Constitutive expression of calpain II in the rat uterus during pregnancy and involution

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Partial genomic clones for the 80 kDa subunits of rat calpains I and II have been isolated. Some exons have been located and sequenced, and used to synthesize RNA probes specific for each isoenzyme. The levels of total DNA, soluble protein, calpain II 80 kDa subunit and the mRNA for this subunit were measured in parallel in separate extracts of non-pregnant, pregnant and *post-partum* rat uteri. The amount of total DNA, expressed as mg/g wet wt. of tissue, was found to remain constant throughout this period, except for a slight rise during involution. Calpain I was present in all samples in very small amounts. The amounts of calpain II 80 kDa subunit (measured on immunoblots) and of its mRNA (measured by means of slot-blots) also did not vary, when expressed in terms of units per g wet wt., during the 10-fold growth of the uterus during pregnancy and its *post-partum* involution. It was concluded that expression of calpain II was constitutive in this normal tissue, which is undergoing rapid growth and involution under complex hormonal control.

INTRODUCTION

The calpains are cytoplasmic Ca^{2+} -dependent thiol proteinases, present in essentially all tissues of all higher animals. Their structures and enzymology have by now been fairly well documented [1–4], but their functions are not yet entirely clear, in spite of many suggestions. There are two forms of the enzyme, calpains I and II, whose Ca^{2+} requirements not only differ from each other, but also seem unphysiologically high when measured for the purified enzymes *in vitro*. However, both enzymes become active *in vivo*, at physiological levels of Ca^{2+} , apparently as a result of Ca^{2+} -induced membrane binding and proteolytic auto-activation [3]. A specific calpain inhibitor, calpastatin, is also present in all cells, and further complicates the question of how calpain activity is regulated [4]. Both calpains consist of a large subunit, of approx. 80 kDa in size, which differs slightly between the two forms, and a small subunit, of approx. 30 kDa, which is identical in calpains I and II. The cDNA sequences of various chicken, human, pig and rabbit calpain large and small subunits have been described [5–9], and related to the corresponding proteins. In addition, a third large subunit cDNA sequence has been detected, but the corresponding protein has not yet been isolated [10,11].

A very wide range of proteins have been shown to act as potential or apparent substrates for calpains [1–4], such as cytoskeletal proteins in platelets and neural tissue, protein kinase C, calmodulin-binding proteins, and the *c-mos* oncogene product [12], among many others. It is, however, very difficult to establish which substrates are physiologically valid, and it is equally difficult to understand, and to design experiments to elucidate, how the calpains can direct their activity to certain substrates at specific times in specific tissues, in response to a rise in Ca^{2+} concentration which is the common result of many different stimuli applied to cells.

The half-life of auto-activated calpain following a rise in the intracellular Ca^{2+} concentration appears to be very short, and the activated calpain must be replaced. It would be of interest, therefore, to know how the synthesis of calpain is regulated. We

have previously made some measurements of calpain in the pregnant rat uterus by means of a sandwich e.l.i.s.a., with results indicating a disproportionate increase in calpain activity during pregnancy [13]. These results led us to investigate the regulation of calpain synthesis during pregnancy, measuring calpain protein by immunochemical means and mRNA by means of nucleic acid probes that we have recently isolated for the calpains. The rat uterus undergoes a rapid and roughly 10-fold increase in wet weight during pregnancy, in a period of 21 days, and an even more rapid involution within 5–6 days *post partum* [14]. It therefore provides a useful model of complex hormonal regulation of growth, without any need for possibly unphysiological intervention.

In the present work, the levels of total uterine DNA, calpain II 80 kDa subunit protein and the mRNA for this subunit were measured in parallel in separate extracts of each uterus taken from a series of rats throughout pregnancy and involution, and the measurements were expressed relative to the wet weight of the tissue. The values for immunoreactive calpain did not show the significant increase that we had reported previously, for reasons that are discussed later, and we present evidence to show that the large subunit of calpain II was expressed constitutively in the rat uterus during this period.

