Production of a Unique Antibody Specific for Membrane Ruffles and Its Use to Characterize the Behavior of Two Distinct Types of Ruffles

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Abstract. I have produced a new monoclonal antibody, YF-169, against membrane ruffle specific 55-kD protein. YF-169 stained membrane ruffles of chick embryo fibroblasts so definitely that it enabled clear and reliable analyses of membrane ruffles. Fibroblasts organized two distinct types of membrane ruffles. One type of the ruffles were transiently formed in serum-starved cells (Type I) when stimulated by serum or platelet-derived growth factor. After spontaneous degradation of Type I ruffles, the other type of ruffles containing many microspikes were gradually organized at leading edges (Type II). The formation of Type I ruffles was not affected by either nocodazole, a microtubule-disrupting drug, or taxol, a microtubule-stabilizing reagent. However, Type II ruffles were entirely destroyed not only by nocodazole but also by taxol, suggesting that regulated organization of microtubule network is important to maintain Type II ruffles. H8, a protein kinase inhibitor prevented the spontaneous degradation of Type I ruffles and also reduced the destructive effect of nocodazole on Type II ruffles without affecting microtubule-disrupting activity. Protein kinases may be involved in the degradation processes of both types of ruffles. W7, a calmodulin antagonist, strongly inhibited Type I ruffle formation and completely destroyed Type II ruffles. W7 was also found to induce a remarkable change of 55-kD protein localization. After degradation of Type II ruffles, most of 55-kD protein was incorporated into newly formed unusual thick fibers. These results suggest that regulated organization of microtubule network is not necessary to form Type I ruffles but is important to maintain Type II ruffles, while calmodulin function is essential for both types of membrane ruffles.

MIGRATING fibroblasts form membrane ruffles at their leading edges. This type of ruffle is generally believed to play a fundamental role in directed migration. Furthermore, it has been known that membrane ruffle formation as well as directed migration are highly dependent on microtubule integrity. Microtubule-disrupting drugs abolish normal localization of membrane ruffles to leading edges and inhibit directed migration (Vasiliev et al., 1970; Goldman, 1971; Gail and Beene, 1971). Little is known, however, about the nature of interaction between microtubules and membrane ruffles. Do microtubules support membrane ruffles through mechanical interaction? Membrane ruffle skeletons are mainly composed of microfilaments and microfilament-associated proteins. Microtubule associated proteins (Selden and Pollard, 1983; Arakawa and Frieden, 1984) as well as intermediate filaments (Hollenbeck et al., 1989; Goldman et al., 1986) have been shown to associate with both microtubules and microfilaments. Based on this evidence, it is possible that microtubules may support membrane ruffles mechanically, but there have been no reports showing mechanical support by microtubules so far. There may be another possibility that microtubules indirectly interact with membrane ruffles through other factors or reactions. Crossin and Carney (1981) demonstrated that depolymerization of microtubules could induce DNA synthesis in fibroblasts in the absence of any growth factors. Their result suggests that microtubule depolymerization can induce certain biochemical reactions in cells.

It has been shown that the other type of ruffles are induced in serum-deprived cells by the stimulation with serum or growth factors (Chinkers et al., 1979; Goshima et al., 1984; Mellström et al., 1988). In this article, this type of membrane ruffle is called Type I, being distinguished from those formed at leading edges of migrating cells, which are called Type II. Type I ruffles develop along nearly the entire margin of the cell, and the formation is always transient. After sudden formation, they are spontaneously eliminated within 30 min or so. The regulatory mechanisms of organization and disorganization of Type I ruffles are poorly understood, and the differences between Type I and Type II ruffles have not been investigated in detail.

Most of studies on membrane ruffle formation have been carried out by means of phase contrast microscopy (Myrdal and Auersperg, 1986; Abercrombie et al., 1970; Dipasquale, 1975) or actin staining with fluorescent dye-conjugated phalloidin (Mellström et al., 1988). Although phase contrast microscopy offers the advantage that one can observe ruffling of living cells, it seems doubtful whether one can detect all membrane ruffles. It is also difficult to detect fine ruffles of fibroblasts by staining with phalloidin, because such ruffles

do not always contain large amounts of polymerized actin. I have produced a unique monoclonal antibody, YF-169, which clearly and specifically stains the membrane ruffles of chick embryo fibroblasts (CEF)[1], and made it possible to detect even fine ruffles which were scarcely stained with phalloidin. Using YF-169, I have investigated the effects of microtubule-disrupting reagents and inhibitors of calmodulin and protein kinase on the two distinct types of membrane ruffles. I report here that calmodulin is essential for both types of ruffles and regulated formation of microtubule network is important for Type II ruffles, and that protein kinases may be involved in the degradation processes of both types of ruffles.

Materials and Methods

Materials

H7, H8, and W7 were purchased from Seikagaku Kogyo Co. (Tokyo). PMA, taxol, cytochalasin D, PDGF, and EGF were from Sigma Chemical Co. (St. Louis, MO), and nocodazole was from Aldrich Chemical Co. (Milwaukee, WI). Fibronectin was prepared from human plasma as described by Hayashi and Yamada (1982). Polyclonal antibodies against tubulin and vimentin were raised by immunization of rabbits. Tubulin was obtained from brains (from chick embryos) by repeated polymerization and depolymerization. Vimentin was prepared from cultured CEF as described by Zackroff et al. (1984). These proteins were electrophoretically purified and then injected to rabbits subcutaneously. The antibodies were affinity-purified as described previously (Talian et al., 1983), and confirmed to be monospecific by Western immunoblotting.

Cell Culture

CEF were prepared from 9-d embryos by trypsinization. The cells were maintained in DME supplemented with 10% FCS, 50 mcg/ml streptomycin, 50 U/ml penicillin, and 2 mg/ml glutamine in an atmosphere of 5% CO_2 and 95% air at 37°C. The cells from third to fifth passage were used for experiments. For immunofluorescence microscopy, the cells were plated on fibronectin-coated glass coverslips (9 × 12 mm). Serum-starved CEF were plated on the coverslips after washing with DME, and then incubated in DME for 18 h.

