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11. The ability to grow at restrictive temperature is not due to recombination or gene conversion. Using allele-specific polymerase chain reaction primers, we could routinely detect both alleles in the diploids.
12. Exponential growth rates were measured in rich (YEP-glucose) medium at the restrictive temperature (37°C). Many heteroallelic diploid strains including *cmd1-226/cmd1-228, cmd1-226/cmd1-239, cmd1-226/CMD1, cmd1-228/cmd1-239, cmd1-228/CMD1*, and *cmd1-239/CMD1* have growth rates (1.9 to 2.0 hour doubling times) the same as those of the wild-type diploid control (1.9 hours). The other heteroallelic strains have slightly slower growth rates: *cmd1-226/cmd1-233* (2.4 hours), *cmd1-228/cmd1-233* (2.4 hours), *cmd1-239/cmd1-233* (2.3 hours), and *cmd1-233/CMD1* (2.2 hours).
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Agmatine: An Endogenous Clonidine-Displacing Substance in the Brain

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Clonidine, an antihypertensive drug, binds to α_2-adrenergic and imidazoline receptors. The endogenous ligand for imidazoline receptors may be a clonidine-displacing substance, a small molecule isolated from bovine brain. This clonidine-displacing substance was purified and determined by mass spectroscopy to be agmatine (decarboxylated arginine), heretofore not detected in brain. Agmatine binds to α_2-adrenergic and imidazoline receptors and stimulates release of catecholamines from adrenal chromaffin cells. Its biosynthetic enzyme, arginine decarboxylase, is present in brain. Agmatine, locally synthesized, is an endogenous agonist at imidazoline receptors, a noncatecholamine ligand at α_2-adrenergic receptors and may act as a neurotransmitter.

In 1984, Atlas and Burstein (*1*) partially purified a substance from calf brain that displaced binding of clonidine, an antihypertensive drug, to α_2-adrenergic receptors of rat brain. This clonidine-displacing substance (CDS) was not a catecholamine nor a peptide and had an estimated mass of 520 daltons. It was subsequently discovered that CDS, like clonidine, would also bind to a nonadrenergic binding site, the imidazoline receptor (I receptor) (*2–4*), which is believed to mediate the hypotensive actions of clonidine and allied agents (*5*). I receptors exist in two major subclasses, I_1 and I_2 (*6*), which differ from α_2-adrenergic receptors and each other with respect to selectivity for ligands, structure, and regional, cellular, and subcellular localizations (*7, 8*). The endogenous ligand of I receptors is not known. However, the fact that CDS binds to all I receptors (*3, 8*) and is bioactive (*9, 10*) suggested that CDS may fill this role, and we sought to establish its structure.

We partially purified CDS from bovine brain using displacement of [^{3}H]*p*-aminoclonidine ([^{3}H]PAC) from rat cerebral cortical membranes to track its activity (*8, 11*). Fresh bovine brains were homogenized in chilled water and centrifuged and the supernatant was precipitated in 70% ethanol. After condensation on a rotatory evaporator and centrifugation, the supernatant was passed through a Dowex 50 (H^+) column eluting with 3 N hydrochloric acid. This step was followed by C_{18} reversed-phase high-performance liquid chromatography (HPLC) eluting with acetic acid (25 mM). Further purification (*12*) was achieved with size-exclusion HPLC with a Bio-Rad SEC 125 column from which CDS was eluted in a fraction that indicated that its molecular mass was less than 300 daltons. CDS tested positive with a ninhydrin spray reagent on thin-layer chromatography. At this stage, CDS contained no ultraviolet chromophore over 200 nm, was a highly polar low molecular weight compound containing an amine functionality, and was contained in a mixture of compounds.

