromaffin cells displayed a uniform FITC-fluorescence throughout the cell body except in the nucleus (Fig. 5), indicating that the injected material readily diffused throughout the cytoplasm during the course of the experiment. Injection of FITC-dextran alone did not modify the distribution of p36. In resting cells p36 was essentially found in the cytoplasm as revealed by the yellow colocalization of rhodamine and FITC (Fig. 5 A) and was translocated to the cell periphery

in cells stimulated with nicotine (Fig. 5 B). Microinjection of the p36<sub>[15–26]</sub> peptide did not significantly modify the cytosolic distribution of p36 in resting cells (Fig. 5 C). However, as illustrated in Fig. 5 D, microinjection of the p36<sub>[15–26]</sub> peptide completely blocked the translocation of p36 in nicotine-stimulated chromaffin cells. This inhibitory effect was observed in the entire population of microinjected cells but translocation did occur in neighboring cells which had not been microinjected. These observations indicate that the p36<sub>[15–26]</sub> peptide was able to interfere with the translocation of endogenous p36 in stimulated chromaffin cells. Microinjection of the [Ala<sup>17</sup>, Ala<sup>21</sup>, Ala<sup>25</sup>]p36<sub>[15–26]</sub> peptide did not modify the intracellular distribution of p36 in either resting (Fig. 5 E) or in stimulated chromaffin cells (Fig. 5 F), indicating that Ser→Ala substitutions were sufficient to alter the efficacy of the p36<sub>[15–26]</sub> peptide to block the translocation of p36.

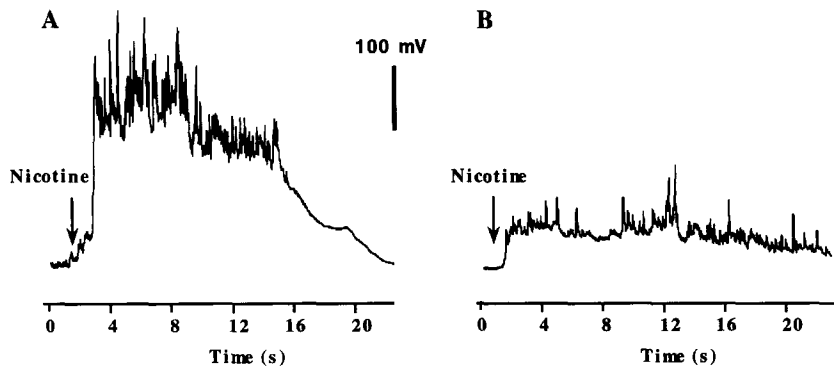
To probe the effect of the p36<sub>[15–26]</sub> peptide on cell secretory activity, we combined microinjection with amperometry to monitor real time exocytosis from individual chromaffin cells (Wightman et al., 1991; Chow et al., 1992). Chromaffin cells were microinjected with FITC-dextran alone or in combination with either the p36<sub>[15–26]</sub> peptide or the [Ala<sup>17</sup>, Ala<sup>21</sup>, Ala<sup>25</sup>]p36<sub>[15–26]</sub> peptide. Cells were then stimulated by a local application of nicotine or a depolarizing concentration of K<sup>+</sup> and the exocytotic release of catecholamines was estimated by measuring the oxidation current recorded with a carbon-fiber microelectrode placed adjacent to a single microinjected cell. In control experiments, we examined the secretory response to nicotine and high K<sup>+</sup> from cells microinjected with FITC-dextran. Experiments were performed on different culture preparations in which 25 microinjected cells were compared to 25 untreated cells. Microinjection of FITC-dextran did not significantly modify the current response to either nicotine or high K<sup>+</sup> stimulation (data not shown). Fig. 6 shows a representative experiment illustrating the effect of the p36<sub>[15–26]</sub> peptide on the amperometric response to nicotine. The local application of nicotine in the cell medium evoked a rapid increase in the oxidation current which usually lasted 15–20 s and consisted of a broad secretion envelope on which were superimposed sharp spikes. Previous studies have demonstrated that these spikes correspond to the exocytotic release of catecholamines from individual storage granules at the cell surface in close apposition with the carbon-fiber electrode (Leszczyszyn et al., 1990; Wightman et al., 1991). Microinjection of p36<sub>[15–26]</sub> peptide into the cytoplasm of chromaffin cells produced a strong inhibition of the amperometric response evoked by nicotine (Fig. 6). The amplitude of the secretion, quantified by integrating the area below the current curve, was inhibited by ~50% in cells microinjected with 100 μM peptide compared to control cells (Table I). Microinjecting more of p36<sub>[15–26]</sub> peptide (500 μM) only slightly enhanced this inhibitory effect (data not shown), suggesting that the peptide concentration was not a limiting factor in our experiments. A similar 50% inhibition of the amperometric response was also observed when secretion was triggered with a depolarizing concentration of K<sup>+</sup> (Table I), showing that the p36<sub>[15–26]</sub> peptide did not block the exocytotic machinery by interfering with the nicotinic receptor. Since the [Ala<sup>17</sup>, Ala<sup>21</sup>, Ala<sup>25</sup>]p36<sub>[15–26]</sub> peptide





**Figure 5.** Effect of the synthetic p36<sub>[15-26]</sub> peptide on the intracellular distribution of p36 in resting and stimulated chromaffin cell. Chromaffin cells were microinjected with fluorescein-conjugated dextran alone (*A* and *B*), or fluorescein-dextran containing 100  $\mu$ M of the synthetic p36<sub>[15-26]</sub> peptide (*C* and *D*), or fluorescein-dextran containing 100  $\mu$ M of the synthetic [Ala<sup>17</sup>, Ala<sup>21</sup>, Ala<sup>25</sup>]p36<sub>[15-26]</sub> peptide (*E* and *F*). After recovery, cells were incubated in Locke's solution in the absence (*A*, *C*, and *E*) or presence of 10  $\mu$ M nicotine (*B*, *D*, and *F*). Cells were then fixed and subsequently immunostained with polyclonal rabbit antibodies to p36 and rhodamine-conjugated anti-rabbit antibodies. Images obtained in the rhodamine (p36) and fluorescein (dextran) channels were recorded simultaneously in the same optical section by a double exposure procedure. The yellow-orange staining corresponds to areas where rhodamine and fluorescein signals are superimposed. Microinjection of the p36<sub>[15-26]</sub> peptide does not significantly modify the distribution of p36 in resting chromaffin cells (compare *C* and *A*) but completely inhibits the translocation of p36 observed in nicotine-stimulated chromaffin cells (compare *D* and *B*). In contrast, the [Ala<sup>17</sup>, Ala<sup>21</sup>, Ala<sup>25</sup>]p36<sub>[15-26]</sub> peptide does not interfere with the translocation of p36 in stimulated cells (compare *F* and *B*).





**Figure 6.** Electrochemical measurement of catecholamine secretion from single chromaffin cells. Typical amperometric response from chromaffin cells microinjected with fluorescein-conjugated dextran (A) or fluorescein-dextran in combination with 100  $\mu\text{M}$  synthetic p36<sub>[15-26]</sub> peptide (B). 30 min after microinjection, a carbon-fiber electrode was placed in contact with each single cell and nicotine (100  $\mu\text{M}$ ) was applied locally for 5 s with a glass micropipette (arrow). Oxidation current for catecholamines at the carbon-fiber electrode was monitored at + 0.6 V. Stimulation with nicotine evokes a rapid increase in the oxidation current accompanied with a series of sharp spikes. Microinjection of the synthetic p36<sub>[15-26]</sub> peptide reduces by 58% the amplitude of secretion estimated by integrating the surface area below the current curve.

did not modify the secretory activity in response to nicotine stimulation (Table I), it seems likely that the p36<sub>[15-26]</sub> peptide inhibits secretion by preventing the translocation of p36 to the subplasmalemmal region. This finding strongly suggests that the translocation of p36 may be an essential event of exocytosis in chromaffin cells.