Production of YF-169 Monoclonal Antibody

YF-169 was originally produced by hybridization of rat Y3 myeloma cells and rat spleen cells immunized with cells from livers (from chick embryos). Hepatic cells were obtained from 10-d embryos by collagenase treatment. After 24 h culture, the cells were detached by the treatment with EGTA, suspended in PBS, emulsified with an equal volume of Freund's complete adjuvant, and then injected intraperitoneally into Sprague-Dawley rats. 2 wk later, the rats were boosted with an emulsion of hepatic cells and Freund's incomplete adjuvant. 2 wk later, the rats received their last booster with hepatic cells in PBS. Spleen cells from the immunized rats were hybridized with Y3 myeloma cells as reported previously (Furth et al., 1982). Supernatants from the hybridoma cultures were screened by immunofluorescence microscopy using CEF. Several monoclonal antibodies were found to stain membrane ruffles. Among them, only YF-169 stained ruffles exclusively. Others stained both membrane ruffles and stress fibers. YF-169, an IgM monoclonal antibody, was prepared from supernatant of serum-free culture. The antigen corresponding to YF-169 was determined by Western immunoblotting using a whole extract of CEF. Nitrocellulose sheets were stained by serial reactions with YF-169, biotin-labeled rabbit antibody against rat IgG (Zymed Labs, Inc., San Francisco, CA) which reacted with light chains of rat IgM, peroxidase-conjugated avidin (Zymed Labs), and 4-chloro-2-naphthol.

Fluorescence Microscopy

Cells on coverslips were fixed for 10 min in a freshly prepared solution of 4% formaldehyde, 0.15 M NaCl, 5 mM EGTA, 5 mM $MgCl_2$, and 40 mM Pipes, at pH 7.0, followed by permeabilization with 0.4% Triton X-100. In some cases, permeabilization was done simultaneously with fixation in order to sufficiently extract cytosol and to enable antibodies to penetrate inside the cells. By this treatment, membrane ruffles were not preserved so well, but stress fibers were fixed enough. After blocking with 5% skim milk and 0.1 M glycine, the cells were double-labeled as follows. Some cells were stained with rhodamine-conjugated phalloidin (Sigma Chemical Co.), then reacted with YF-169, and then stained with fluorescein-conjugated rabbit antibody against rat IgG (Cappel Laboratories, Malvern, PA). Other cells were reacted with a mixture of YF-169 and antitubulin antibody (or antivimentin antibody), stained with rhodamine-conjugated goat antibody against rabbit IgG (Cappel Laboratories), and then stained with fluorescein-conjugated rabbit antibody against rat IgG. The staining was done with optimum concentration of antibodies which had been determined by titration. The coverslips were mounted in 95% glycerol containing 3 mg/ml *n*-propyl gallate and 10 mM Tris-HCl of pH 8.0. Cells were photographed on an Olympus fluorescence microscope (model BHS-RFK) with an Olympus DApo 40 × UV objective.

Results

Characterization of YF-169

To detect the antigen of YF-169, Western immunoblotting was carried out using a whole extract of CEF. YF-169 reacted with a single band of 55 kD (Fig. 1).

YF-169 clearly stained the two distinct types of membrane ruffles, i.e., Type I ruffle shown in Fig. 2 *C* and Type II ruffle shown in Fig. 2 *E*. In the case of Type II ruffles, microspikes were particularly stained by the antibody, although these structures were stained very faintly with rhodamine-phalloidin (Fig. 2, *E* and *F*). YF-169 scarcely stained stress fibers or focal adhesion plaques. Similar results were obtained using well-extracted cells. The staining pattern of YF-169

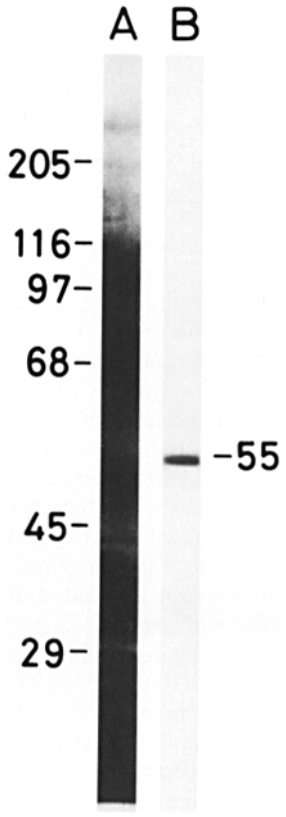

Figure 1. Identification of YF-169 antigen by immunoblotting. Whole extract of CEF was applied to SDS-gel electrophoresis, and then transferred onto nitrocellulose sheet. After the sheet was cut into narrow strips, each strip was stained with amidoblack (lane *A*) or YF-169 (lane *B*) as described in Materials and Methods. YF-169 reacted with a single band of 55 kD. Numbers on the left indicate the positions of molecular weight markers.

1. *Abbreviation used in this paper*: CEF, chick embryo fibroblast.