Further attempts to purify CDS directly were unsuccessful, and we therefore isolated CDS in its derivatized form (*13*). Derivatization of the enriched active fraction with 9-fluorenylmethyl chloroformate (FMOC-Cl) generated a mixture of compounds stable under most purification conditions. Although binding was lost on derivatization, it was regained after base hydrolysis to release the free amine. Sequential C_{18}-column chromatography of the derivatized mixture, which monitored the biological activity enrichment by release of free amine from a subsample of each fraction, yielded a single peak on C_{18} HPLC that coeluted with authentic FMOC-agmatine. Subjecting the natural purified FMOC-CDS derivative to electrospray mass spectroscopy–mass spectroscopy (MSMS) analysis gave results identical to those obtained for authentic FMOC-agmatine, which confirms that agmatine is the CDS compound isolated (Fig. 1). We quantitated the amount of agmatine in weighed amounts of bovine brain by derivatization to FMOC-Cl of a fraction (based on 200 U of CDS activity). The quantity of FMOC-agmatine present was determined from a standard curve generated by plotting ultraviolet intensity as a function of FMOC-agmatine concentration. In this way, the amount of agmatine in bovine brain was found to be 1.5 to 3.0 nmol per gram of tissue (0.2 to 0.4 μg/g),

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which indicates that agmatine is responsible for most of the CDS activity in the purified sample. Although the molecular mass of crude CDS has been estimated from gel filtration and dialysis to be approximately 500 daltons (*1*), the molecular mass of agmatine is 130 daltons. The apparently larger molecular weight of crude CDS is probably a result of agmatine that has aggregated because of its strong polarity.

We compared the binding of agmatine and CDS to α_2-adrenergic and I receptors of the I_1 and I_2 subclasses by standard ligand-binding assays (*11*). We determined binding to α_2-adrenergic receptors by measuring displacement of [^{3}H]PAC from membranes of bovine frontal cortex (*2*); binding to I_1 imidazoline sites was determined by competition with [^{3}H]PAC, with the use of membranes of bovine ventrolateral medulla (VLM) in the presence of 10 μM epinephrine (to specifically block α_2-adrenergic sites) (*2*); and interactions with I_2 receptors were determined by binding of [^{3}H]idazoxan to membranes of bovine adrenal chromaffin cells (*8*).

In cerebral cortex, agmatine, like CDS, displaced [^{3}H]PAC from α_2-adrenergic receptors (Fig. 2). Both curves were sigmoidal, with a Hill slope close to unity. The inhibitory constant (IC_{50}) of CDS in this tissue (4 μM) by definition (*14*) represents 1 U of CDS activity. At the I_1 receptor, the displacement curves for [^{3}H]PAC produced by agmatine and CDS were also both monophasic (Hill slope = 1), with IC_{50} values of 0.7 μM and 0.6 U, respectively. These values are lower than that obtained for the α_2-adrenergic binding sites, which indicates a higher affinity at the I_1 receptor (*3*). The structures of agmatine, clonidine, and idazoxan are shown in Fig. 3.

At the I_2 binding sites in adrenal chromaffin cell membranes, the IC_{50} values for agmatine and CDS were 1 μM and 0.8 U, respectively, and the inhibition curves were shallow, with a Hill slope of 0.5 (Fig. 2). Comparable shallow inhibition curves have been reported for some drugs that displace [^{3}H]idazoxan to I_2 sites in chromaffin cells and other tissues (*15*), probably reflecting the fact that I_2 binding sites exist in interactive or interconvertible forms (*15*).

Bovine adrenal chromaffin cells were incubated with varying concentrations of agmatine, and we measured the release of norepinephrine and epinephrine by standard procedures (*10*). Like CDS (*10*) (Fig. 4), agmatine elicited a concentration-dependent release of both norepinephrine and epinephrine with a median effective concentration of 5 μM (Fig. 4). Because chromaffin cells express I receptors but not α_2-adrenergic receptors (*8*), these results indicate that agmatine is an agonist at this receptor and is bioactive.

We investigated whether rat brain expresses arginine decarboxylase, the enzyme synthesizing agmatine in bacteria, plants, some parasites, and marine invertebrates (*16–19*). Homogenates of whole rat brains were freshly prepared and separated into soluble and membrane fractions by centrifugation at 30,000*g* for 30 min (*20*). Enzyme