#### *Ca<sup>2+</sup> Dependence for the Translocation of p36 in Stimulated Chromaffin Cells*

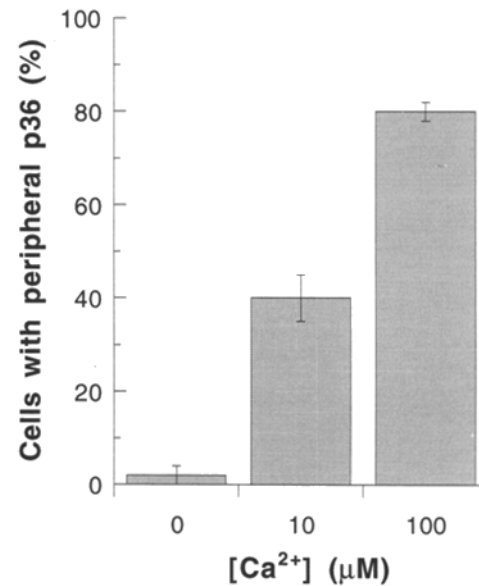
The calcium sensitivity of the p36 translocation was assessed in SLO-permeabilized chromaffin cells since permeabilized cell models offer the opportunity to control the ionic composition of the cytoplasm (Sontag et al., 1988). SLO-permeabilized chromaffin cells were stimulated for 5 min with either 10  $\mu\text{M}$  or 100  $\mu\text{M}$  free Ca<sup>2+</sup>. This range of calcium concentration corresponds to the predicted level of Ca<sup>2+</sup> present in the active subplasmalemmal zone during exocytosis (Llinas et al., 1992; Neher and Zucker, 1993). Cells were then fixed and double-labeled with anti-p36 and anti-DBH antibodies. Chromaffin cells were

counted in randomly selected areas of the coverslips to estimate the ratio of cells displaying a peripheral p36 labeling. p36 was primarily found in the cytosol in resting conditions when SLO-permeabilized chromaffin cells were incubated in a calcium-free medium (Fig. 7). Surprisingly, stimulation with 10  $\mu\text{M}$  free calcium triggered the translocation of p36 to the cell periphery in only 40  $\pm$  5% of the cultured chromaffin cells (Fig. 7). The remaining cells presented a diffuse labeling pattern over the whole cell body indicating that p36 was retained into the cytoplasm. However, the translocation of p36 required higher levels of calcium in this subpopulation of chromaffin cells since we observed that the entire population of SLO-permeabilized

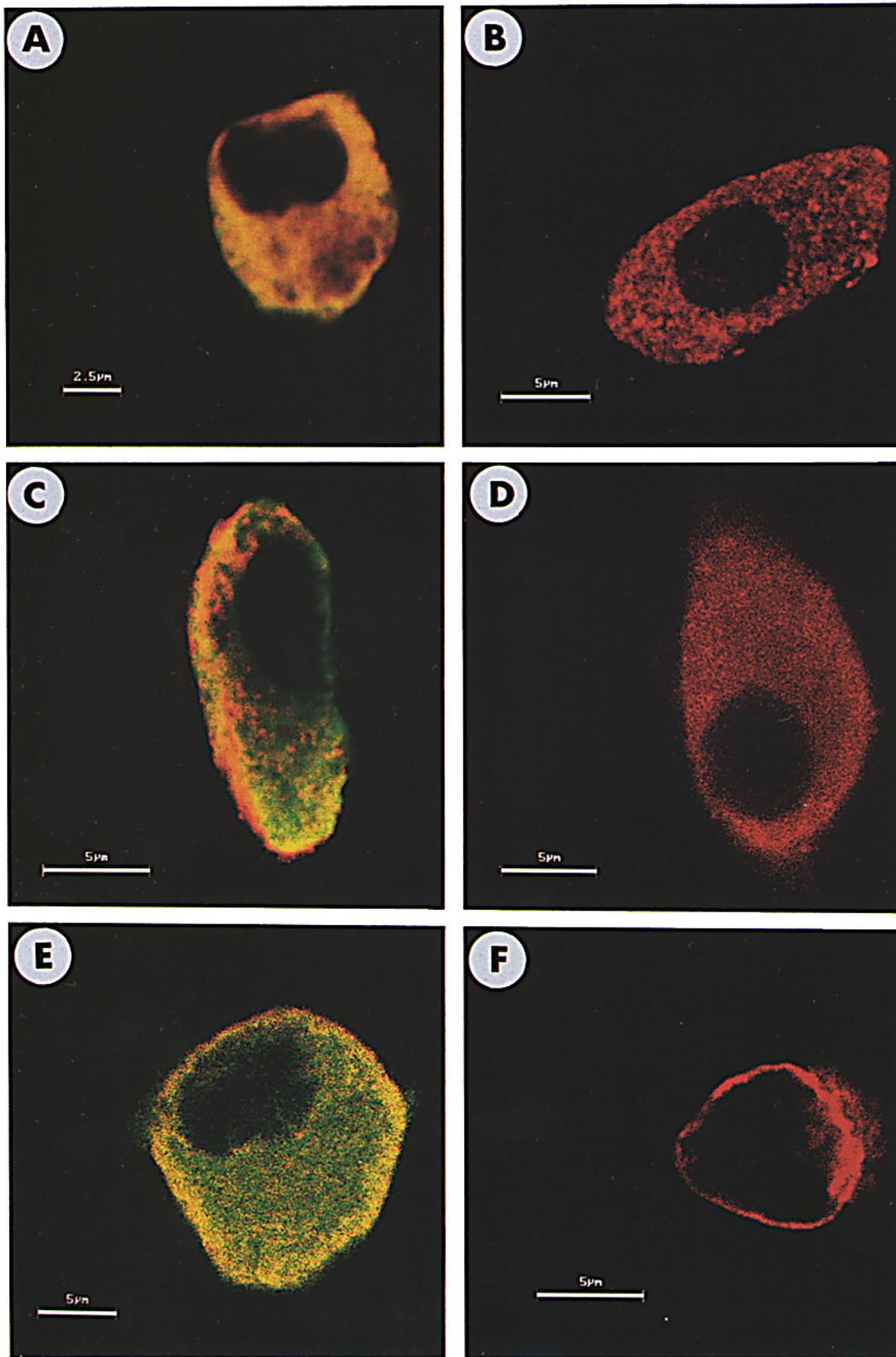
**Table I.** Effect of the Synthetic p36<sub>[15-26]</sub> and [Ala<sup>17</sup>, Ala<sup>21</sup>, Ala<sup>25</sup>]p36<sub>[15-26]</sub> Peptides on Catecholamine Secretion from Single Chromaffin Cells

	Nicotine		High K <sup>+</sup>	
	Exp 1	Exp 2	Exp 3	Exp 4
Control (pA-sec)	1129 $\pm$ 97	1005 $\pm$ 108	423 $\pm$ 67	715 $\pm$ 118
[Ala <sup>17</sup> , Ala <sup>21</sup> , Ala <sup>25</sup> ]p36 <sub>[15-26]</sub> (pA/sec)	1200 $\pm$ 96	1094 $\pm$ 90		
p36 <sub>[15-26]</sub> (pA-sec)	448 $\pm$ 48	562 $\pm$ 114	233 $\pm$ 27	380 $\pm$ 48
Percent inhibition	60	56	52	47

Chromaffin cells were microinjected with water (control) or with either 100  $\mu\text{M}$  of p36<sub>[15-26]</sub> peptide or 100  $\mu\text{M}$  of [Ala<sup>17</sup>, Ala<sup>21</sup>, Ala<sup>25</sup>]p36<sub>[15-26]</sub> peptide in water. 30 min after microinjection, cells were stimulated with a local application of 100  $\mu\text{M}$  nicotine (Experiments 1 and 2) or 100 mM K<sup>+</sup> (Experiments 3 and 4). The amperometric response was integrated to obtain the total catecholamine secretion expressed in pA/sec. Results obtained in four experiments performed in different dishes with two different culture preparations are presented. Data are the means of 25 cells/group from the same dish  $\pm$  SEM. The absolute values were compared between control and peptide-injected chromaffin cells with paired *t* tests. The p36<sub>[15-26]</sub> peptide significantly inhibited the amplitude of the secretory response to nicotine and high K<sup>+</sup> in every microinjected cell with *P* < 0.0025 in each experiment.

