pared as described by G. Cathala *et al.* [*DNA (N.Y.)* **2**, 329 (1983)]. A cDNA library in λgt10 was prepared and ~700,000 clones were plated and screened without amplification [T. Huynh, R. Young, R. Davis, in *DNA Cloning, A Practical Approach*, D. Glover, Ed. (IRL, Oxford, 1985), vol. 1, p. 49]. A 500-bp fragment of mouse LPL cDNA was ^{32}P-labeled and hybridized with duplicate nitrocellulose filters in 5× SSC (standard saline citrate), 50 m*M* sodium phosphate (*p*H 6.8), 5× Denhardt's solution, 20% formamide, 10% dextran sulfate, and 20 μg/ml of boiled, sonicated salmon sperm DNA at 42°C. The filters were then washed in 2× SSC and 0.1% SDS at 55°C, and exposed to x-ray film. About 180 duplicate positive clones appeared from which DNA of nine clones was purified for detailed analysis.
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16. We thank T. Olivecrona for communication of unpublished data, P. Kern for obtaining adipose tissue, and D. Drayna for initial contributions to this project. This research was supported by Genentech, Inc., NIH grant HL28481, the American Heart Association, Greater Los Angeles Affiliate (grant 492-TG13), and the Veterans Administration. A.J.L. is an established investigator of the American Heart Association.

10 December 1986; accepted 17 February 1987

Ubiquitin Is a Component of Paired Helical Filaments in Alzheimer's Disease

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Paired helical filaments (PHF), which constitute a distinct type of pathological neuronal fiber, are the principal constituent of neurofibrillary tangles that occur in the brain of patients with Alzheimer's disease. Their insolubility in sodium dodecyl sulfate and urea has prevented the analysis of their subunit composition by gel electrophoresis. A monoclonal antibody (DF2) was isolated that specifically labeled PHF at both the light and electron microscopic levels. It labeled a small polypeptide (5 kilodaltons) that was shown to be ubiquitin in immunoblots of the soluble fraction of brain homogenates. To obtain direct evidence that ubiquitin is a component of PHF, PHF were treated with concentrated formic acid and digested with lysylendopeptidase; ubiquitin-derived peptides were then identified by reversed-phase high-performance liquid chromatography. Two fragments in the PHF digest were identified as derived from ubiquitin by protein sequencing. This procedure should make possible definitive identification of other PHF components.

THE PROGRESSIVE FORMATION OF unusual neuronal fibers characterizes Alzheimer's disease (AD), which is the most common cause of intellectual failure in aged humans. These fibers are composed of two 10-nm filaments wound into a helix with a half-periodicity of 80 nm and are thus termed paired helical filaments (PHF) (*1*). They accumulate as large perikaryal masses, called neurofibrillary tangles (NFT), or occur as small bundles in dystrophic neurites that form plaques in AD brain. The nature of PHF currently draws much attention because of their unusual morphology, their apparent specificity to human brain, and a possible correlation between their concentration in the brain and the degree of dementia (*2*).

The first step to understanding the origin and nature of PHF should be the identification of their components, but available data conflict. This fact is attributable to the insolubility of PHF (*3*), which has prevented the application of conventional analytical methods. Another approach has been to search for normal soluble proteins, possible precursors of the PHF, that are immunoreactive with polyclonal antibodies to PHF (*4*). This approach has led to the immunochemical identification of the microtubule-associated phosphoprotein, tau, as the major antigenic determinant of PHF (*5*). By means of a similar strategy, we have characterized one monoclonal antibody to PHF whose specificity differs from that of polyclonal PHF antibodies and have found that it recognizes ubiquitin. Furthermore, we have developed a new procedure to digest PHF proteolytically and identified ubiquitin-derived fragments by protein sequencing. This approach will be generally applicable to the analysis of other components of PHF.

PHF were purified in the presence of sodium dodecyl sulfate (SDS) according to a previous method, with minor modifications (*4*); the resultant fractions are referred to as SDS-PHF. SDS-PHF were emulsified with complete or incomplete Freund's adjuvant and injected into a Lewis rat. Three days after the last intravenous injection, rat spleen cells were fused with Sp 2/0-Ag14 mouse myeloma cells. Within eleven 96-well plates, 276 wells were positive by enzyme-linked immunosorbent assay (ELISA) for cells secreting rat immunoglobulin. The second screening for PHF-reactive antibodies with sonicated PHF in an ELISA gave 43 positive wells. Of these 43 wells, 3 wells showed strong reactivities with NFT. After cloning by limiting dilution, one hybridoma (DF2) was established. DF2 monoclonal antibody was of the immunoglobulin M class by the Ouchterlony double-diffusion test.