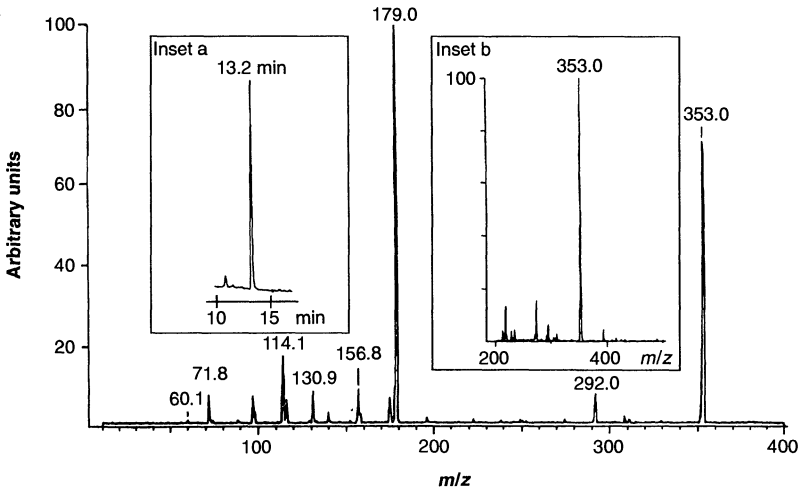

Fig. 1. Purification of CDS. (**Inset a**) The HPLC profile of purified FMOC-CDS with a C_{18} column (250 mm by 10 mm) (YMC) with a 65/35 to 20/80 dilution of the trifluoroacetic acid (0.1%) acetonitrile gradient over 20 min. The retention time was identical to that obtained for authentic FMOC-agmatine. (**Inset b**) The MS profile of FMOC-CDS with an MH^+ ion of 353 daltons. (**Main figure**) The parent ion from inset b at 353 daltons was passed through a second mass spectrometer, giving the fragmentation pattern shown. The mass spectral results were identical to those obtained for authentic FMOC-agmatine. MS and MSMS were performed with electrospray ionization with a Finnigan MAT TSQ 700 mass spectrometer; *m/z*, mass-to-charge ratio.

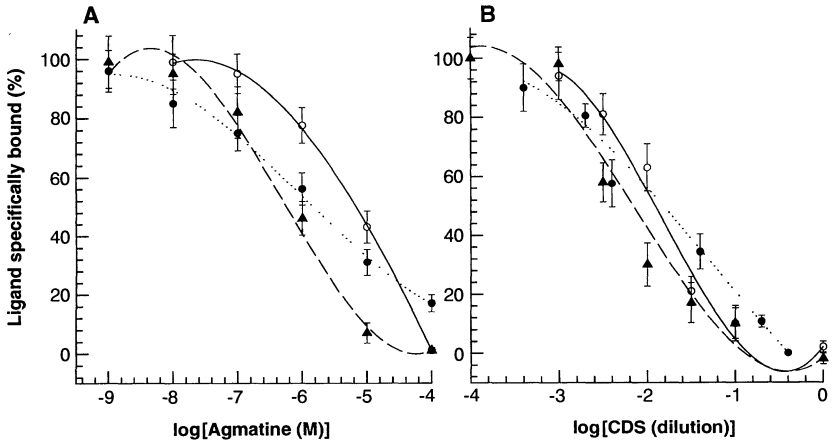

Fig. 2. Effect of agmatine (**A**) (Sigma) and purified CDS (**B**) on α_2-adrenergic and imidazoline receptor binding (*11*). Binding to α_2-adrenergic receptors was measured by the displacement of [^{3}H]PAC from membranes prepared from rat cerebral cortex (○); the binding to I_1 imidazoline receptors was measured by competition with [^{3}H]PAC to membranes of bovine VLM in the presence of 10 mM epinephrine to specifically block α_2-adrenergic sites (▲); and the interaction with I_2 receptors was measured by analysis of competition of [^{3}H]idazoxan binding to membranes of bovine adrenal chromaffin cells (●). Values are the mean ± SEM of three experiments done in triplicate.

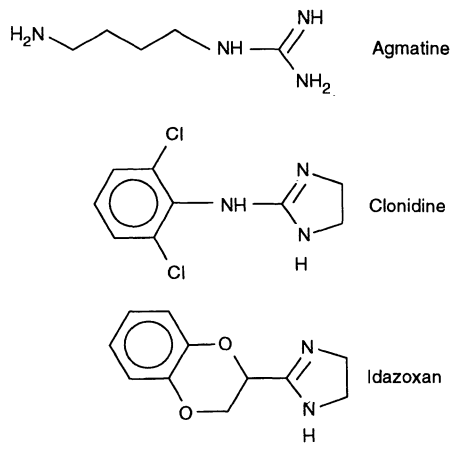

Fig. 3. Structures of agmatine, clonidine, and idazoxan.

activity was assayed by the method of Wu and Morris (*19*), which measures conversion of L-[1-^{14}C]arginine to $^{14}CO_2$.