MATERIALS AND METHODS

Materials

A monoclonal antibody to human calpain I, and a polyclonal antibody to rat calpain II, both of which are specific for the 80 kDa subunits of their respective calpains, have been described previously [15].

Restriction digestions, agarose gels and gel blotting, and sub-cloning into M13 and pGEM3Z (Promega Biochemicals, Madison, WI, U.S.A.) vectors were carried out by standard procedures [16–18]. Sequencing of M13 single-stranded DNA and of pGEM3Z double-stranded DNA following denaturation was performed by the dideoxy method with [^{32}P]dATP on 7 M-

Abbreviations used: DEPC, diethyl pyrocarbonate; SSC, 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0.

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The nucleotide sequence data reported here will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X51772 and X51773.

urea/6% and 8% polyacrylamide gels, using Sequenase (U.S. Biochemicals, Cleveland, OH, U.S.A.) and the protocols supplied by the manufacturer. Sequencing primers were supplied by Dr. K. Deugau, Queen's University Oligonucleotide Synthesis Service.

Nucleic acid probes for rat calpain I and II mRNA

Rabbit calpain cDNA probes were very kindly supplied by Dr. K. Suzuki as pBR322 plasmids pLU 1001 and pLM 1006 [5]. These contained DNA sequences specifying the C-terminal portions and 3'-untranslated regions of mRNA for the large subunits of rabbit calpains I and II respectively. The 1.2 kb *Pst*I/*Hind*III fragment from pLM 1006, containing 87% coding sequence and 13% 3'-untranslated sequence, was prepared for use as a hybridization probe by elution from an agarose gel and labelled by nick-translation.

A rat genomic library was prepared by partial digestion of rat DNA with *Mbo*I, followed by size selection of 15–20 kb fragments by sucrose-density centrifugation, and ligation into *Bam*HI-digested arms of the bacteriophage λ EMBL3 [19]. The recombinant phage were plated on *Escherichia coli* K802 cells at a density of approx. 2.5×10^4 plaque-forming units (p.f.u.)/15 cm LB agar plate, and the phage DNA was transferred to 0.45 μ m nitrocellulose discs (Schleicher and Schuell, Keene, NH, U.S.A.). The filters were then placed sequentially for 5 min each on Whatman 3MM paper saturated with 1.5 M-NaCl/0.5 M-NaOH, 1.5 M-NaCl/0.5 M-Tris-HCl (pH 8.0), and $6 \times$ SSC, pH 7.0, and then air-dried and baked under vacuum for 2 h at 80 °C. Prehybridization and hybridization with the rabbit calpain II probe were performed for 16 h at 55 °C in 1 M-NaCl/1% (w/v) SDS/10% (w/v) dextran sulphate/heat-denatured herring testis DNA (100 μ g/ml). After hybridization, the filters were washed twice for 5 min each in $2 \times$ SSC at 20 °C and once for 45 min in $0.1 \times$ SSC/0.1% SDS at 55 °C, and autoradiographed. Positive plaques were picked, re-plated and re-screened until plaque-pure, and then large-scale phage preparations were made by liquid culture, with two final CsCl step gradient centrifugations. It is shown below that the EMBL3 clone 10-1, which hybridized strongly to the rabbit calpain II cDNA probe, is derived from the gene for the rat calpain II large subunit, and that clone 7-1, which gave a significantly weaker signal under identical conditions, derives from the gene for rat calpain I.

RNA probes were prepared [16] spanning exons 12 and 13 of rat calpain I from EMBL3 clone 7-1, and spanning exon 10 of rat calpain II from clone 10-1, by amplification with the polymerase chain reaction [20] and blunt-end cloning of the amplified material into the *Hinc*II site of pGEM3Z. The products were double-strand sequenced in both directions to establish the orientation and sequence of the inserts. After appropriate linearization, sense and anti-sense [32 P]RNA was generated by *in vitro* transcription, using T7 and SP6 polymerases.