We have confirmed specific binding of DF2 to PHF in several ways. (i) DF2 stained virtually all isolated NFT prepared under nondenaturing conditions (*4*). The numerous very small tangles observed after staining with DF2 resembled those stained with the polyclonal antibodies to PHF (Fig. 1A). (ii) Tangles isolated by extensive extraction with SDS/ME (2-mercaptoethanol) (*4*) were also intensely stained with DF2, suggesting that a DF2-defined epitope is tightly bound to PHF and not dissociated in SDS. (iii) DF2 decorated PHF, as shown by immunoelectron microscopy (Fig. 1B). Immunogold particles conjugated with DF2 were observed on PHF, but not on such contaminating elements in the PHF fractions as collagen fibers, membranous structures, or other organelles. (iv) DF2 stained both neurofibrillary tangles and senile plaque neurites in formalin-fixed AD brain sections; fine neurites in the cerebral cortex were also immunostained (Fig. 1C). However, unlike polyclonal PHF antibodies (*4*), DF2 definitely stained the background of the tissue sections in both AD and normal brains. The absence of such background staining in the isolated NFT preparations could be ascribed to a lack of soluble DF2-immunoreactive proteins since these tangles were obtained by sedimentation through a sucrose layer (*4*). These observations sug-

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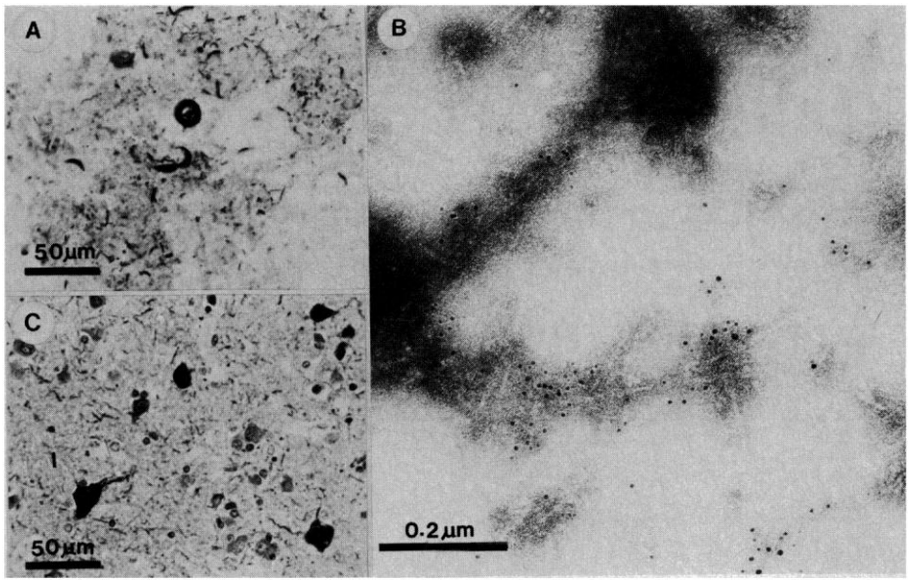

Fig. 1. Specific binding of DF2 to NFT and PHF. (**A**) NFT prepared under nondenaturing conditions and stained with DF2 by the avidin-biotin method (Vectastain, Vector). (**B**) DF2 binding to PHF as demonstrated by immunoelectron microscopy. SDS-PHF were placed on carbon-coated copper grids. Grids were floated on 20% goat serum and transferred to DF2-containing culture medium. After being rinsed with 1% goat serum, they were incubated with anti-rat immunoglobulin conjugated with gold particles (diameter, 5 nm). Grids were rinsed and stained with 5% uranyl acetate, dried, and viewed at 75 kV. (**C**) Formalin-fixed AD brain section stained by the avidin-biotin method.