Arginine decarboxylase activity was present in rat brain (Table 1). The amount of enzyme, which was inhibited by its end product agmatine (*16*), was increased threefold in the membrane fraction, whereas its activity in the cytoplasmic fraction did not differ from that of a boiled blank. The localization to membranes was similar to the distribution of the enzyme in bacterial cell walls and of an atypical ornithine decarboxylase localized to cell membranes in *Caenorhabditis elegans* (*18*). Our results demonstrate that agmatine and arginine decarboxylase, its biosynthetic enzyme, are present in the mammalian brain. This presence of arginine decarboxylase, the enzyme involved in the rate-limiting step in agmatine biosynthesis in plants and bacteria (*16–19*), suggests that the source of the amine is local synthesis, not food or enteric bacteria.

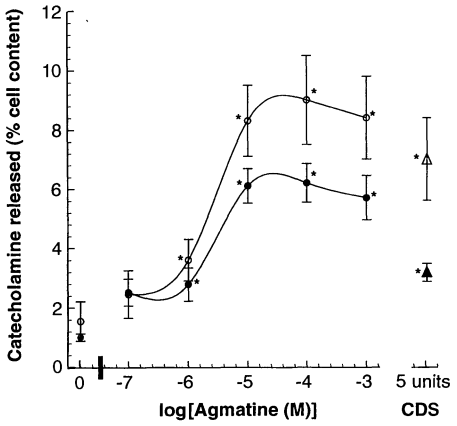

Fig. 4. Effect of agmatine on catecholamine release from bovine adrenal chromaffin cells. Cells (1 × 10^6) were incubated with agmatine or CDS for 10 min at 37°C in Krebs-Ringer buffer, and the amount of epinephrine (● or ▲) and norepinephrine (○ or △) released into the buffer and in the cells was measured by HPLC. Results are expressed as percent of cell content released. Values are from two experiments done in triplicate. Asterisks indicate $P < 0.01$ compared to the control.

Table 1. Arginine decarboxylase activity in rat brain. The activity was measured in 450 μl of 25% whole brain homogenate, soluble fraction, or membranes with 0.4 μCi of L-[1-^{14}C]arginine. Nonenzymatic decarboxylation (no enzyme controls) was subtracted from the total activity. Values are the mean ± SEM of three experiments.

Brain fraction	$^{14}CO_2$ released (pmol hour^{-1} g^{-1})
Brain whole homogenate	720 ± 20
Homogenate + agmatine (1 mM)	25 ± 10
Membrane fraction	1,810 ± 95
Soluble fraction	170 ± 58
Membrane fraction after boiling for 3 min	40 ± 12
Escherichia coli enzyme (0.125 U)	10,247 ± 145

Agmatine also shares properties of CDS. Both agents bind to α_2-adrenergic and I receptors with comparable kinetics and release catecholamines on a dose-dependent basis from adrenal chromaffin cells. These observations are consistent with agmatine being an endogenous ligand for I receptors. It may be possible that agmatine, like other bioactive amines, is a neurotransmitter or neuromodulator. Consistent with this possibility are the facts that (i) in whole brain the concentration of agmatine (0.2 to 0.4 μg/g) is comparable to that of norepinephrine (0.5 μg/g) or dopamine (0.5 μg/g) (*21*); (ii) its affinity for α_2-adrenergic (4 μM) and I_1 and I_2 receptors (0.7 to 1.0 μM) is in a comparable range to that of other neurotransmitters [for example, norepinephrine has an IC_{50} of 3 μM for α_1- and 0.8 μM for α_2-adrenergic receptors in rat brain (*22*)]; and (iii) like other transmitters, CDS is unequally distributed in brain (*23*) and is concentrated in synaptosomes (*10, 23*). Until now, agmatine has been considered to be only a precursor of putrescine and thereby of other polyamines (*16*). However, our findings raise the possibility that agmatine may be a biologically active molecule in its own right. In support of this are older observations that agmatine can block the nicotinic cation channel in sympathetic ganglion and adrenal chromaffin cells (*24*), can stimulate release of insulin and increase uptake of ^{45}Ca in pancreatic islet cells (*25*), and is a potent antilipolytic agent in rat adipocytes (*26*). Whether agmatine is also a precursor of polyamines in brain remains to be established.