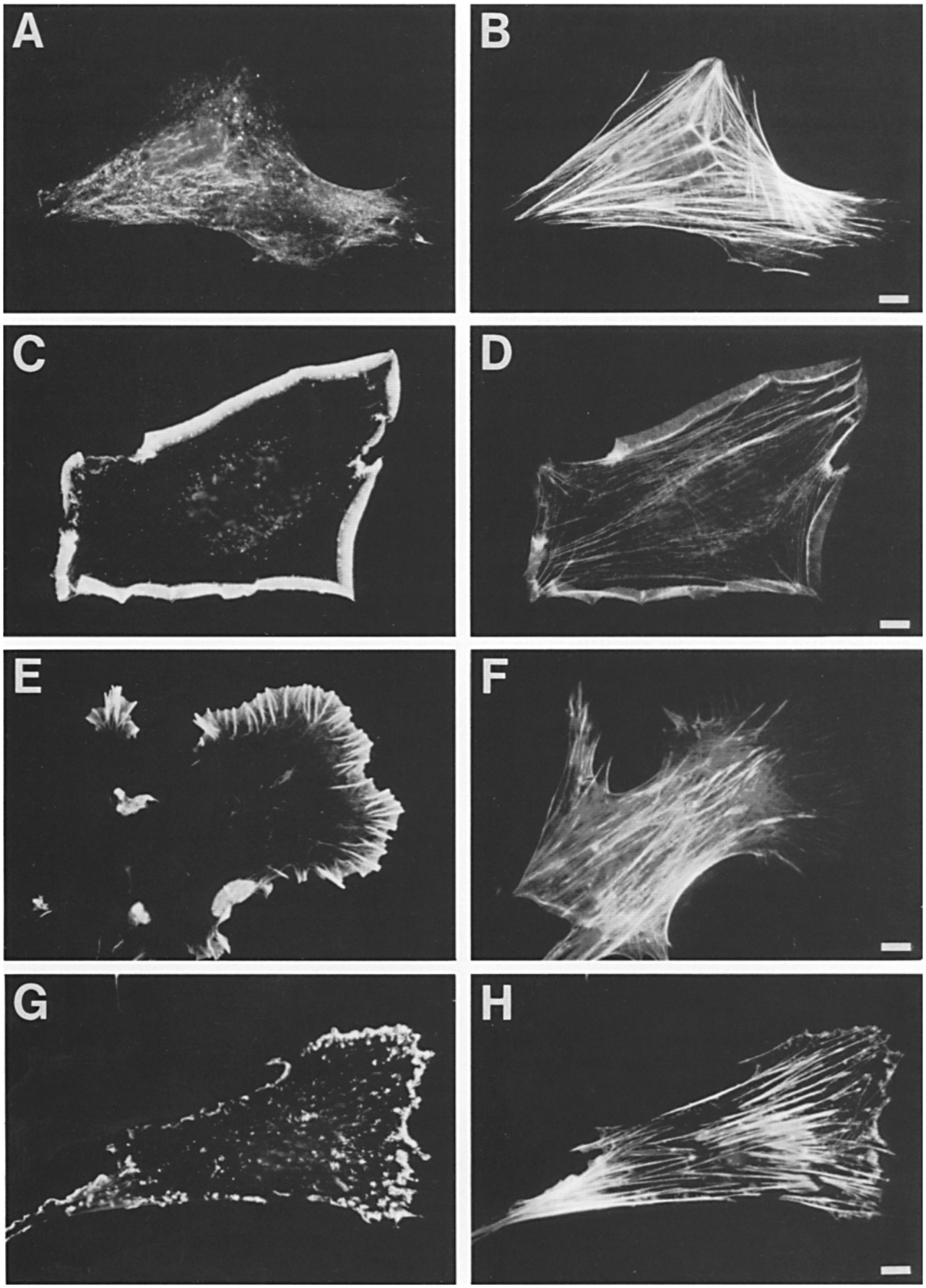

Figure 2. Type I and Type II ruffles revealed by YF-169 and effect of cytochalasin D on Type II ruffles. Serum-deprived cells (*A* and *B*) were stimulated for 4 min by FCS at final concentration of 20% (*C* and *D*). Cells were incubated for 15 h in the presence of 15% FCS (*E* and *F*), and then treated with 4 μM cytochalasin D for 10 min (*G* and *H*). Membrane ruffles shown in *C* and *E* correspond to Type I and Type II, respectively. The cells were double-stained with YF-169 (*A*, *C*, *E*, and *G*) and rhodamine-phalloidin (*B*, *D*, *F*, and *H*). Bars, 10 μm.

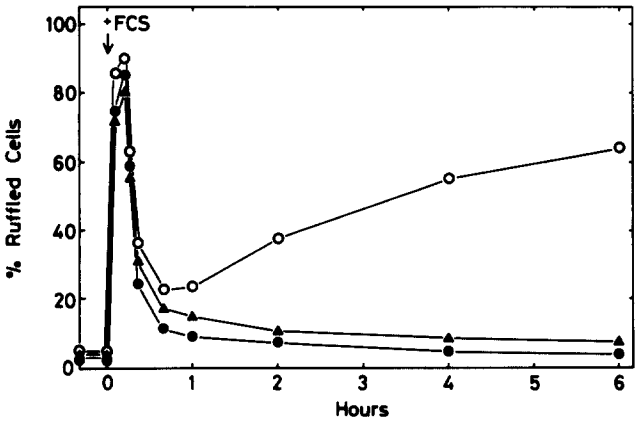

Figure 3. Serial formations of Type I and Type II ruffles and the effects of nocodazole and taxol on them. Serum-starved cells were divided into three groups. After addition of 20% FCS, first group of the cells were incubated for various times and then fixed. The second and third groups of the cells were also incubated for various times in the presence of serum, but treated with 35 μM nocodazole or with 25 μM taxol for 10 min before fixation. The time in the figure indicates the periods from the addition of serum. Some cells were treated with the drugs before addition of serum, but others were done after addition of serum. After staining with YF-169 and antitubulin antibody, number of cells with and without membrane ruffles were counted. The vertical axis represents percentage of cells having membrane ruffles. Each point of the figure was obtained by counting more than 300 cells. (○) Control; (●) treated with nocodazole; (▲) treated with taxol. Although ruffled cells were counted without distinguishing their types, the first peak corresponds only to Type I ruffles. In the period from 1 to 1.5 h, Type I was mixed with Type II, and after 1.5 h, Type II was the major species.

Table I. Effects of Various Drugs on the Formation of Type I and Type II Ruffles

Drugs (μM)	Ruffled cells	
	Type I	Type II
	%	
Control	82.7 ± 3.4 (100)*	71.8 ± 4.3 (100)
Nocodazole (35)	78.0 ± 3.7 (94.3)	3.2 ± 2.3 (4.5)
Nocodazole (35)		
+ H8 (10)	ND	22.6 ± 6.8 (31.5)
+ H8 (50)	81.1 ± 3.3 (98.1)	46.3 ± 5.2 (64.5)
+ H8 (100)	ND	52.1 ± 6.5 (72.6)
Nocodazole (35)		
+ H7 (50)	80.4 ± 2.5 (97.2)	21.4 ± 5.6 (29.8)
+ H7 (100)	ND	37.7 ± 3.6 (64.5)
+ H7 (200)	ND	48.6 ± 4.2 (67.7)
H8 (50)	83.4 ± 3.9 (101)	72.3 ± 4.6 (101)
H7 (50)	80.6 ± 2.9 (97.5)	70.6 ± 3.2 (98.3)
W7 (50)	0.6 ± 0.4 (0.7)	3.3 ± 2.1 (4.6)
PMA (0.05)	6.4 ± 2.3 (7.7)‡	8.1 ± 1.7 (11.3)‡
PMA (0.05)		
+ H7 (200)	75.6 ± 3.8 (91.4)	65.8 ± 4.8 (91.6)
Taxol (25)	78.8 ± 1.7 (95.3)	4.7 ± 1.3 (6.5)
Taxol (25)		
+ H8 (100)	78.4 ± 3.6 (94.8)	24.5 ± 4.2 (34.1)