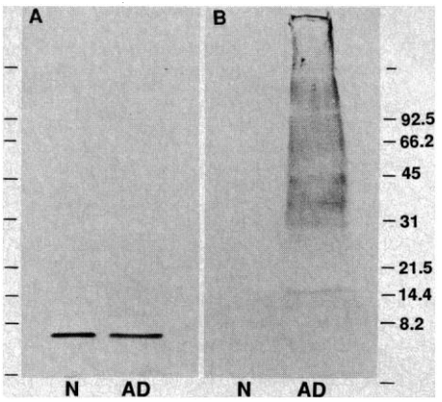

Fig. 2. Immunoblots of (**A**) the tris-saline (TS)–soluble and (**B**) Sarkosyl-insoluble fractions from AD and normal (N) brains. Brain homogenates (20% w/v) in TS (50 m*M* tris HCl, *p*H 7.6, 0.15*M* NaCl) were spun at 100,000*g* for 30 minutes. The pellets were extracted with 1% Sarkosyl as described (*5*). Sarkosyl-insoluble pellets were resuspended with TS. The fractions were separated by electrophoresis on 15% polyacrylamide gels and blotted onto a nitrocellulose sheet (Schleicher & Schuell; pore size, 0.2 μm). Blots were incubated overnight with DF2-containing culture medium at 4°C and immunostained by the avidin-biotin method. Numbers are apparent molecular sizes in kilodaltons.

gest that a DF2-reactive antigen was present in the soluble fractions of normal and AD brain homogenates.

Tris-saline and Sarkosyl-insoluble fractions from AD and normal brains were examined by immunoblotting with DF2 (Fig. 2). A gel-excluded protein band and a diffuse smear of immunoreactivity in the running gel were stained in Sarkosyl-insoluble AD fractions, but no reaction was seen in blots of Sarkosyl-insoluble materials from normal brain (Fig. 2B). The pattern of staining resembled that previously observed with the polyclonal PHF antibodies (*4*). DF2 strongly labeled a 5-kD polypeptide in the soluble fractions from both AD and normal brains (Fig. 2A), a finding compatible with the diffuse background staining of tissue sections. Thus, although DF2 and the polyclonal antibodies to PHF share some characteristics including high specificity to PHF and the staining of diffuse smears in AD blots, it is evident that DF2 differs from the polyclonals in its specificity for a normal 5-kD protein. We postulated that the 5-kD protein and a certain portion of PHF share common antigenic determinants, possibly homologous amino acid sequences. To obtain more information about the antigenic determinants of PHF that DF2 recognizes, we purified the 5-kD protein from high-speed supernatants of normal human brain homogenates (Fig. 3). The partial amino

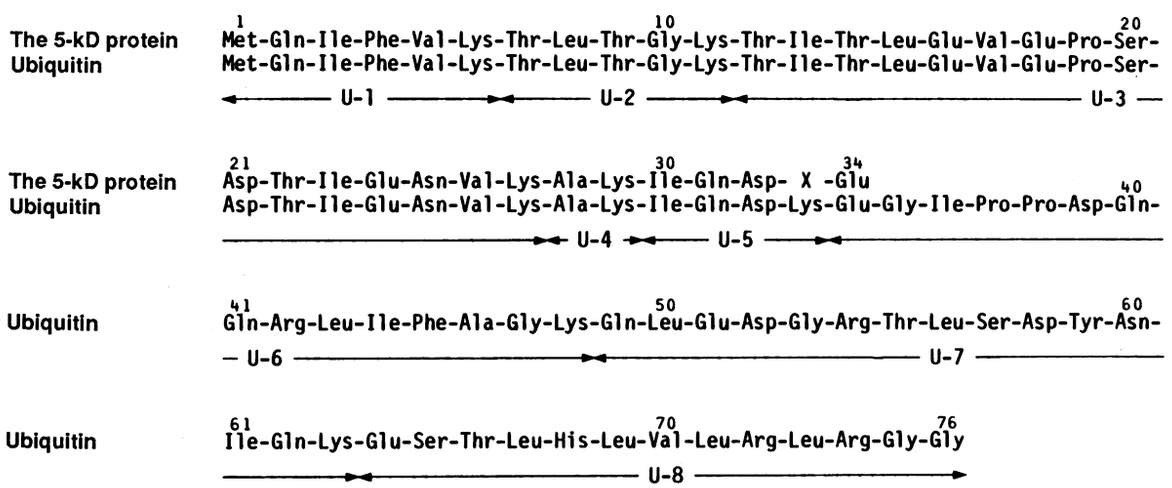

Fig. 3. Amino acid sequence of the 5-kD protein and ubiquitin. The 5-kD protein was purified as follows: Normal human brains were homogenized with five volumes of TS and then ultracentrifuged at 100,000*g* for 60 minutes. The supernatant was fractionated with ammonium sulfate (70 to 100%) and on a Sephadex G-50 column. Fractions containing the 5-kD protein were combined and concentrated. At this step, the 5-kD protein was a major species in the fraction as judged by SDS–polyacrylamide gel electrophoresis and immunoblotting. This crude fraction was further purified by RPHPLC. The column (Ultrasphere ODS, Beckman) was developed with a linear gradient of 0 to 60% acetonitrile in 0.1% trifluoroacetic acid. The HPLC-purified 5-kD protein was applied to a gas-phase sequenator (ABI, model 470A), and its partial amino acid sequence was determined. Since ubiquitin contains seven lysine residues, treatment with lysylendopeptidase produces eight fragments of ubiquitin (U-1 to U-8) (Fig. 4).