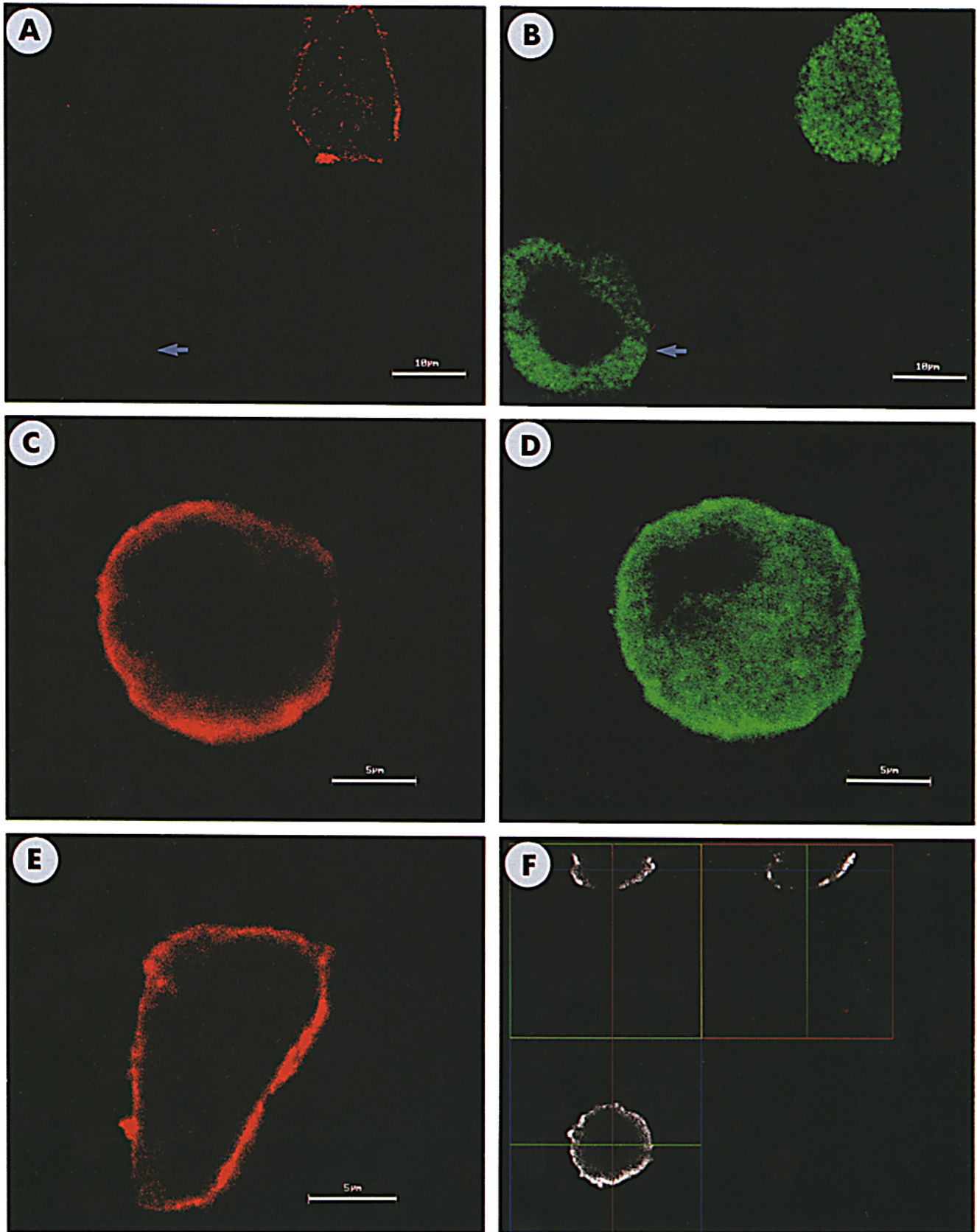


**Figure 7.** Translocation of p36 in SLO-permeabilized chromaffin cells: dose response to calcium. SLO-permeabilized cells were incubated 5 min in permeabilizing medium containing the indicated concentrations of free calcium. Cells were then fixed and labeled with rat anti-DBH antibodies and rabbit anti-p36 antibodies. Immunoreactivity was detected with fluorescein-conjugated anti-rat and rhodamine-conjugated anti-rabbit immunoglobulins. The values represented the percentage of chromaffin cells with a subplasmalemmal labeling of p36 in a randomly selected transect covering the entire width of the coverslip. Counts were performed in duplicate on samples taken from three different experiments. The values are presented as mean  $\pm$  SD.



**Figure 8.** Distribution of p36 in SLO-permeabilized adrenergic cells. Cells were permeabilized with SLO and incubated 5 min in permeabilizing medium in the absence of calcium (*A* and *B*) or in the presence of 10  $\mu\text{M}$  (*C* and *D*) or 100  $\mu\text{M}$  free calcium (*E* and *F*). Cells were then fixed and stained with anti-p36 antibodies detected by rhodamine-conjugated immunoglobulins in combination with anti-PNMT antibodies revealed with fluorescein immunoglobulins. Images were taken through the center of the nucleus and recorded simultaneously from a single focal plane. The yellow-orange staining corresponds to the regions where fluorescein (PNMT) and rhodamine (p36) signals are superimposed. Stimulation with 10  $\mu\text{M}$  free calcium triggers the translocation of p36 in adrenergic PNMT-labeled chromaffin cells (*C*) but p36 remains in the cytosol in noradrenergic cells unlabeled with PNMT antibodies (*D*). Stimulation with 100  $\mu\text{M}$  free calcium produces the translocation of p36 in both PNMT-positive and PNMT-negative chromaffin cells (*E* and *F*).





**Figure 9.** Subcellular localization of p11 in resting cells (*A, B, C, and D*) and nicotine-stimulated (*E and F*) chromaffin cells. Double immunofluorescence confocal micrographs with anti-p11 antibodies detected with rhodamine anti-mouse antibodies (*A, C, and E*) and either anti-chromogranin A antibodies visualized with fluorescein anti-rabbit (*B*) or anti-PNMT antibodies visualized with fluorescein anti-sheep antibodies (*D*). Single optical sections were taken through the center of the nucleus. Note that some chromogranin A-labeled chromaffin cells are completely devoid of p11 in the cell body (*A and B, arrowheads*). p11 is present in the periphery of adrenergic PNMT-labeled chromaffin cells (*C and D*). Orthogonal sections of p11 labeling in nicotine-stimulated cells obtained from analysis of serial optical images (0.2 μm) are shown in *F*.

chromaffin cells displayed a subplasmalemmal staining with the anti-p36 antibodies when the stimulation was evoked with 100  $\mu\text{M}$  free  $\text{Ca}^{2+}$  (Fig. 7). These findings confirm that the translocation of p36 is a calcium-dependent event in chromaffin cells. In addition, they reveal a certain degree of heterogeneity in the  $\text{Ca}^{2+}$  affinity of the p36 translocation event within the chromaffin cell population.