Probes for 7SL RNA and actin mRNA

A pUC8 plasmid containing 125 bp of unique sequence from human 7SL cDNA was kindly supplied by Dr. E. Ullu [21], and an actin probe was already available [22]. The plasmids were grown up and purified by standard methods, and labelled by nick-translation.

Tissue preparation

Non-pregnant and timed-pregnant rats (Sprague-Dawley; Canadian Breeding Farms and Laboratories, Charles River, Montreal, Quebec, Canada) were killed by decapitation, and quickly drained of blood. As rapidly as possible, the uteri were removed, pinned to a cork board, lightly blotted, freed of fat and

other tissue, cut open lengthwise, and the products of conception were removed. The uterine tissue was not otherwise cut or scraped, so that the endometrial layer was included in subsequent extracts, in contrast with our earlier practice [14]. The wet weight of the uterus was recorded, and samples were taken for the various assays. For most uteri, sufficient tissue was available to take 2×0.5 g for mRNA extraction, 2×0.1 g for estimation of total DNA and 1×0.5 g for calpain immunoblotting. For non-pregnant uteri and those in late involution, the tissue samples were necessarily smaller.

Isolation of mRNA

Total rat uterine RNA was extracted by homogenization of the uterine sample (0.5 g) in 10 vol. of acid guanidinium thiocyanate/phenol/chloroform (5:5:1, by vol.) [23] at room temperature with a Brinkman Polytron for 45 s at two-thirds of maximum speed, with the addition of 50 μ l of Antifoam A emulsion (Sigma Chemical Co., St. Louis, MO, U.S.A.), in a 15 ml glass tube. This homogenate was then processed as described [23] with two propan-2-ol precipitations of the RNA. The final pellet was dissolved in 0.5% SDS in diethyl pyrocarbonate (DEPC)-treated water, and the dissolved RNA was measured by its absorption at 260 nm and 280 nm. The quality of the extracted RNA was checked by inspection of the 18 S and 28 S ribosomal RNA bands on an ethidium bromide-stained agarose gel.

For slot-blotting [18], RNA samples were diluted in a denaturing solution of 4 vol. 37% formaldehyde/6 vol. of $20 \times$ SSC, and heated at 65 °C for 15 min. The blots were prepared in pairs with identical arrays of RNA samples, and then exposed to different probes. Samples of 100 μ l estimated to contain 3 μ g (for 7SL RNA assays) or 20 μ g (for calpain mRNA assays) of total RNA were added to duplicate wells of the slot-blot apparatus fitted with a Bio Trace RP nylon membrane (Gelman Sciences, Montreal, Quebec, Canada), with no or minimal suction, followed by washing with 2×100 μ l of $10 \times$ SSC. The blots were air-dried and baked for 1 h under vacuum at 80 °C. Prehybridization and hybridization for 16–20 h were performed in 50% (v/v) formamide/0.2% (w/v) polyvinylpyrrolidone/0.2% (w/v) Ficoll 400/5 $\times$ SSC/0.5% SDS/50 mM-sodium phosphate (pH 6.5)/heat-denatured herring testis DNA (100 μ g/ml) at 45 °C for the 7SL cDNA probe and at 65 °C for the calpain II RNA probe. After hybridization, the blots were washed for 2×30 min in $0.1 \times$ SSC/0.1% SDS at 45 °C or 65 °C as above. The blots were autoradiographed for approx. 4 h for the 7SL RNA and overnight for the calpain mRNA, and the autoradiograms were quantified on a Hoefer Model GS-300 densitometer with an integration program. The duplicate integrated densitometer values were averaged, and the results were expressed as ratios of the results with different probes on the two identical blots. The observed densitometer values were linearly proportional to the amount of RNA applied to the blots, within the range used.