acid sequence of the 5-kD protein was determined from the amino terminus to the 34th position (Fig. 3). By computer-aided homology search, this amino terminal sequence was found to be identical to that of ubiquitin. Hence, the 5-kD protein recognized by DF2 is ubiquitin, a 76-residue polypeptide (*6*).

We did not know whether ubiquitin itself is incorporated into PHF since it was possible that other proteins contain similar sequences reactive with DF2. Therefore, we have developed a method for direct identification of ubiquitin sequence in PHF. Although SDS-PHF are not readily solubilized even with harsh solvents and are highly resistant to various proteases (*7*), the initial treatment of PHF with concentrated formic acid allowed apparently complete digestion of PHF with lysylendopeptidase, a highly specific protease for Lys-X (*8*). PHF-enriched fractions from AD brains and identically prepared fractions from normal brains (*9*) were subjected to this treatment: released peptides from both fractions were separated by reversed-phase high-performance liquid chromatography (RPHPLC). Several peaks (Fig. 4A) were not seen in the control sample, which suggests that they contained PHF-derived peptides. Those peaks do not represent all of the peptides from PHF. Other peaks probably also contain PHF peptides. Purified ubiquitin was similarly treated and also separated by RPHPLC (Fig. 4B). PHF peptide peaks P-1 and P-2, which precisely coeluted with ubiquitin peaks U-6 and U-3, respectively, were subjected to further analysis on a second RPHPLC column. P-1 was resolved into two peaks, one of which (P-1a) contained the U-6 sequence by protein sequencing. P-2 gave a single peptide that was shown to be U-3. The partial amino acid sequences of P-1a and P-2 peptides were as follows: P-1a: Glu-Gly-Ile-Pro-Pro-Asp-Gln-Gln-Arg-Leu-Ile-Phe-Ala-Gly; P-2: Thr-Ile-Thr-Leu-Glu-Val-Glu-Pro-Ser. Thus, the presence of the two independent peptides derived from ubiquitin was unambiguously confirmed in the peptide mixtures derived from PHF, which indicates that ubiquitin is a component of PHF.