To determine the nature of the chromaffin cells which preferentially translocate p36 at low calcium concentrations, SLO-permeabilized chromaffin cells were stimulated with 10  $\mu\text{M}$  free  $\text{Ca}^{2+}$  and double-labeled with anti-p36 antibodies and anti-phenylethanolamine *N*-methyl transferase (PNMT) which labels only adrenergic chromaffin cells (Verhofstad et al., 1985). Fig. 8 illustrates the staining pattern obtained by visualizing the labeling of p36 and PNMT together in the same section. In resting SLO-permeabilized cells, p36 and PNMT, were both localized in the cytoplasm of adrenergic cells as suggested by the yellow color observed in the whole cell body except in the nucleus (Fig. 8 A). In cells stimulated with 10  $\mu\text{M}$  free  $\text{Ca}^{2+}$ , the colocalization of p36 and PNMT (yellow) was reduced to the cell periphery (Fig. 8 C), indicating that the translocation of p36 to the subplasmalemmal region did occur in adrenergic chromaffin cells. In contrast, p36 remained in the cytosol in noradrenergic chromaffin cells which were not labeled by the anti-PNMT antibodies (Fig. 8 D). However, stimulation with 100  $\mu\text{M}$  free  $\text{Ca}^{2+}$  produced translocation of p36 to the cell periphery in both PNMT-positive and PNMT-negative chromaffin cells (Fig. 8, E and F). In other words, the translocation of p36 displayed a higher sensitivity to calcium in adrenergic chromaffin cells. p36 also translocated in noradrenergic cells but showed a calcium affinity one order of magnitude lower than that observed in adrenergic cells.

### Subcellular Localization of p11

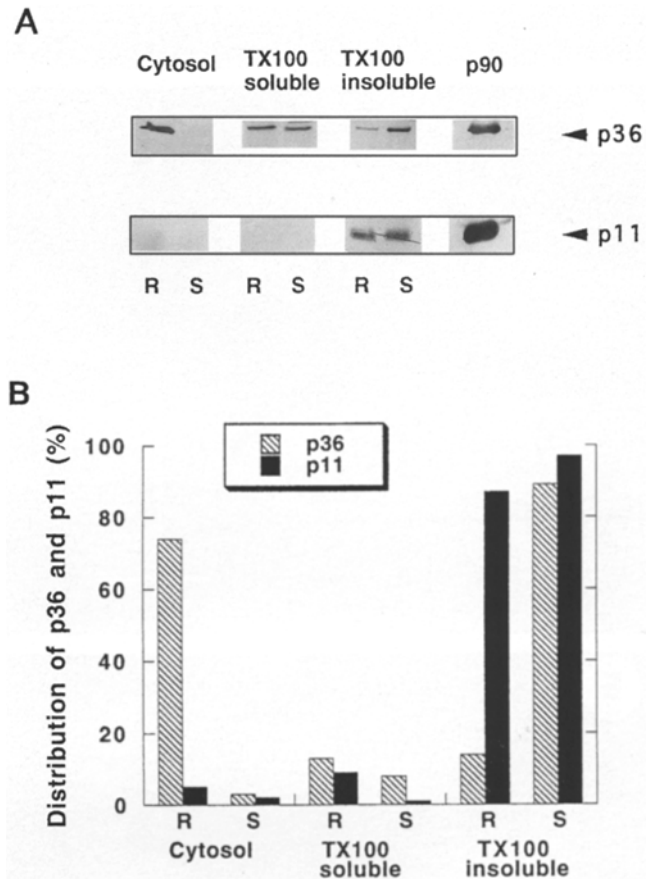
The subcellular localization of the 11-kD light chain of annexin II was analyzed with a monoclonal anti-p11 antibody in both resting cells and nicotine-stimulated chromaffin cells. As illustrated in Fig. 9, p11 was localized in the subplasmalemmal region (Fig. 9 A) in chromaffin cells visualized with anti-chromogranin A antibodies (Fig. 9 B). However, some chromogranin A-positive cells were not labeled with the anti-p11 antibody, suggesting that the presence of p11 may be restricted to a subpopulation of chromaffin cells.

To examine whether p11 was restricted to adrenergic or noradrenergic chromaffin cells, we performed double immunofluorescent labeling experiments with anti-p11 monoclonal antibodies used in combination with anti-PNMT antibodies. We found that adrenergic chromaffin cells identified as such by their immunoreactivity with the anti-PNMT antibodies expressed p11 in the peripheral region of the cell (Fig. 9, C and D). In contrast, chromaffin cells unlabeled with PNMT antibodies were never stained with anti-p11 antibodies. These observations indicate that p11 was selectively expressed in adrenergic and not in noradrenergic chromaffin cells.

The distribution of p11 remained unchanged in chromaffin cells stimulated with nicotine (Fig. 9 E). p11 was detected exclusively in the cell periphery as seen by the

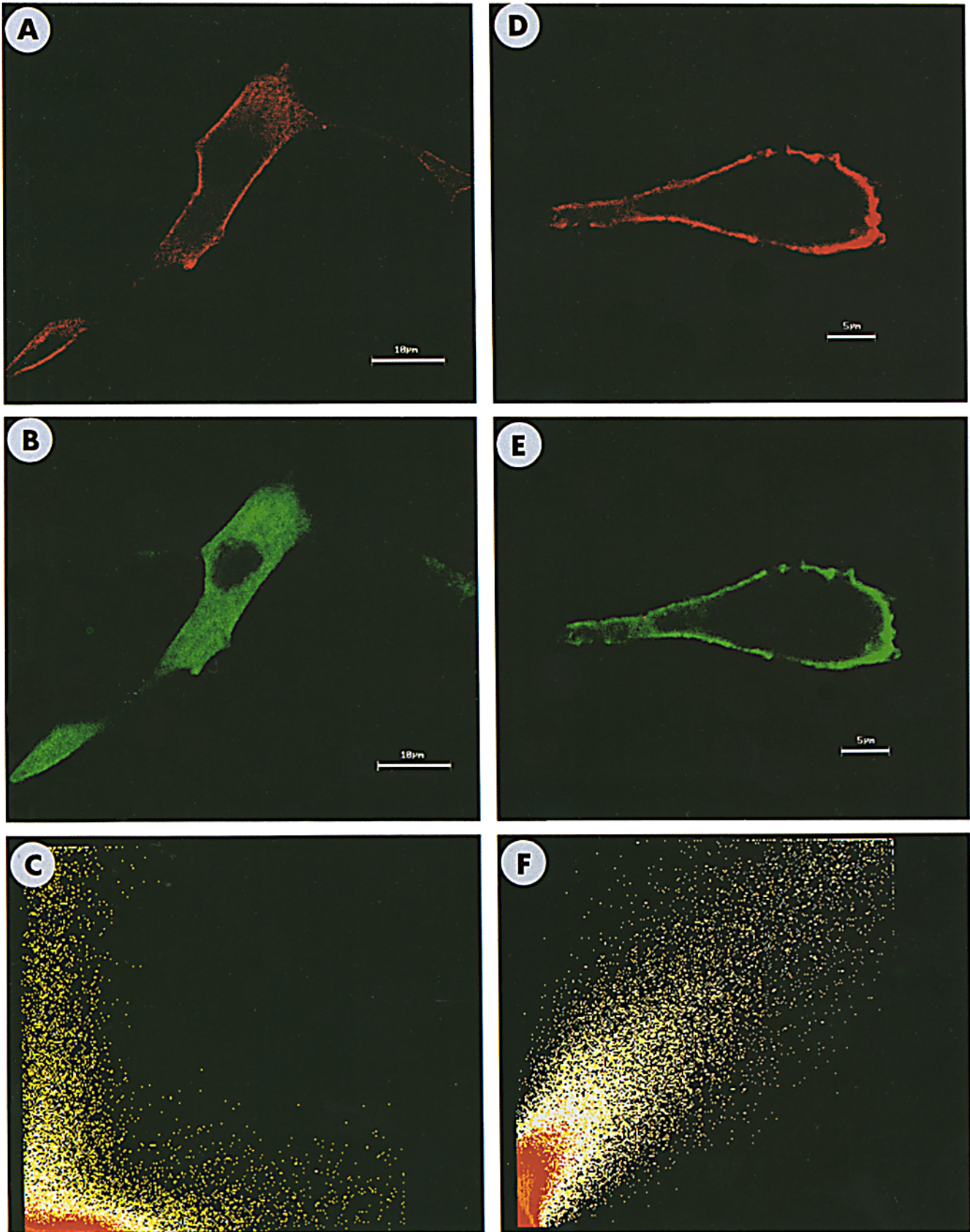
3D-imaging of section series (Fig. 9 F). These observations were confirmed biochemically by subcellular fractionation experiments and detection of p11 and p36 on Western blots (Fig. 10). In both resting and stimulated cells, p11 was present only in the Triton X-100 insoluble cytoskeleton. On the other hand, p36 translocated from the cytosol to the Triton X-100 insoluble fraction upon nicotine stimulation (Fig. 10). Thus, p11 which was virtually absent from the cytosol might represent the Triton X-100 insoluble target protein to which p36 translocated in stimulated adrenergic chromaffin cells.