Measurement of total DNA

Uterine tissue (0.1 g) was homogenized with a Virtis Tissuemizer at room temperature in 4 ml of 1% SDS/10 mM-Tris/HCl/5 mM-EDTA (pH 7.5) containing 100 μ g of proteinase K/ml (Boehringer-Mannheim, Dorval, Quebec, Canada) for 3×30 s at three-quarters of maximum speed, and the homogenates were incubated at 42 °C overnight [24]. No further extraction or precipitation of the DNA was found to be necessary. The samples were stored at 20 °C. DNA in 20 μ l portions of these extracts was measured by means of the fluorescence enhancement at 458 nm of Hoechst dye H33258 (1 μ g/ml in 2 ml of 2 M-

NaCl/50 mM sodium phosphate, pH 7.0), using a standard curve of calf thymus DNA [25]. As controls, it was shown that serial dilution of uterine extracts gave consistently proportional values, and that addition of standard DNA to the extracts gave the expected additive values. Although SDS has been reported to interfere with this assay [26], the presence in our study of SDS at a final concentration of 0.01% (w/v) did not interfere, given one modification. It was found that a small amount of precipitate was formed on addition of the SDS-containing tissue extracts to the dye solution; the mixtures were therefore left at 4 °C overnight and centrifuged at 5000 *g* for 20 min before taking readings.

Extraction of calpain

Uterine tissue (0.5 g, or 0.1 g for smaller uteri) was homogenized at 4 °C with a Brinkman Polytron for 2 × 30 s at two-thirds of maximum speed in 2 ml of 5 mM-EDTA/10 mM-2-mercaptoethanol/20 mM-Tris/HCl, pH 7.8, containing 50 µg of phenylmethanesulphonyl fluoride/ml. The crude homogenates were centrifuged at 13000 *g* for 15 min in an Eppendorf microcentrifuge at 4 °C; portions of the supernatants were mixed with one-quarter volume of 5 × concentrated SDS gel sample buffer [27] and heated at 95 °C for 10 min. These samples were stored at 4 °C.

Measurement of extracted protein

The protein content of the tissue extracts in SDS gel sample buffer was measured by the turbidity at 400 nm of the mixture resulting from addition of 20 µl portions of the extracts to 1 ml of water, followed by addition of 0.5 ml of 50% trichloroacetic acid [28]. A standard curve was constructed with BSA dissolved in SDS gel sample buffer. This procedure was used because the high concentrations of SDS and 2-mercaptoethanol in gel sample buffer precluded the use of the Lowry or Bradford protein methods.

Immunoblot quantification of calpain protein

Sets of four 14 cm × 12 cm SDS/10% acrylamide gels were run simultaneously at 35 V overnight with up to 40 uterine samples, with equal loading of 50–60 µg of total protein per well. The upper halves of the gels, containing the 80 kDa subunit, were electroblotted in pairs on to an Immobilon P membrane (Millipore Corp., Bedford, MA, U.S.A.) for 6 h at 35 V. The resulting blots were blocked in 50% fresh skim milk/50% buffer A [0.05 M-NaCl, 10 mM-EDTA, 0.5% Tween 20, 0.1% (w/v) BSA, 0.05 M-Tris/HCl, pH 7.8] for 1–2 h, and then both blots, bearing 30–40 calpain samples, were incubated together overnight in 20 ml of skim milk/buffer A with 0.1 ml of polyclonal anti-(rat calpain II) antibody. After thorough washing, the blots were incubated in 20 ml of buffer A with 2 µCi of ¹²⁵I-Protein A (2–10 µCi/µg) for 5–10 h, followed by further washing and autoradiography. The radioactive areas of the blots located from the autoradiograph, together with control areas, were cut out and their radioactivity was measured in a γ-radiation counter. The observed radioactivity was linearly proportional to the amount of protein loaded, within the range used.

Values are given as means ± S.D. To ascertain the level of significance of differences between groups, Student's unpaired *t* test was used.