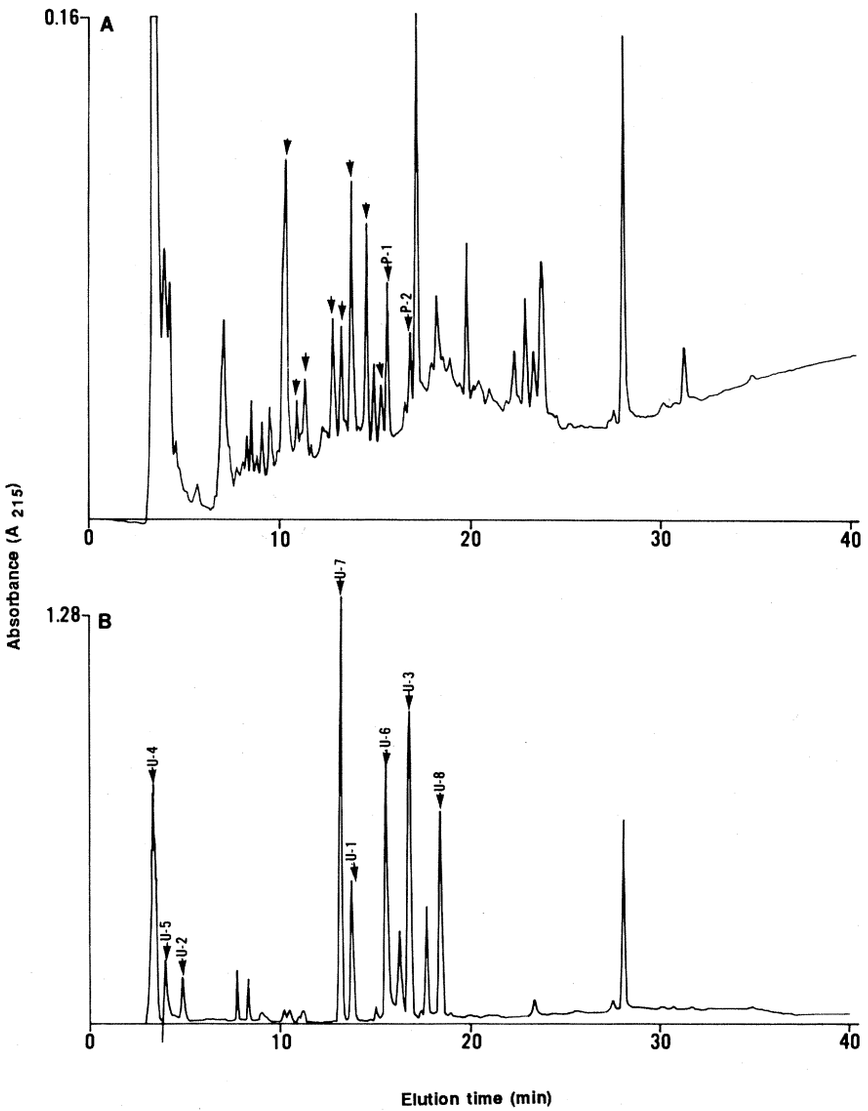

Fig. 4. HPLC peptide profiles. (**A**) SDS-PHF (0.3 mg) were dialyzed against 70% formic acid for 48 hours at 4°C, to remove bound SDS (this step is important to ensure reproducibility of RPHPLC). After incubation for 16 hours at 30°C in the presence of cyanogen bromide (CNBr) (0.5 mg), PHF were lyophilized and dissolved in 50 m*M* tris, *p*H 9.0, 5*M* urea (CNBr cleavage is not essential for the subsequent enzymatic digestion). The suspension was digested with purified lysylendopeptidase (1.6 μg). Peptides were then separated on Bakerbond Butyl (C4) with a linear gradient of 0 to 80% acetonitrile-isopropanol (3:7) in 0.1% trifluoroacetic acid. Peptides were detected by absorbance at 215 nm. Identically prepared fractions from control brains were subjected to the same treatment and separation by RPHPLC. Peaks that were not seen in control fractions are indicated by arrows. (**B**) An identical digest of purified ubiquitin was also separated by RPHPLC. Each major peptide was analyzed for amino acid composition and partial amino terminal sequence, and, on the basis of these data, the eight proteolytic fragments of ubiquitin were identified as U-1 to U-8. P-1 and P-2 were then further purified on Aquapore Octyl with a linear gradient of 0 to 40% acetonitrile in 0.1% trifluoroacetic acid. P-1 was resolved into two peaks (P-1a and P-1b), whereas P-2 gave only a single peak.

We do not know whether ubiquitin is a major component of the PHF. It is possible that it is only a minor component since DF2 binds to distinct sites on PHF with an apparent periodicity of 80 nm (*10*). However, we cannot exclude the possibility that some of the ubiquitin epitopes in PHF are not accessible to antibodies. On the other hand, a rough estimation of ubiquitin content in PHF based on the HPLC profiles (Fig. 4) suggests that ubiquitin is not a minor constituent of PHF. In this respect, further studies will be needed with PHF of higher purity.

Ubiquitin, a protein that has been highly conserved during the course of evolution (*11*), exists in cells in both a free form and a conjugated form. Ubiquitin is an element required for an adenosine triphosphate (ATP)–dependent proteolytic system that is probably responsible for a highly selective breakdown of abnormal or short-lived intracellular proteins (*12*). Ubiquitin activated by ATP is covalently linked to the target protein via an isopeptide bond between its carboxyl terminal glycine and ϵ-amino groups of lysine residues in the substrate. Once proteins are ubiquitinated, they are rapidly degraded by an ATP-dependent protease.

Immunochemical evidence favors the existence of a conjugated form of ubiquitin in PHF since affinity-purified antibodies specific for conjugated ubiquitin (*13*) specifically decorated NFT, as DF2 did, at the light microscopic level and SDS-PHF at the elec-

tron microscopic level (*10*). In contrast to DF2, the polyclonal antibodies to ubiquitin showed no significant background staining in tissue sections. It is possible that DF2 reacts with both free and conjugated forms of ubiquitin (*14*), whereas the affinity-purified antibodies preferentially recognize a conjugated form. Although the target or targets of ubiquitin in PHF are unknown, it may be that phosphorylated forms of tau that are undetectable in control brains (*5*) are linked to ubiquitin in PHF. Smearing on immunoblots could be attributed to ubiquitination (*15*) of altered forms of phosphorylated tau in PHF.