To test this possibility, we used double immunofluorescent experiments with anti-p36 and anti-p11 antibodies. p11 labeling was revealed with anti-mouse antibodies conjugated to rhodamine (Fig. 11, A and D) and p36 with



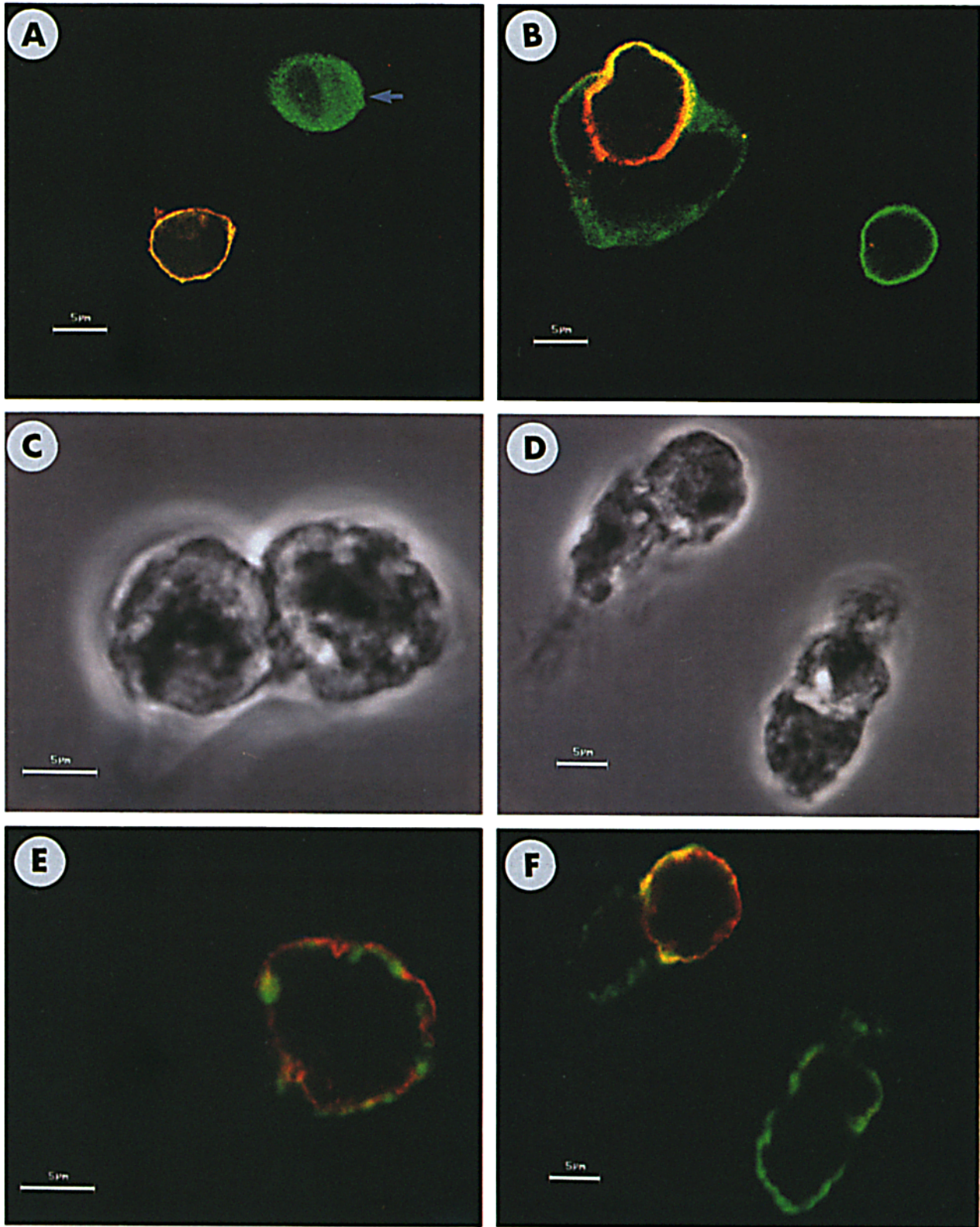
**Figure 10.** (A) Immunodetection of p11 and p36 in subcellular fractions from resting and stimulated chromaffin cells. Cultured chromaffin cells were incubated with Locke's solution in the presence (stimulated cells, S) or absence (resting cells, R) of nicotine and then processed to separate the cytosol and the Triton X-100 soluble and insoluble fractions as described in Materials and Methods. Proteins were separated by monodimensional (15% acrylamide) gel electrophoresis, transferred to nitrocellulose sheets and blots were subsequently incubated with anti-p36 and anti-p11 antibodies. Immunolabeled bands were revealed with secondary antibodies conjugated to alkaline phosphatase. (B) Blots were subsequently incubated with  $^{125}\text{I}$ -labeled protein A and the radioactivity associated with the p11 and p36 immunoreactive bands was estimated with a phosphorimager. Data are expressed as percentages of the radioactivity found in the three fractions (100%).





**Figure 11.** Subcellular distribution of p11 and p36 in resting (A, B, and C) and nicotine-stimulated (D, E, and F) chromaffin cells. Chromaffin cells were double labeled with monoclonal anti-p11 antibodies revealed by rhodamine anti-mouse antibodies (A and D) in combination with polyclonal rabbit antibodies to p36 detected with fluorescein anti-rabbit antibodies (B and E). Optical sections were taken through the center of the nucleus. C and F show the two dimensional scatter histograms of gray values obtained from p36 and p11 labelings recorded simultaneously in the same section by a double exposure procedure. The vertical axis represents the intensity recorded in the fluorescein channel (p36) scaled in standardized arbitrary units 1-255. The horizontal axis represents the intensity recorded in the rhodamine channel (p11). The dots localized diagonally correspond to the colocalization of p36 with p11. The two subunits of annexin II are colocalized in stimulated chromaffin cells.