Type I ruffles were induced by the stimulation with 20% FCS for 4 min after the cells had been treated with each drug or with each two drugs. Type II ruffles were induced after incubation with 15% FCS for 15 h, and then the cells were treated with each drug or with each two drugs. Incubation times were 10 min for nocodazole, W7, and taxol, and 30 min for H8, H7, and PMA. When cells were treated with two drugs, they were done first with H8 or H7, and then done with nocodazole, PMA, or taxol. After staining with YF-169 and anti-tubulin antibody, cells with and without membrane ruffles were counted. Values are mean ± SD from ~3–5 experiments.

* Values in parentheses represent relative percentages when control values are regarded as 100%.

‡ PMA did not degrade membrane ruffles like nocodazole, but obviously exerted influence on them and caused their shrinkage. Such shrunken ruffles were distinguished from normal ones, and the cells having shrunken ruffles were not counted in the group of ruffled cells.

was completely disrupted by the treatment with cytochalasin D (Fig. 2 *G*). The disruption seemed to be completed before substantial disappearance of stress fibers, suggesting that 55-kD protein-containing ruffle structures are much more sensitive to cytochalasin D than stress fibers. In the following experiments, membrane ruffles were observed by the staining with YF-169.

Serial Formations of Type I and Type II Ruffles and Effects of Nocodazole and Taxol on Them

Serum-starved CEF did not have any membrane ruffles, and YF-169 did not stain any particular structures of the cells (Fig. 2 *A*). The cells organized Type I ruffles along nearly entire margins when serum was added to the culture (Fig. 2 *C*). No microspikes were observed at Type I ruffles. Similar responses were induced by 40 ng/ml PDGF, but not by 60 ng/ml EGF (data not shown). Most of Type I ruffles were spontaneously eliminated within 30 min (Fig. 3). After elimination of Type I ruffles, another type of ruffles, Type II, were gradually organized at leading edges (Fig. 3). Most of Type II ruffles contained many microspikes.

Type I ruffles could be induced by the stimulation with serum or PDGF even after the cells had been treated with nocodazole, by which microtubules had been totally disrupted (Figs. 3 and 4, *A* and *B*, Table I). Type I ruffle formation was not affected by taxol either, and taxol-treated cells contained a lot of short microtubules instead of normal microtubule networks (Figs. 3 and 4, *C* and *D*, Table I).

Unlike Type I ruffles, almost all Type II ruffles were readily destroyed by nocodazole (Figs. 3 and 4 *E*, Table I). This was one of the large differences between Type I and Type II ruffles. In addition to nocodazole, taxol was found to have a strong destructive effect on Type II ruffles. Almost all Type II ruffles were degraded by the treatment with taxol (Figs. 3 and 4 *G*, Table I). Taxol-treated cells seemed to have higher density of microtubules than those of normal cells.

Effects of Protein Kinase Inhibitors on Membrane Ruffle Degradation

After overnight incubation with nocodazole, up to 40% of cells reorganized membrane ruffles even without microtubules (Fig. 5, *A* and *B*). At that time, vimentin filaments collapsed around nucleus (Fig. 5 *D*). These ruffles were classified as Type II, because they had polarity and microspikes. This result suggested that Type II ruffles did not need any mechanical support by microtubules or by intermediate filaments, and therefore, that some microtubule-dependent factors or reactions might be involved in the degradation pro-