RESULTS

Characterization of the rat calpain genomic clones

Restriction mapping and hybridization of blots of restriction digests of the rat genomic clones with rabbit calpain II cDNA,

and subsequent cross-hybridization with fragments of the clones, showed that several genomic clones (including 10-1) were overlapping segments of the same region of rat genomic DNA, covering a total of approx. 25 kb. One other rat genomic clone (7-1) showed different restriction and hybridization patterns. Restriction maps of 7-1 and 10-1 are shown in Fig. 1, in which are indicated those fragments which hybridized to the rabbit calpain cDNA, and the positions of the exons which have so far been located. Exons 12–15 of rat calpain I and exons 5–21 of rat calpain II (80 kDa subunits) have been sequenced [28a], and are numbered here on the basis of the assumption (so far rigorously maintained) that they correspond to those established for chicken calpain [6], since chicken calpain is the only one whose genomic structure has been described. The rat calpain II exon 10 DNA sequence showed 89.4% identity with human calpain II cDNA, and 67.6% with human calpain I cDNA [7,8]. Conversely, the DNA sequences of rat calpain I exons 12–15 showed 84.1% identity with human calpain I cDNA, and 63.2% with human calpain II cDNA. The deduced amino acid sequence identities were closely parallel to these values, and the comparisons of rat and rabbit calpains also showed very similar patterns.

Rat RNA preparations, either total RNA or poly(A)⁺ RNA, were electrophoresed, blotted and probed both with rabbit calpain II cDNA, and with an anti-sense RNA probe derived from exon 10 of rat calpain II. The results (Fig. 2) showed that a single band of approx. 3.5 kb was detected by the RNA probe in rat lung poly(A)⁺ RNA and in total RNA preparations from several tissues. This band agreed in size both with that detected by the rabbit calpain II cDNA probe and with that described for calpain II large subunit mRNA in other species [5,29,30].

Genomic blots were also done of total rat DNA and of the isolated EMBL3 clones, and were probed both with rabbit calpain II cDNA and with various fragments of the isolated clones. The results (not shown) were fully consistent with the suggestion that the isolated clones represent portions of the rat calpain genes for calpain I and II, and that there exists only one copy (or at the most very few copies) of these genes per rat genome. These probes did not detect any unexplained band which might be derived from the p94 form of rat calpain [10,11].

Uterine DNA and protein levels

The variations of wet weight and total DNA content of the rat uterus throughout pregnancy and involution are illustrated in Fig. 3. Although the DNA content per uterus increased 8–10-fold during pregnancy, there was no statistically significant

Fig. 1. Restriction maps of the rat calpain genomic clones in EMBL3

(a) Clone 7-1, rat calpain I; (b) clone 10-1, rat calpain II. Rat genomic DNA was partially digested with *Mbo*I and cloned into *Bam*HI-digested EMBL3 arms. The rat DNA is oriented 5' to 3' with respect to the sense strand of exon sequences. Portions of rat DNA which hybridized with a 1.2 kb *Pst*II/*Hind*III C-terminal fragment of rabbit calpain II cDNA are indicated by hatching, and the positions and approximate sizes of the rat calpain exons so far detected are shown by black bars. B, *Bam*HI; E, *Eco*RI; S, *Sal*I.

Fig. 2. Northern blot analysis of rat RNA

(a) Poly(A)⁺ RNA from rat lung (7 µg), and (b) total RNA (40 µg) from rat lung (lane 1), kidney (lane 2) and uterus (lane 3) were run on an agarose gel, transferred to a Gene Screen Plus nylon membrane, and probed with a ³²P-labelled anti-sense RNA for exon 10 of rat calpain II. The positions of the ribosomal 18 S and 28 S RNAs are indicated.

Table 1. Amounts of DNA in rat uterine homogenates, and of soluble protein in uterine extract supernatants

The values are expressed in units/g wet wt. of uterus (means ± S.D.). For the DNA measurements there were three or four uteri at most of the time points, two separate tissue pieces were extracted per uterus and each extract was assayed in quadruplicate; the S.D.s therefore include 24–32 values, but represent only three or four animals. For protein values, one tissue piece was extracted per animal and each extract was assayed at least in triplicate, so that the S.D.s include 9–12 values, representing three or four animals.