**Figure 12.** Subcellular distribution of p11 and p36 in SLO-permeabilized chromaffin cells stimulated with either 10  $\mu\text{M}$  (A) or 100  $\mu\text{M}$  (B) free calcium. Double immunofluorescence labeling with p11 antibodies detected with rhodamine anti-mouse immunoglobulins and p36 antibodies revealed with fluorescein anti-rabbit immunoglobulins. The optical sections are taken through the center of the nucleus. Images corresponding to p36 and p11 labeling are shown simultaneously in the same focal plane. The yellow staining corresponds to areas where rhodamine (p11) and fluorescein (p36) colocalize. Stimulation with 10  $\mu\text{M}$  free calcium triggers the translocation of p36 to the cell periphery only in p11-labeled chromaffin cells (A). Note the diffuse staining pattern obtained with p36 in a chromaffin cell (*arrowhead*) which does not contain p11 (A). In contrast, stimulation with 100  $\mu\text{M}$  free calcium produces the translocation of p36 in both

anti-rabbit antibodies conjugated to fluorescein (Fig. 11, *B* and *E*). In resting chromaffin cells, p11 was present exclusively in the subplasmalemmal network (Fig. 11 *A*), whereas p36 was distributed throughout the cytoplasm (Fig. 11 *B*). The cytofluorogram representing the pixels obtained from the superimposition of the two fluorescent images of p36 and p11 confirmed that the two proteins were not colocalized (Fig. 11 *C*). Stimulation of chromaffin cells with 10  $\mu\text{M}$  nicotine triggered the translocation of p36 to the subplasmalemmal region (Fig. 11 *E*), whereas p11 remained localized beneath the plasma membrane (Fig. 11 *D*). The cytofluorogram obtained from the superimposed fluorescent images revealed the colocalization of the two subunits of annexin II (Fig. 11 *F*). This observation is in line with the idea that p11 may be the docking ligand for p36 in the subplasmalemmal region in stimulated adrenergic cells.

### ***p11 Determines the Calcium-Sensitivity of Both the p36 Translocation and the Exocytotic Process in Chromaffin Cells***

Since we had previously observed that the translocation of p36 was more sensitive to calcium in adrenergic chromaffin cells, we investigated the possible correlation between the presence of p11 in the subplasmalemmal region and the calcium sensitivity of the p36 translocation. Therefore SLO-permeabilized chromaffin cells were stimulated with either 10  $\mu\text{M}$  or 100  $\mu\text{M}$  free calcium, fixed, and then stained with both anti-p11 (rhodamine) and anti-p36 (fluorescein) antibodies to examine the translocation of p36 in cells expressing p11. Stimulation with low  $\text{Ca}^{2+}$  did not trigger the translocation of p36 in cells which did not contain p11. Fluorescein labeling remained diffuse and cytoplasmic revealing the presence of p36 in the cytosol in cells which were not stained with the anti-p11 antibodies (Fig. 12 *A*, *arrowhead*). Conversely, cells with a yellow staining pattern corresponding to the colocalization of p36 with p11 were immunolabeled exclusively in the subplasmalemmal region (Fig. 12 *A*). Thus, low calcium triggered the translocation of p36 only in adrenergic cells expressing p11. When SLO-permeabilized chromaffin cells were stimulated with 100  $\mu\text{M}$  free  $\text{Ca}^{2+}$ , p36 was translocated to the plasma membrane in the entire chromaffin cell population and peripheral p36 labeling was observed in all chromaffin cells, even in those which did not express p11 (Fig. 12 *B*).

To complete this study, we examined whether the presence of p11 in the cell periphery, which apparently correlated with a higher calcium sensitivity of the p36 translocation, also conferred a higher affinity for calcium to the exocytotic release from adrenergic cells. Exocytosis can be visualized by immunofluorescence in living cells with anti-

DBH antibodies present in the cell incubation medium (Perrin and Aunis, 1985; Sontag et al., 1988). DBH, which is exclusively located on the inner face of secretory granule membranes, is exposed on the cell surface during exocytosis. Thus, the secretory activity can be evaluated by the appearance of fluorescent patches at the cell surface corresponding to DBH immunoreactivity. To correlate the calcium sensitivity of exocytosis with the presence of p11 in the cell periphery, SLO-permeabilized chromaffin cells were stimulated with either 10 or 100  $\mu\text{M}$  free calcium in the presence of anti-DBH antibodies (fluorescein). Cells were then washed rapidly, fixed, and processed for immunofluorescence with anti-p11 antibodies (rhodamine). Unstimulated SLO-permeabilized cells showed no fluorescent surface patches corresponding to DBH (data not shown), confirming that DBH labeling is a valid marker for calcium-evoked exocytotic activity. Stimulation of chromaffin cells with 10  $\mu\text{M}$  free calcium triggered the appearance of a patchy pattern of fluorescein surface staining corresponding to DBH immunoreactivity, in cells which expressed p11 as revealed by a subplasmalemmal labeling in the rhodamine channel (Fig. 12, *C* and *E*). When cells in randomly selected areas were counted, more than 80% of the chromaffin cells expressing p11 secreted catecholamines in response to 10  $\mu\text{M}$  free calcium. In contrast, cells which were not stained with the p11 antibodies were never labeled with DBH under these conditions of stimulation (Fig. 12, *C* and *E*). Fluorescent DBH patches were only observed in p11-negative chromaffin cells when cells were stimulated with 100  $\mu\text{M}$  free calcium (Fig. 12, *D* and *F*). Note that in this case, fluorescent DBH patches were observed in both p11-positive and p11-negative chromaffin cells (Fig. 12 *F*). Taken together these observations strongly suggest that exocytosis from adrenergic and noradrenergic chromaffin cells occurs with distinct calcium affinities which seem to be related to the presence of p11 in the subplasmalemmal region. Adrenergic cells expressing p11 seem to be activated by a 10-fold lower concentration of cytosolic calcium than noradrenergic cells which do not contain p11.

### ***Discussion***

Several lines of evidence suggest that annexin II may be a key protein involved in membrane fusion events occurring at various steps of the intracellular secretory pathway. For instance, annexin II plays a major role in the fusion and distribution of early endosomes (Emans et al., 1993; Harder and Gerke, 1993) and has been implicated in the various fusion stages that occur in the transcytotic pathway of hepatocytes (Wilton et al., 1994). In view of its cal-

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p11-negative and p11-positive chromaffin cells (*B*). (*C–F*) Double immunofluorescence assay of exocytosis in p11 positive and p11 negative chromaffin cells. SLO-permeabilized chromaffin cells were stimulated with either 10  $\mu\text{M}$  (*C* and *E*) or 100  $\mu\text{M}$  (*D* and *F*) free  $\text{Ca}^{2+}$ . Chromaffin cells expressing p11 were identified by their immunoreactivity to p11 antibodies detected with anti-mouse immunoglobulins conjugated to rhodamine. Secretion was revealed by immunodetection of the secretory granule membrane marker DBH at the cell surface with fluorescein-conjugated antibodies. (*C* and *D*) Phase contrast micrographs and (*E* and *F*) the corresponding images obtained in the rhodamine (p11) and fluorescein (DBH) channels recorded simultaneously in the same section. The yellow-orange color corresponds to areas where fluorescein and rhodamine signals are superimposed. Positive secretory response of chromaffin cells which do not contain p11 in the subplasmalemmal region are observed only in response to 100  $\mu\text{M}$  free calcium (*D* and *F*). At low calcium, only p11 containing chromaffin cells seem to be exocytosis competent (*C* and *E*).

cium-dependent phospholipid-binding properties, annexin II has also been proposed as a candidate for a role in  $\text{Ca}^{2+}$ -regulated exocytosis. Annexin II can aggregate isolated secretory granules in the presence of micromolar calcium and fuse them after addition of arachidonic acid (Drust and Creutz, 1988). Ultrastructural studies performed on chromaffin cells indicate that the protein forms filamentous cross-links between secretory granules and plasma membranes in stimulated cells (Nakata et al., 1990). Furthermore, the reintroduction of purified annexin II partially restores secretion in permeabilized chromaffin cells (Ali et al., 1989; Ali and Burgoyne, 1990; Sarafian et al., 1991). However, the function of annexin II in regulated exocytosis remains debated since the protein only retards the loss of secretion in permeabilized chromaffin cells (Sarafian et al., 1991) and is apparently unable to reconstitute exocytosis after the cells have lost their ability to secrete (Sarafian et al., 1991; Wu and Wagner, 1991).