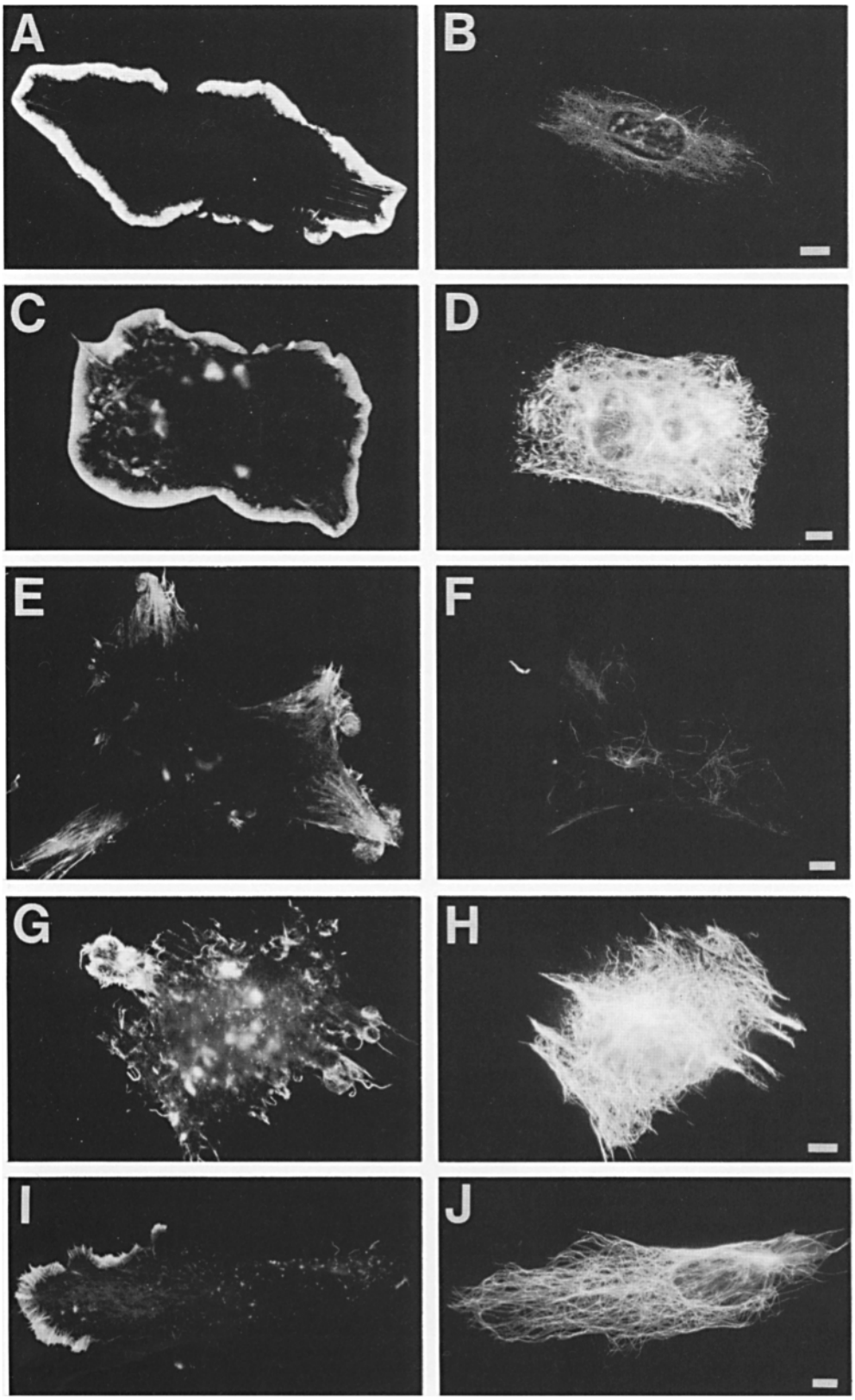

Figure 4. Effects of nocodazole and taxol on Type I and Type II ruffles. Serum-deprived cells were treated for 10 min with 35 μM nocodazole (*A* and *B*) or with 25 μM taxol (*C* and *D*), and then stimulated with 20% FCS for 4 min. Cells were incubated for 15 h in the presence of 15% FCS, and then treated for 10 min with 35 μM nocodazole (*E* and *F*) or with 25 μM taxol (*G* and *H*). Normal microtubule network is shown as a reference (*J*). The cells were double-stained with YF-169 (*A*, *C*, *E*, *G*, and *I*) and anti-tubulin antibody (*B*, *D*, *F*, *H*, and *J*). Type I ruffle formation was not affected by either nocodazole or taxol, whereas Type II ruffles were quite sensitive to both of the drugs. Bars, 10 μm.

cesses of Type II ruffles by nocodazole. A possibility of protein kinase was examined using protein kinase inhibitors, H8 and H7. H8 significantly reduced the destructive effect of nocodazole on Type II ruffles in a dose-dependent manner (Table I), suggesting that unknown microtubule-dependent protein kinase (or kinases) was involved in the degradation of Type II ruffles. H8 did not affect microtubule-disrupting activity of nocodazole. H7 also reduced the destructive effect of nocodazole on Type II ruffles, but the reducing activity of H7 was lower than that of H8 (Table I). Stress fibers were