Day of pregnancy or days post partum	No. of animals	DNA (mg/g wet wt.)	Extracted protein (mg/g wet wt.)
0	3	9.58 ± 0.89	28.4 ± 7.1
9	3	6.23 ± 1.10	11.6 ± 2.5
15	3	8.82 ± 0.77	28.6 ± 3.3
17	3	7.59 ± 0.61	34.7 ± 3.3
19	4	8.05 ± 1.12	30.4 ± 2.7
21 (birth)	4	8.53 ± 0.98*	33.4 ± 1.8
1	3	10.14 ± 0.59	37.2 ± 2.2
2	3	8.88 ± 0.96	38.0 ± 4.0
4	3	12.60 ± 1.21*	30.9 ± 3.9
6	1	13.8†	29.5†

* $P < 0.001$ for comparison between day 21 of pregnancy and 4 days post partum.

† Mean of several observations on one animal.

variation in the levels of DNA when the data were expressed in mg of DNA/g wet wt. of tissue, except for a slight increase at 4–6 days post partum (Table 1).

The amounts of protein that were observed in the supernatants recovered after homogenization of the uterus in a low-ionic-strength buffer and centrifugation are also shown in Table 1. These values were essentially constant, except for an anomalous result on day 9, and a rise of about 10% on days 1 and 2 post partum.

Calpain II mRNA levels

The yield of total RNA, calculated from the u.v. absorption values and averaged for all of the uterine RNA extracts, was

Fig. 3. Wet weight of rat uterus, and total DNA content of uterus, during pregnancy and involution

The wet weight (●) is given in g, and the DNA content (○) is given in mg/uterus. The S.D.s for three or four animals at each time point are shown, except when they were too small. There was only one animal at 6 days post partum.

Table 2. Ratios of calpain II mRNA to 7SL RNA

Duplicate samples of rat uterus total RNA were applied to slots on each of two blots, which were subsequently probed with a rat calpain II exon 10 anti-sense RNA probe and with 7SL cDNA respectively. The integrated densitometer readings for the duplicate samples were averaged and divided by the corresponding average from the other blot (calpain II mRNA/7SL RNA). Each value in the Table represents the mean ratio ± S.D. for at least four observations on at least two tissue extracts.

Day of pregnancy or days post partum	Expt...	Ratio		
		I	II	III
0		0.85 ± 0.22	0.94 ± 0.05	0.87 ± 0.08
9		1.41 ± 0.13	1.12 ± 0.09	0.91 ± 0.21
15		1.69 ± 0.48	1.31 ± 0.16	0.91 ± 0.45
17		0.86 ± 0.12	0.39 ± 0.06	0.37 ± 0.07
19		1.01 ± 0.43	0.62 ± 0.30	0.47 ± 0.21
21 (birth)		1.90 ± 0.71	1.02 ± 0.07	0.74 ± 0.15
1		0.80 ± 0.19	0.54 ± 0.03	0.43 ± 0.02
2		0.83 ± 0.06	0.58 ± 0.03	0.36 ± 0.01
4		1.06 ± 0.12	0.84 ± 0.07	0.57 ± 0.07
6		1.25 ± 0.12	0.89 ± 0.03	0.70 ± 0.06
Mean ± S.D.		1.14 ± 0.44	0.86 ± 0.32	0.65 ± 0.29

2.70 ± 0.63 mg/g wet wt. (mean ± S.D., $n = 47$), and no trends were evident in the individual values with respect to time. The fluorescence intensities of the 28 S and 18 S ribosomal RNA bands on an ethidium bromide-stained gel were not as high as expected from the absorption values in some cases, suggesting that some degradation had occurred, most significantly on days 17 and 19 of pregnancy, and on days 1 and 2 post partum.