### *Translocation of p36 in Stimulated Chromaffin Cells*

To get further insights into the possible involvement of annexin II in calcium-regulated exocytosis, we focused here on the intracellular localization of p36 and its cellular ligand p11 in resting and stimulated chromaffin cells. Based on subcellular fractionation techniques and immunological detection, previous studies have suggested the translocation of annexins from the cytosol to either subcellular organelles or to the plasma membrane in activated secretory cells (Sarafian et al., 1991; Sjölin et al., 1994). However, the techniques used in these studies to identify the protein in specific subcellular compartments required procedures that disrupt the cell integrity and might cause artefactual redistributions. In neutrophils, LeCabe and Maridonneau-Parini (1994) analyzed the subcellular localization of annexin III by conventional immunofluorescence microscopy and found that the protein translocated to the cell periphery upon cell stimulation. Here we combined immunocytochemistry with confocal microscopy to perform a detailed quantitative analysis of the intracellular distribution of p36 in resting and nicotine-stimulated chromaffin cells. Our results demonstrate that p36, which is essentially cytoplasmic in resting chromaffin cells, is translocated to the subplasmalemmal region upon nicotinic stimulation. Subcellular fractionation and immunoprecipitation experiments confirmed the translocation of p36 in response to nicotine and further indicated that p36 was predominantly present in a Triton X-100 insoluble fraction in stimulated chromaffin cells.

To probe the role of p36 in the exocytotic event, we performed microinjection experiments and monitored exocytosis from single chromaffin cells. We previously observed that activation of PKC triggers the binding of cytosolic p36 to cell membranes in permeabilized chromaffin cells (Sarafian et al., 1991). One of the serine phosphorylation sites for PKC has been identified in position 25 in the  $\text{NH}_2$ -terminal tail of p36 (Gould et al., 1986). Since Ali and Burgoyne (1990) previously reported that a synthetic peptide corresponding to the  $\text{NH}_2$ -terminal 15 residues of p36 containing the binding site for the p11 light chain (Glenney et al., 1986; Johnsson et al., 1986) was without effect on exocytosis, we decided to microinject the p36<sub>[15-26]</sub> peptide

containing the phosphorylation sites for PKC and pp60src, i.e., serine 25 and tyrosine 23, respectively. The effect of this p36<sub>[15-26]</sub> peptide on the translocation of p36 in stimulated chromaffin cells was assessed by immunocytochemistry and confocal microscopy. We found that the translocation of p36 to the cell periphery was completely blocked in chromaffin cells microinjected with this peptide. Because the synthetic p36<sub>[15-26]</sub> peptide corresponds to the domain of p36 which contains the phosphorylation sites, we assume that the peptide may prevent the phosphorylation of the endogenous protein. Indeed, we observed that stimulation with nicotine triggered the phosphorylation of p36 in agreement with a previous study (Drust and Creutz, 1988). It is interesting to note that  $^{32}\text{P}$ -labeled p36 was essentially found in the Triton X-100 insoluble fraction, suggesting that translocation of p36 may occur before phosphorylation in stimulated cells. Accordingly, immunocytochemical staining and confocal analysis with a monoclonal antibody against PKC revealed that PKC was predominantly found in the subplasmalemmal region in chromaffin cells (data not shown). This suggested that phosphorylation of p36 by PKC may require the previous translocation of cytosolic p36 to the cell periphery. Thus, a possible interpretation for the inhibiting effect of the p36<sub>[15-26]</sub> peptide on the translocation of p36 is that the peptide prevents the phosphorylation of p36 and may thereby destabilize the interaction of p36 with the subplasmalemmal network. In agreement, data obtained in permeabilized chromaffin cells show that activation of PKC promotes the association of endogenous p36 to a membrane-bound compartment (Sarafian et al., 1991). It is also interesting to mention that a similar sequence was recently suggested in a human T cell line in which annexin II translocated first to the plasma membrane and then was phosphorylated by PKC upon cell stimulation (Dubois et al., 1995). Annexin II must be phosphorylated by PKC to reconstitute secretion in permeabilized chromaffin cells (Sarafian et al., 1991). On the other hand, previous studies indicate that phosphorylated annexin II is unable to aggregate purified chromaffin granules, thus questioning the function of the protein in exocytosis (Johnstone et al., 1992; Wang and Creutz, 1992). The properties of annexin II phosphorylated by PKC have been recently reinvestigated in details by Regnouf et al. (1995). As previously described, phosphorylated annexin II was found unable to aggregate chromaffin granules at physiological  $\text{Ca}^{2+}$  concentrations. However, Regnouf et al. (1995) demonstrated that phosphorylation by PKC triggers the fusion of granules preaggregated by unphosphorylated annexin II. In other words, annexin II is not a fusogenic protein but phosphorylation by PKC renders it fusogenic. This finding taken together with the present observations suggesting that annexin II is translocated and phosphorylated in the subplasmalemmal region in stimulated chromaffin cells, offers strong support for a role of phosphorylated annexin II in the final fusion step of exocytosis.

The secretory activity of chromaffin cells microinjected with the p36<sub>[15-26]</sub> peptide was examined by amperometry using carbon-fiber microelectrodes as a sensor for real time detection of catecholamine release from a single cell (Leszczyszyn et al., 1991; Wightman et al., 1991; Chow et al., 1992). We found that the exocytotic response to either



nicotine or high  $K^+$  was strongly inhibited by the p36<sub>[15-26]</sub> peptide, an observation which supports the idea that the translocation of p36 to the plasma membrane is an essential event in the sequence leading to exocytosis. However, despite the fact that the translocation of p36 was totally inhibited in cells microinjected with the p36<sub>[15-26]</sub> peptide, a residual secretory activity was systematically measured in cells stimulated with either nicotine or  $K^+$ . Since we could not further reduce the secretory activity by increasing the amount of peptide microinjected into the cytosol, we postulated the existence of a subpopulation of secretory granules that can be released by a mechanism which does not require the translocation of p36. Regulated secretion involves both  $Ca^{2+}$  and ATP. Studies in diverse secretory systems (Holz et al., 1989; Lumpert et al., 1990; Hay and Martin, 1992; Vitale et al., 1994) indicate that ATP acts before  $Ca^{2+}$  by priming the exocytotic apparatus. An attractive speculation is that the ATP-dependent priming step involves the translocation and the phosphorylation of p36. Thus, the component of secretion that occurs in microinjected cells having an impaired translocation of p36 may correspond to the release of primed granules that undergo the final ATP-independent fusion step upon cell stimulation. Alternatively, we cannot exclude the occurrence of distinct parallel pathways in the exocytotic machinery. Indeed separate mechanisms of action involving either annexin II or the *N*-ethylmaleimide-sensitive fusion protein (NSF) and its soluble attachment proteins (SNAPs) have been suggested for endosome-endosome fusion (Mayorga et al., 1994). In epithelial cells, the exocytotic vesicular transport to the apical plasma membrane seems to involve a member of the annexin family (Fiedler et al., 1995), whereas NSF, SNAP, and Rab proteins operate in the basolateral transport (Ikonen et al., 1995). In neutrophils, secretion from two distinct granule populations is modulated differently by calcium and separately regulated by distinct proteins of the annexin family (Lew et al., 1986; Sjölin et al., 1994).