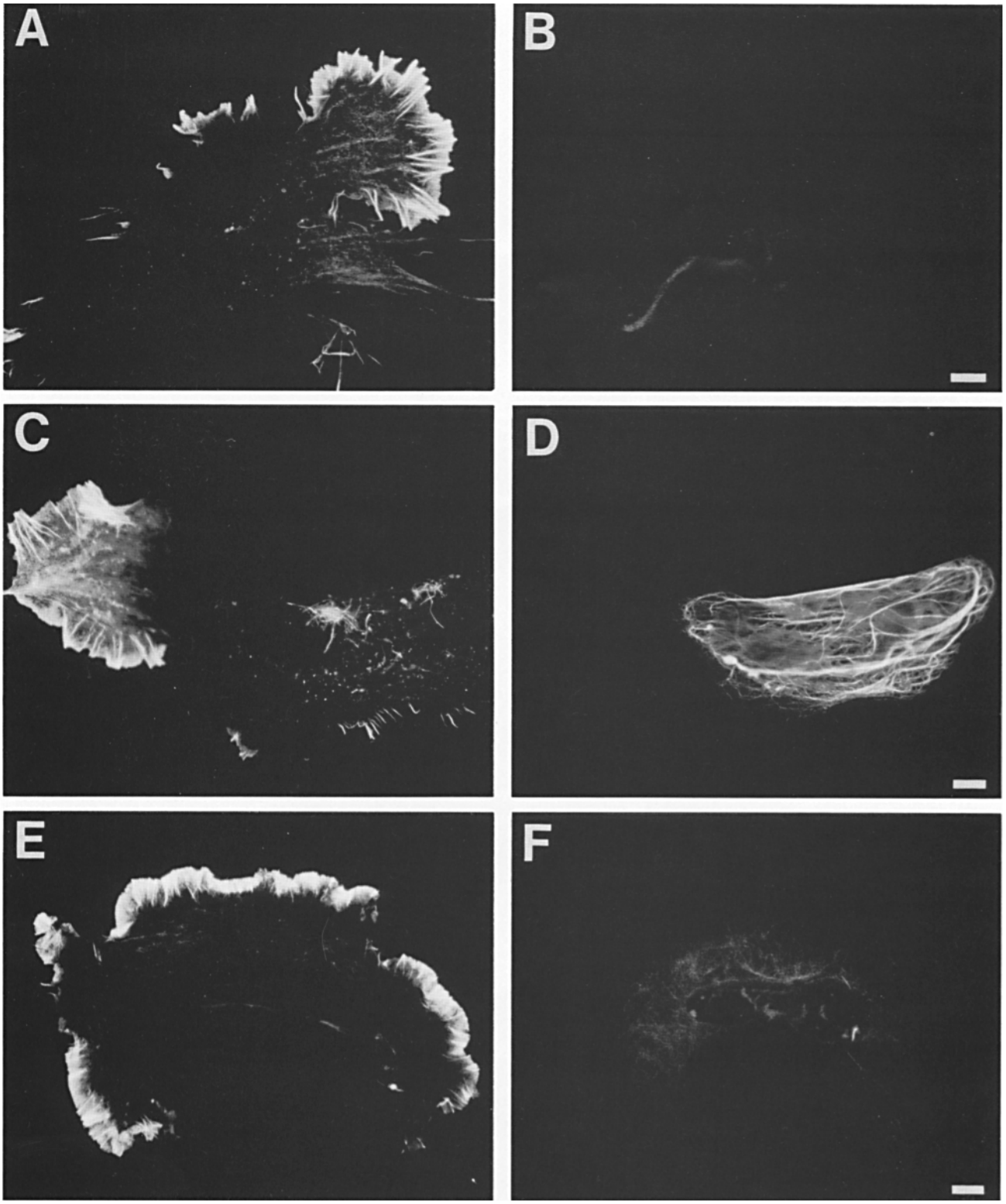

Figure 5. Regeneration of Type II ruffles after long incubation with nocodazole and suppression of nocodazole-induced degradation of Type II ruffles by H8. Cells were incubated for 15 h in the presence of 15% FCS, and then treated with 10 μM nocodazole for 15 h (*A–D*). After long incubation with nocodazole, Type II ruffles were reorganized in spite of the complete depolymerization of microtubules and the collapse of intermediate filaments. After incubation with 15% FCS for 15 h, cells were treated with 100 μM H8 for 30 min, and then treated with 35 μM nocodazole for 10 min (*E* and *F*). Type II ruffles still remained even after most microtubules had been disrupted. The cells were double-stained with YF-169 (*A*, *C*, and *E*), and antitubulin antibody (*B* and *F*) or antivimentin antibody (*D*). Bars, 10 μm.

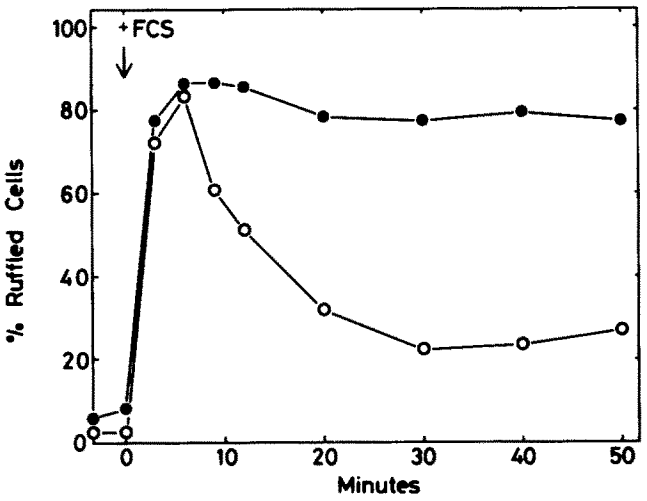

Figure 6. Effect of H8 on the spontaneous elimination of Type I ruffles. Type I ruffles were induced as described in Fig. 4 legend after 30 min incubation with and without 100 μM H8. Number of cells with and without membrane ruffles were counted after staining with YF-169 and antitubulin antibody. The vertical axis represents percentage of cells having membrane ruffles. The time in the figure indicates the periods from the addition of serum. (○) Control; (●) treated with H8. H8 prevented the spontaneous elimination of Type I ruffles.

diminished by H8 or H7, and cell shape was modified. However, Type II ruffles were still maintained in many cells in the absence of microtubules (Fig. 5 *E*). The concentration of H8 required for 50% suppression of nocodazole-induced Type II ruffle degradation was roughly estimated as 25 μM. This value was higher than that for protein kinase A (1.2 μM) or protein kinase C (15 μM), but was lower than that for myosin light chain kinase (68 μM) (Hidaka et al., 1984). H8 moderately reduced the destructive effect of taxol on Type II ruffles (Table I).

Above results implied that the spontaneous degradation of Type I ruffles might also be caused by protein kinase. As a matter of fact, H8 prevented the degradation of Type I ruffles too (Fig. 6), suggesting that the spontaneous degradation of Type I ruffles was induced by protein kinase.

The effect of PMA, a protein kinase C activator, on membrane ruffles was compared with that of nocodazole in order to examine the similarity between protein kinase C and unknown protein kinase involved in membrane ruffle degradation. PMA did not degrade membrane ruffles like nocodazole, but caused shrinkage of both types of ruffles (Fig. 7). In PMA-treated cells, the average width ± SD of Type I ruffles was 2.1 ± 0.7 μm, and that of Type II was 2.7 ± 0.8 μm, whereas in normal cells, the average width ± SD of Type I ruffles was 5.7 ± 1.8 μm, and that of Type II was 7.1 ± 2.2 μm.

Effect of Calmodulin Antagonist on Membrane Ruffles

W7, a calmodulin antagonist, completely inhibited the formation of Type I ruffles (Fig. 8 *A*, Table I) in a way different from PMA. W7-treated cells did not form any traces of membrane ruffles. W7 also degraded Type II ruffles within 10 min (Fig. 8 *C*). Stress fibers were eliminated, but instead of that, unusual thick fibers were organized in many cells after the treatment with W7 for 60 min (Fig. 8 *E*). They were heavily stained with YF-169, but scarcely stained with rhodamine-phalloidin. They were not colocalized with either microtubules or intermediate filaments (data not shown). The concentration of W7 used in this experiment was 50 μM which is the same as that required for 50% inhibition of myosin light chain kinase (Hidaka et al., 1980).

Discussion

I have produced a monoclonal antibody, YF-169, which is specific for 55-kD protein. YF-169 stained membrane ruffles heavily and exclusively, so it was very useful to observe membrane ruffles. YF-169 made it possible to investigate the formation of membrane ruffles in detail.

Molecular weight and localization of 55-kD YF-169 antigen are similar to those of 55-kD actin-bundling protein reported by Yamashiro-Matsumura and Matsumura (1986). One difference is that their 55-kD protein is localized on stress fibers as well as microspikes whereas YF-169 antigen seems to be absent on stress fibers, although it is hard to completely exclude the possibility of the presence of 55-kD YF-169 antigen on stress fibers. The function of 55-kD YF-169 antigen remains to be solved.

CEF serially organized Type I and Type II ruffles after stimulation with serum. These two types of ruffles differed from each other in respect of morphology and sensitivities to both microtubule-disrupting and -stabilizing drugs. Type I ruffles were formed along nearly entire margins without microspikes, while Type II ruffles were organized at leading edges with many microspikes. Type I ruffle formation was not affected either by nocodazole or by taxol, but Type II ruffles were readily destroyed by either of these drugs. It is probable that the formation of Type I ruffle is independent of microtubules but the maintenance of Type II ruffle is highly dependent on them.