In order to assay all tissue extracts in duplicate, over a period of 3 weeks three pairs of slot blots, each containing several samples from every day of the study, were exposed to the 7SL cDNA probe or to the calpain II RNA probe. Fresh probes were prepared for each blot. The results in Table 2 are the ratios of the averaged densitometer values, i.e. rat calpain II/7SL RNA. In the measurements of 7SL RNA, equal amounts of total RNA from each sample, calculated from the absorption values, gave highly uniform densitometer readings on the three blots probed with the 7SL cDNA; the mean values on each of the three blots

Table 3. Quantification of rat calpain II 80 kDa subunit by means of immunoblotting using a polyclonal antibody to rat calpain II and ¹²⁵I-Protein A

The values are given as c.p.m./ μ g of protein applied to the gel (means $\pm$ S.D.). For each experiment (I–IV), the number of replicate gel tracks included in each value is given in parentheses. The vertical columns of data cannot strictly be compared with each other.

Day of pregnancy or days post partum	Expt...	80 kDa subunit (c.p.m./ μ g)			
		I (4)	II (5)	III (3)	IV (4)
0					25.9 $\pm$ 8.2†
9		47.4*	29.3 $\pm$ 3.8		
15		46.1 $\pm$ 6.7	32.2 $\pm$ 2.7	31.0 $\pm$ 0.8	36.5 $\pm$ 1.7
17		35.9 $\pm$ 7.6	29.0*	22.0 $\pm$ 2.1	28.3 $\pm$ 3.0
19		46.9 $\pm$ 4.0	30.8 $\pm$ 2.9	26.1 $\pm$ 4.3	33.8 $\pm$ 2.2
21 (birth)		53.7 $\pm$ 7.4	33.7 $\pm$ 2.0	38.2 $\pm$ 5.4	
1		55.4 $\pm$ 6.2	34.7 $\pm$ 3.6	29.7 $\pm$ 1.7	
2		44.5 $\pm$ 3.5	29.6 $\pm$ 3.4	26.2 $\pm$ 3.2	
4			38.5 $\pm$ 7.3	26.4 $\pm$ 5.6	
6		54.5 $\pm$ 6.0	34.9 $\pm$ 3.0	30.0 $\pm$ 0.6	

* Mean of two observations on one animal.

† Mean of six observations on three animals.

were within 1 % of each other, and the S.D.s were 14 %, 18 % and 28 % of the means. In contrast, the mean densitometer values on each blot probed with the calpain II RNA probe differed considerably (in the proportions 3.1:2.3:1.8), and the S.D.s were about 30 % of the means. The ratios calculated for the three blots cannot therefore be compared with each other. The means and S.D.s of all the ratios derived from each pair of blots are also given in Table 2, at the bottom of each column.

In addition to these measurements with 7SL RNA, the ratio of rat calpain II mRNA to actin mRNA was estimated on one pair of Northern blots run with samples of total RNA from every day of the study. In this case the two autoradiograms were measured with a video densitometer and an integration program. The resulting ratios had a mean and S.D. of 0.95 ± 0.37 .

Immunoblotting of calpain II

For quantitative immunoblotting, the use of a polyclonal antibody to the 80 kDa subunit of calpain II and ¹²⁵I-Protein A, followed by measurement of the radioactivity on excised pieces of the blotted membrane, was found to give linear and consistent values, and was superior to densitometry either of autoradiographs or of coloured bands obtained with enzyme-linked second antibodies. It was found both for crude extracts and for pure standard calpain that the observed radioactivity on the blots was linearly proportional to the amount of protein applied to the gel, showing that other tissue components did not interfere. In practice, equal protein loading per track resulted in equal amounts of bound radioactivity, permitting reliable comparison. It should be noted that the specificity and sensitivity of the polyclonal anti-(rat calpain II) antibody is such that only the calpain II 80 kDa subunit, and not that of calpain I, was detected. The amounts of rat calpain I in the uterine extracts detectable on immunoblots with a monoclonal antibody to human calpain I were small, and calpain I was not studied further.