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11. Using [^{3}H]PAC as a ligand for each, we prepared membranes from bovine frontal cortex, for measurement of α_2-adrenergic receptors (*2*), and from VLM, for detection of I_1 binding sites (*2*); using [^{3}H]idazoxan (*8*), we prepared adrenal chromaffin cells for detection of I_2 receptors. Ligand-binding assays were carried out as described (*2, 8*).
12. Four fresh bovine brains were obtained on ice from a local slaughterhouse and cleaned of dura, blood vessels, and cerebellum. The remaining tissue (about 1500 g) was minced and homogenized in 3 volumes (w/v) of ice-cold distilled water by a Polytron (Brinkmann; setting 8 for 25 s). The homogenate was centrifuged at 5000*g* for 35 min at 4°C. The supernatant was brought to a concentration of 70% ethanol by the addition of dry ice-cold ethanol while stirring. After 30 min, the material was recentrifuged at 2000*g* for 10 min. The alcoholic-supernatant was conducted on a rotatory evaporator at 35°C until the volume was reduced to about 400 ml of aqueous solution. This solution was centrifuged in an ultracentrifuge at 50,000*g* for 45 min. The supernatant was applied to a Dowex (H^+) 50X8-400 column (Sigma), washed with 50 mM HCl, and eluted with 3 N HCl. Active fractions were dried under vacuum, dissolved in water, and subjected to a series of HPLC steps. The first was the use of an Econosil C_{18} reversed-phase column (22.5 mm, inner diameter; 250 mm, length) (Alltech), in which 2 ml of sample was injected each time and eluted isocratically with 25 mM acetic acid. The active fractions were combined, dried, and rechromatographed on the same column, but with 5 mM HCl as the eluent. The next was a phenyl column (10 mm, inner diameter) from which the active fractions were eluted with 5 mM HCl. The active fractions were dried and run again on a Microsorb phenyl column (10 mm, inner diameter; 250 mm, length) (Rainin). The final purification step was by size-exclusion HPLC (Bio-Rad SEC 125 column) with elution by 10% ethanol.
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20. We used rat brains for detection of arginine decarboxylase because the enzyme activity of bovine brains rapidly declines after death. Whole rat brains were homogenized in 3 volumes (w/v) of ice-cold water and 0.5 ml of the whole homogenate was used for the assay. The homogenate was centrifuged at 30,000*g* for 30 min, and the supernatant and pellet were separated. The membrane pellet was resuspended in water to the same volume as the supernatant, and both fractions were assayed for enzyme activity. The assay mixture consists of 5 mM tris-HCl buffer (pH 8.7), 0.8 mM $MgSO_4$, 0.05 mM pyridoxal phosphate, 0.1 mM L-arginine, and 0.4 μCi of L-[1-^{14}C]arginine (specific activity, 50 mCi/mmol). The reaction was carried out in glass tubes with the center well inserted into tightly closed rubber stop-

pers. The center wells have filter paper strips moistened with methylbenzethonium hydroxide to trap $^{14}CO_2$. Homogenate was added to start the incubation for 1 hour at 37°C, and the reaction was terminated by the addition of 40% trichloroacetic acid. After further incubation for 20 min at 37°C, the filter paper strips were transferred to scintillation vials and the radioactivity determined by liquid scintillation counting. Commercially available *Escherichia coli* enzyme (Sigma) was used to verify the assay conditions.

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Reduction in Viscosity of Cystic Fibrosis Sputum in Vitro by Gelsolin

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Obstruction of airways by viscous sputum causes lung damage in patients with cystic fibrosis (CF). Sputum samples from CF patients were shown to contain filamentous actin. Human plasma gelsolin, a protein that severs actin filaments, rapidly decreased the viscosity of CF sputum samples in vitro. Gc globulin and deoxyribonuclease I, proteins that sequester monomeric actin but do not sever actin filaments, were less efficient than gelsolin in diminishing sputum viscosity. These results suggest that gelsolin may have therapeutic potential as a mucolytic agent in CF patients.