It has been shown that membrane ruffles (Type II) are highly dependent on microtubule integrity (Goldman, 1971; Gail and Beene, 1971), and I confirmed their results. However, it has been unclear how microtubules are concerned in the maintenance of Type II ruffles. The present experiments of overnight incubation with nocodazole became a cue to solve the question. Almost all membrane ruffles were eliminated once by the treatment with nocodazole for 10 min, but after prolonged incubation with nocodazole, a certain number of cells regenerated Type II ruffles even in the absence of microtubules. Intermediate filaments remained collapsing around nucleus. These results show that membrane ruffles do not need mechanical support by microtubules or by intermediate filaments. When the cells were treated with nocodazole, Type II ruffles might be destroyed by unknown factors which were probably activated by the depolymerization of microtubules. The regeneration of Type II ruffles after long incubation with nocodazole may be explained by down-regulation of such membrane ruffle-degrading factors.

Protein kinase inhibitors, H8 and H7, significantly reduced the destructive effect of nocodazole on Type II ruffles. This result suggests that protein kinase (or kinases) may be the membrane ruffle-degrading factor itself or closely related to the degrading system. H8 also prevented the spon-

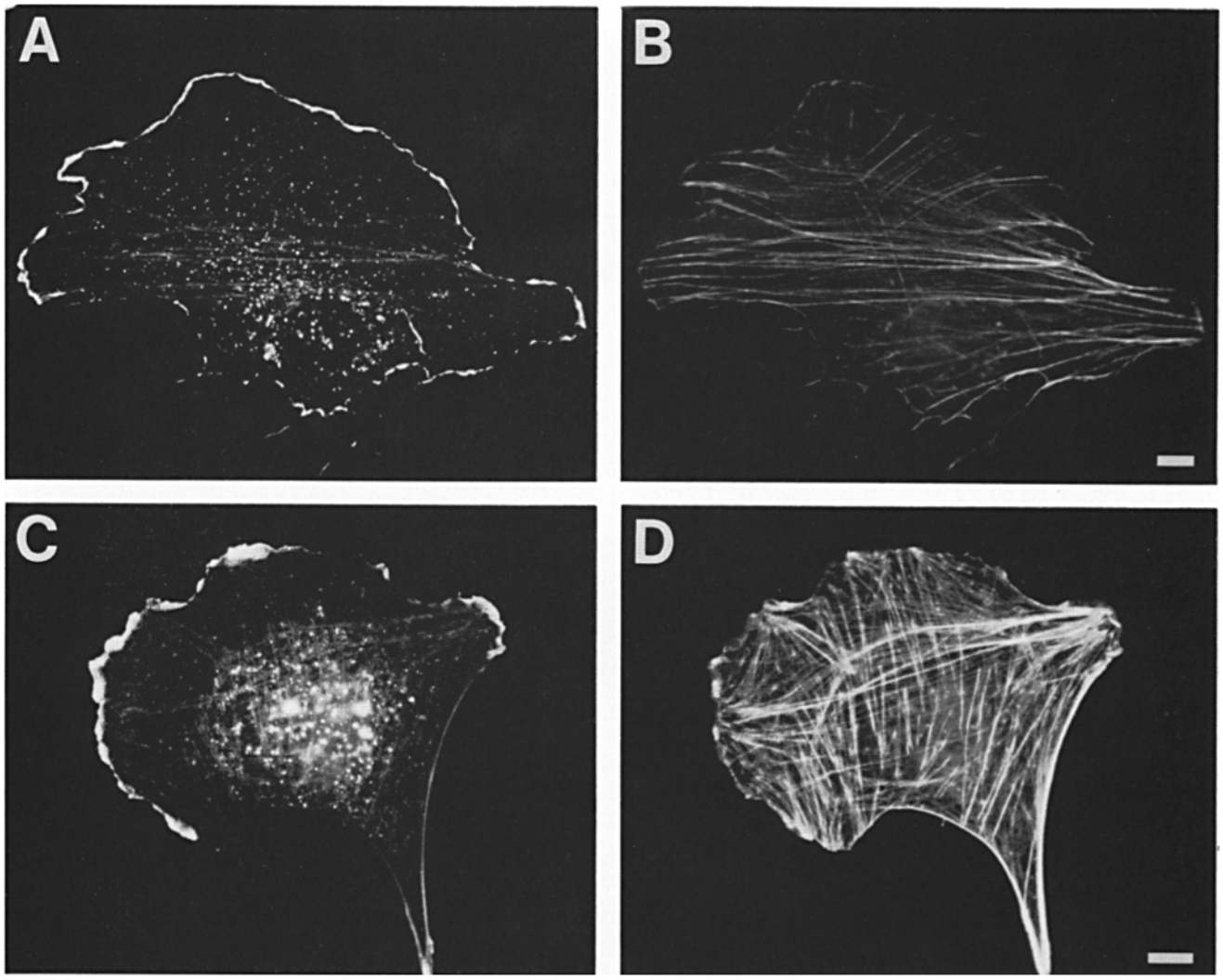

Figure 7. Effect of PMA on Type I and Type II ruffles. Serum-starved cells were treated with 50 nM PMA for 30 min, and then stimulated with 20% FCS for 4 min (*A* and *B*). After incubation with 15% FCS for 15 h, cells were treated with 50 nM PMA for 30 min (*C* and *D*). Cells were double-labeled with YF-169 (*A* and *C*) and rhodamine-phalloidin (*B* and *D*). PMA caused shrinkage of both types of ruffles. Bars, 10 μm.

taneous degradation of Type I ruffles. Protein kinase may be involved in this degradation too. Identifications of the unknown protein kinases and their substrates will be the next subject.

Taxol, a microtubule-stabilizing reagent (Schiff et al., 1979), did not affect Type I ruffle formation, but strongly destroyed Type II ruffles. This result suggests that dynamic behavior of microtubules such as dynamic instability (Mitchison and Kirshner, 1984) may also be important to maintain Type II ruffles. The destructive effect of taxol on Type II ruffles was not strongly but moderately suppressed by H8, so it is uncertain at present whether protein kinase is involved in taxol-induced degradation of Type II ruffles.