Our data suggest that PHF are not inert elements within degenerating neurons; tangle-bearing neurons are actively responding by ubiquitinating PHF proteins. The failure of degradation of PHF in spite of ubiquitination could be due to a defective ATP-dependent protease or to an unusual resistance of PHF to the protease (*7*).

Recently, the ubiquitin and ATP-dependent proteolysis has been highlighted in connection with the heat-shock response. The overloading of the proteolysis system by abnormal proteins seems to activate heat-shock genes (*16*). Thus, one may speculate that accumulated PHF would trigger the expression of heat-shock genes in tangle-bearing neurons. Recently, constitutive expression of heat-shock proteins was observed in indirect flight muscles of certain *Drosophila* mutants that produce abnormal forms of actin III (*17*). In view of these data, it is tempting to speculate that PHF-bearing neurons could express heat-shock proteins constitutively at least at certain stages of neuronal degeneration. If so, AD-specific neuronal proteins that are currently being intensively searched for in numerous laboratories might be found among heat-shock proteins.

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14. The epitope of DF2 was shown by means of the dot immunobinding method and ubiquitin fragments to reside in U-6. The two synthetic peptides:

$$\overset{32}{\text{Asp-Lys-Glu-Gly-Ile-Pro-Pro-}}\overset{39}{\text{Asp}}$$

and

$$\overset{45}{\text{Phe-Ala-Gly-Lys-Gln-Leu-Glu-Asp-}}\overset{53}{\text{Gly}}$$

did not react with DF2. Hence we speculate further that the epitope of DF2 would be

$$\overset{40}{\text{Gln-Gln-Arg-Leu-}}\overset{44}{\text{Ile}}\text{ in U-6}$$

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18. We thank A. L. Haas for providing affinity-purified antibodies to ubiquitin, D. J. Selkoe for helpful comments, and T. Seki for technical assistance. Supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan.

17 September 1986; accepted 16 January 1987

Regional Changes in Calcium Underlying Contraction of Single Smooth Muscle Cells

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The role of calcium in regulating the contractile state of smooth muscle has been investigated by measuring calcium and contraction in single smooth muscle cells with the calcium-sensitive dye fura-2 and the digital imaging microscope. The concentration of free calcium in the cytoplasm increased after stimulation of the cells by depolarization with high potassium or by application of carbachol. Changes in calcium always preceded contraction. The increase in calcium induced by these stimuli was limited to less than 1 μ*M*. Calcium within the nucleus was also subject to a limitation of its rise during contraction. Intranuclear calcium rose from 200 n*M* at rest to no more than 300 n*M* while cytoplasmic calcium rose to over 700 n*M*. These apparent ceilings for both cytoplasmic and intranuclear calcium may result either from negative feedback of calcium on cytoplasmic and nuclear calcium channel gating mechanisms, respectively, or from the presence of calcium pumps that are strongly activated at the calcium ceilings.

MEASUREMENTS OF INTRACELLULAR free calcium concentrations ($[Ca^{2+}]_i$) in smooth muscle have been performed in a number of ways in intact tissues (*1*) and in populations of isolated cells (*2, 3*). In general, $[Ca^{2+}]_i$ is correlated with the extent of contraction, although several exceptions have been reported (*4*). The exceptions may reflect Ca^{2+}-independent mechanisms that control contraction of smooth muscle or, alternatively, heterogeneity in the response of cells in a multicellular population that may produce an apparent dissociation of contractile state from Ca^{2+}. The development of a new class of fluorescent indicators (*5*) that produce a large Ca^{2+}-sensitive signal provides the opportunity to circumvent these uncertainties by permitting the measurement of Ca^{2+} and contraction in the same cell. Moreover, the use of the dye fura-2 in conjunction with the digital imaging microscope allows measurement of $[Ca^{2+}]_i$ with subcellular spatial resolution (*6*). Here we use this approach to assess the magnitude and kinetics of changes in Ca^{2+} that take place in different regions of single smooth muscle cells as they contract and then relax after exposure to excitatory stimuli (*7*).

The smooth muscle cells used in these studies were isolated by enzymatic disaggregation of the stomach muscularis of the toad (*Bufo marinus*) (*8*). $[Ca^{2+}]_i$ was monitored in cells loaded with fura-2 as a change in the

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