Patients with CF accumulate thick secretions in their airways that cause progressive pulmonary destruction (*1*). CF sputum is a complex material, but a major cause of its thick consistency is pus, derived from masses of degenerating leukocytes (*2*). The ability of bovine pancreatic deoxyribonuclease I (DNase I) and, more recently, human recombinant DNase I to liquify CF sputum has been ascribed to enzymatic degradation of leukocyte-derived DNA (*3*). We considered the possibility that filamentous actin might also contribute to the thickness of CF sputum. Actin comprises 10% of total leukocyte protein (*4*) and forms long protease-resistant (*5*) filaments that are highly viscoelastic (*6*). Furthermore, DNase I is known to bind monomeric actin and to slowly depolymerize actin filaments (*7*), which suggests that some of its reported mucolytic effect might be due to actin disaggregation rather than to DNA hydrolysis.

We documented the presence of actin in CF sputum (*8*) by three independent methods. First, we did an immunoblot analysis of denatured CF sputum and identified a polypeptide that was similar in size to actin and that reacted with an actin-specific antibody (Fig. 1, inset) (*9*). Second, we showed that CF sputum eliminated the typical time delay in actin polymerization that has been attributed to cation-induced nucleation of monomeric actin (Fig. 1) (*10*). Nucleation of actin polymerization is a property of the ends of preformed actin filaments, and the increased polymerization rate may be due to the introduction of filaments from the sputum (*10*). Third, we showed that phalloidin, a fungal peptide that specifically ligates filamentous actin, bound to CF sputum.

Analysis of 10 CF sputum samples revealed that the concentration of polymeric actin (*11*) varied widely among samples (ranging from 0.1 to 5 mg/ml) and did not appear to correlate with sample viscosity (Fig. 1B). This is perhaps not surprising, however, as the viscosity of filamentous actin, like that of other linear polymers, depends on the shear rate, the polymer concentration, the orientation of the filaments, and, most importantly, the average filament length. The viscosity of purified filamentous actin, for example, varies with the fifth power of the average filament length (*6*). We reasoned that if filamentous actin contributes substantially to the viscosity of CF sputum, then reducing actin filament length should markedly diminish sputum viscosity.

To investigate the effect of actin filament length on viscosity, we added gelsolin, a protein that rapidly severs noncovalent bonds between monomers within a filament (*12*), to CF sputum samples and then monitored their rheological properties. Prior to gelsolin treatment, the viscosity of 32 CF sputum samples was 322 ± 199 Pa-s (mean ± SD) (*13*). At concentrations of 100 to 500 nM, gelsolin, purified from human plasma (*14*), rapidly diminished the viscosity of these sputum samples by an average of 62 ± 44% (mean ± SD), irrespective of the initial viscosity of the untreated sputum. By contrast, at a concentration of 250 nM, bovine pancreatic DNase I and another actin monomer–binding protein, Gc globulin (a vita-

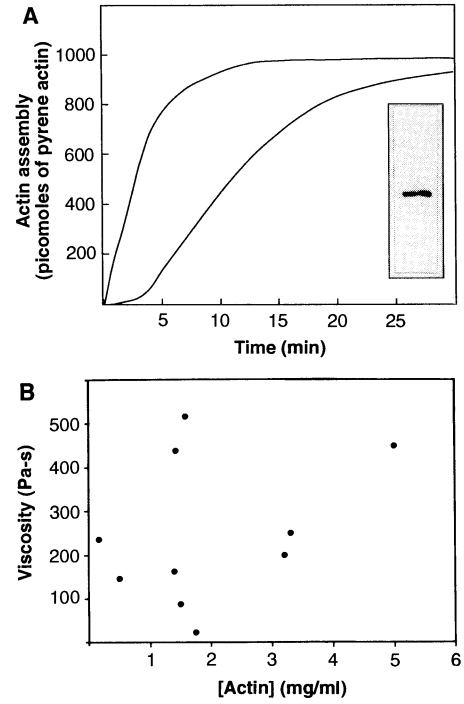

Fig. 1. (**A**) Identification of actin in CF sputum. The graph shows the increase in fluorescence of pyrene actin as the actin polymerized in the absence (lower curve) or presence (upper curve) of extracts of CF sputum. The inset shows an immunoblot of proteins detected in the sputum of two different CF patients by an antibody to actin. (**B**) Analysis of actin concentration and viscosity of 10 CF sputum samples. The concentration of polymeric actin was estimated by adding rhodamine-labeled phalloidin to the sputum samples and measuring specifically bound fluorescence (*11*). The viscosity of the samples was derived from their flow rate in response to a constant stress (*13*).

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