PMA, an activator of protein kinase C (Castagna et al., 1982), caused retraction of both Type I and Type II ruffles, but did not degrade them like nocodazole. This result suggests that protein kinase involved in membrane ruffle degradation differs from protein kinase C.

W7 not only destroyed Type II ruffles but also strongly inhibited the formation of Type I ruffles. It is probable that calmodulin plays an important role in the organization and the maintenance of both types of ruffles. Caldesmon, which binds calmodulin and modulates the binding of other proteins to microfilaments (Sobue et al., 1988), has been shown to be located on membrane ruffles (Bretscher and Lynch, 1985). Calmodulin may regulate the organization of membrane ruffles through interaction with caldesmon or other microfilament-associated proteins. W7 was also found to induce the formation of unusual thick fibers containing 55-kD YF-169 antigen. It seems likely that most of 55-kD protein localized at membrane ruffles was translocated into the newly formed fibers. Calmodulin may have an important influence on the localization of 55-kD protein. W7-induced unusual fibers were scarcely stained with rhodamine-phalloidin, but it is not clear whether actin filaments were absent in the fibers or phalloidin-binding sites were blocked.

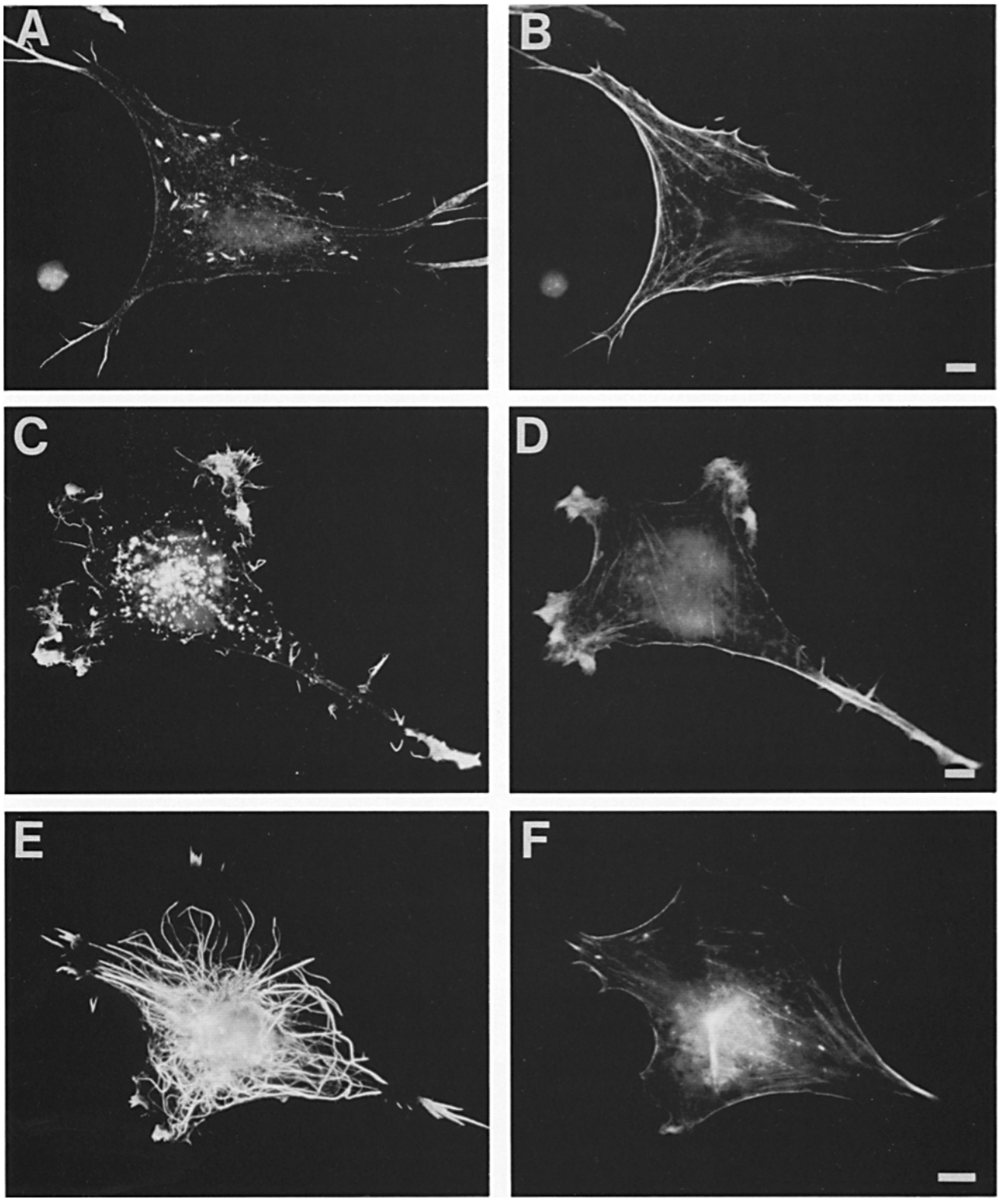

Figure 8. Effect of W7 on Type I and Type II ruffles. Serum-starved cells were treated with 50 μM W7 for 10 min, and then stimulated with 20% FCS for 4 min (*A* and *B*). After incubation with 15% FCS for 15 h, cells were treated with 50 μM W7 for 10 min (*C* and *D*) or 60 min (*E* and *F*). The cells were double-stained with YF-169 (*A*, *C*, and *E*) and rhodamine-phalloidin (*B*, *D*, and *F*). W7 inhibited the formation of Type I ruffles and degraded Type II ruffles. Bars, 10 μm.

In summary, CEF serially formed microtubule-independent Type I and microtubule-dependent Type II ruffles after stimulation with serum or PDGF. Spontaneous degradation of Type I ruffles and nocodazole-induced degradation of Type II ruffles were prevented by protein kinase inhibitors. Calmodulin antagonist inhibited Type I ruffle formation and destroyed Type II ruffles. These results suggest that calmodulin and protein kinases may function in the regulation of membrane ruffle formation.

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