### Role of p11

In many cell types, the intracellular docking ligand of p36 is the S-100-related protein p11 which is always located on the cytoplasmic face of the plasma membrane (Osborn et al., 1988). Sequence analysis of p11 demonstrated a striking homology with the  $\alpha$  subunit of the brain S-100 protein (Glenney and Tack, 1985; Harder et al., 1992). S-100 proteins are low molecular weight acidic proteins of about 100 amino acids, with a highly conserved secondary structure and similarities between individual polypeptides ranging from 23 to 60% identically placed residues (Kligman and Hilt, 1988). This extraordinary conservation of the gene and protein structure together with their expression during developmental processes suggest important physiological role(s) although the *in vivo* functions of S-100 proteins remain unclear (Masiakowski and Shooter, 1990). Here, confocal analysis revealed that in resting chromaffin cells, p11 was present exclusively in the cortical region whereas p36 was distributed throughout the cytoplasm. It is likely that the cytosolic localization of p36 may correspond to the annexin II monomer. Upon nicotinic stimulation, p11 and p36 colocalized underneath the plasma membrane in a

Triton X-100 insoluble compartment. Since the p36<sub>2</sub>-p11<sub>2</sub> complex has a  $K_d$  of  $<3 \times 10^{-8} M^{-1}$  (Johnsson et al., 1988), the areas where p36 colocalized with p11 may reflect the formation of the heterotetramer of annexin II. Thus, the annexin II tetramer (p90) may occur exclusively in the subplasmalemmal region in adrenergic cells. By stimulating the translocation of p36 to a compartment containing p11, nicotine may trigger the formation of the annexin II tetramer near the exocytotic sites. However, further biochemical data are still required to establish the actual molecular interaction between p36 and p11 in stimulated chromaffin cells.

Surprisingly, p11 was not found in the entire population of chromaffin cells. The subpopulation of chromaffin cells which expressed p11 corresponded to the adrenergic PNMT-positive cells. Consistent with this observation, p11 is also not expressed in PC12 cells, a noradrenergic cell line of rat adrenal pheochromocytoma (Masiakowski and Shooter, 1990). The presence of p11 solely in adrenergic chromaffin cells raises the question of the ligand to which p36 translocates upon cell stimulation in noradrenergic cells. Since p36 is a calcium-dependent phospholipid-binding protein, p36 may well associate directly with the plasma membrane lipid bilayer in stimulated noradrenergic cells. Alternatively, it has been reported that S-100 proteins can substitute for p11 in regulating the activities of p36 in cells which do not express p11 (Bianchi et al., 1992). The S-100-related protein calyculin interacts with annexin II in the presence of calcium (Filipek et al., 1991) and seems to be involved in the calcium signaling pathway underlying exocytosis in various secretory cell types (Timmons et al., 1993; Okasaki et al., 1994). The possible presence of calyculin or other S-100-related proteins in chromaffin cells requires further investigation but may well be correlated with the aminergic phenotype. An interesting future undertaking will be to determine whether the monomer vs the tetramer of annexin II may be assigned to a specific step of secretion occurring in one or the other chromaffin cell phenotype.

*In vitro* studies indicate that p90 requires less calcium than p36 to associate with phospholipid bilayers (Evans and Nelsestuen, 1994). p11 behaves as the regulatory chain of annexin II in that it modifies the  $Ca^{2+}$ - and/or the phospholipid-binding properties of p36 (Powell and Glenney, 1987). Since p11 was expressed only in adrenergic chromaffin cells, we compared the calcium sensitivity of the p36 translocation in adrenergic and noradrenergic chromaffin cells. For this purpose, we used permeabilized cells which permit the change of cytosolic  $[Ca^{2+}]_i$  by known amounts. In chromaffin cells, depolarization that raises the average cytosolic  $Ca^{2+}$  concentration to a few micromolars can rapidly elevate  $[Ca^{2+}]_i$  near the exocytotic sites to the 50–200  $\mu M$  range (Cheek et al., 1989; Neher and Zucker, 1993). Therefore, we measured the translocation of p36 in SLO-permeabilized chromaffin cells stimulated with either 10  $\mu M$  or 100  $\mu M$  free  $Ca^{2+}$ . We found that 10  $\mu M$  free  $Ca^{2+}$  triggered the translocation of p36 in adrenergic cells, but 100  $\mu M$  free  $Ca^{2+}$  was required to translocate p36 in both adrenergic and noradrenergic cell types. We then examined whether the calcium sensitivity of the p36 translocation may determine the calcium affinity of the exocytotic pathway. Our observations indicate that low calcium (10  $\mu M$ ) stimulated the exocytotic release prefer-

entially in adrenergic chromaffin cells. In other words, adrenergic cells which possess p11 in the subplasmalemmal region recruit p36 to the cell periphery and activate the exocytotic machinery with a 10-fold higher affinity for calcium than noradrenergic cells which do not contain p11.

It is tempting to correlate these observations suggesting different  $\text{Ca}^{2+}$  requirements for exocytosis in noradrenergic and adrenergic chromaffin cells with the varying calcium affinities reported for the exocytotic pathway in synapses and neuroendocrine cells (for review see Burgoyne and Morgan, 1995). It is now clear from studies involving high-resolution capacitance measurement coupled with flash photolysis of caged calcium that exocytosis in neurons is controlled by a  $\text{Ca}^{2+}$ -binding protein(s) with a low affinity (saturating at  $>100 \mu\text{M}$  free  $\text{Ca}^{2+}$ ), whereas exocytosis in neuroendocrine cells seems to be regulated by a  $\text{Ca}^{2+}$ -binding protein with a  $\text{Ca}^{2+}$  affinity an order of magnitude higher (Thomas et al., 1993; Heidelberger et al., 1994). The nature of the  $\text{Ca}^{2+}$ -binding proteins and how they interact with the components recently identified in the synaptic and secretory exocytotic machinery remain key unresolved issues (Rothman and Orci, 1992; Bennett et al., 1992; Brose et al., 1992; Söllner et al., 1993; Damer and Creutz, 1994). The presence of p11 at the exocytotic sites seems to confer a higher calcium sensitivity to adrenergic chromaffin cells. Thus, an attractive speculation is that the annexin II tetramer may be one of the  $\text{Ca}^{2+}$  sensors by which the final exocytotic stages display different  $\text{Ca}^{2+}$  affinities in synapses and neuroendocrine cells. The distribution of p11 in endocrine and neuroendocrine cells is currently unknown. However, the absence of p11 in the brain has been reported (Zokas and Glenney, 1987) and this observation correlates well with the hypothesis that neurons may not express p11 and require thereby more calcium to trigger the exocytotic machinery.

### Annexin II in Exocytosis

The recent finding that the *N*-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs) assemble into a tight multimolecular complex with SNAP-25, syntaxin, and VAMP/synaptobrevin (the SNAREs) has shed some light on the molecular events underlying the exocytotic process (Söllner et al., 1993). Since synaptobrevin and syntaxin are associated with, respectively, synaptic vesicles and the plasma membrane, it has been suggested that these proteins may provide a molecular basis for docking secretory vesicles at exocytotic sites (for review see Warren, 1993). It should be noted that at present there is no direct functional evidence that NSF does act in regulated exocytosis in neurons, but  $\alpha$ -SNAP has been shown to regulate exocytosis in chromaffin cells (Morgan and Burgoyne, 1995). We demonstrate here that annexin II plays an essential role in exocytosis in chromaffin cells, although its exact function remains to be determined. Since p36 translocates from the cytosol to the subplasmalemmal cytoskeleton, it may be anticipated that the role of annexin is probably not to direct the granules towards the exocytotic sites. Indeed phosphorylated annexin II may participate in exocytosis as a promoter of membrane fusion and the necessary specificity for the fusion process may be provided by the SNARE complex which

docks the secretory granules to the appropriate sites on the plasma membrane. In the model proposed by Söllner et al. (1993), the SNAREs form the vesicle-docking complex, and when secretion is activated by the entry of  $\text{Ca}^{2+}$ ,  $\alpha$ -SNAP binds to the SNAREs in place of synaptotagmin. The soluble NSF is then recruited and hydrolysis of ATP by NSF has been proposed to act as a driving force leading to membrane fusion. However, several pieces of evidence obtained in various secretory cell types indicate that the late fusion step in exocytosis is regulated by calcium and occurs in the absence of ATP, thus questioning the function of NSF as the fusion protein per se. Annexin II is an attractive alternative (Wilson, 1995) since the ability of this phosphoprotein to fuse secretory granules is entirely calcium-dependent.

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