In all, 31 uteri were extracted over ten time points, and it was not feasible to run all of these simultaneously even in duplicate. In order to compare values across the period of pregnancy and involution, using the relatively cumbersome procedure of immunoblotting, sets of blots were run of 3–5 replicates from one

animal at each of 6–8 time points, and of one sample from each of 3–4 animals at each of 6–8 time points (Table 3). These measurements were carried out over several weeks, so that the vertical columns of data in Table 3 cannot be compared. The amounts of protein extracted on day 9 (Table 1) were anomalous, but the derived value of calpain/ μ g of protein for day 9 (Table 3) was nonetheless consistent with values from all the other time points. The data showed that there was no significant change in the immunochemical estimate of rat calpain II when expressed in terms of c.p.m./ μ g of protein applied to the gel. Since the amounts of soluble protein extracted per g wet wt. of tissue were also essentially constant (Table 1), the immunoblot data demonstrate that the amounts of calpain II per g wet wt. were constant throughout pregnancy and involution.

DISCUSSION

Nucleic acid probes for rat calpains I and II

It was felt to be desirable to isolate nucleic acid clones specific for rat calpains I and II for this and future work, in order to discriminate between them, although in the uterus the contribution of calpain I turned out to be very small. Initial attempts to isolate calpain cDNA clones from a rat lung λ gt11 library were not successful, but when the EMBL3 rat genomic library was screened with the rabbit calpain II cDNA fragment, several unambiguously positive clones were immediately observed, and readily plaque-purified. The evidence of DNA sequencing, intron–exon junctions, Northern blots of rat mRNA and Southern blots of total rat DNA showed that the isolated clones were derived from genes for the 80 kDa subunits of rat calpains I and II which are transcribed *in vivo*. Isoenzyme-specific RNA probes could then be synthesized from the available exons and used to measure rat calpain I and II mRNA. Since these uterine measurements were made, we have almost completed a genomic map of rat calpain II 80 kDa subunit, and have isolated partial cDNA clones covering most of it [28 a].

Uterine extractions

It was necessary to assume that various components that we wished to measure were uniformly distributed throughout the whole uterus, so that measurements on extracts of different pieces of a given uterus could be related. To avoid this assumption, it would be necessary to homogenize the whole uterus before taking samples, but this was not possible because of the different extraction conditions required for the several measurements. The wet weight of tissue was used as a baseline for comparisons, since it was not certain that any commonly used marker (DNA, total protein, actin mRNA, 7SL RNA, etc.) would necessarily maintain a constant relationship to wet weight. In practice, however, the total DNA, the total extractable protein and the 7SL RNA were all constant with respect to wet weight, so that any of these parameters could be used as a baseline.

DNA levels

In this set of pregnant and involuting rat uteri, the DNA content, when expressed as mg of DNA/g wet wt., did not vary significantly, except for a significant rise ($P < 0.001$) in late involution, probably attributable to the previously reported hyperinvolution of the *post-partum* uterus [14]. The amounts of DNA extracted from each uterus were much higher than previously reported [14], and the fact that the values of DNA/g wet wt. measured here did not vary was not consistent either with our own earlier measurements or with some of the very much earlier work on this point which had failed to agree on the relative roles

of hyperplasia and hypertrophy in uterine growth. In our previous experiments, the endometrium was scraped off in order to measure myometrial DNA only, which may explain part of the difference, but the main factor is undoubtedly the major improvements in available techniques which have been published since then. The combination of Polytron homogenization and proteinase K digestion in the presence of SDS [24] together with the now well-established DNA method of Labarca & Paigen [25], made it possible to solubilize the tough uterine tissue completely and to measure DNA in the resulting digest without any isolation or precipitation steps, and therefore without significant loss of DNA. Control experiments showed that recovery of added DNA was close to 100%, and the observed DNA values were proportional to the amount of sample taken, so that interference by other tissue components was not a factor. Because of these methodological considerations, it is felt that the DNA values reported here are reliable. One important conclusion is therefore that the growth of the rat uterus in pregnancy occurs very largely by hyperplasia, and that hypertrophy of individual cells is not significant.

Measurement of calpain II mRNA

The guanidinium thiocyanate RNA extraction method [23] is well established, and we have used it successfully for non-pregnant uterus and other rat tissues. Some degradation of the pregnant rat uterus RNA had apparently occurred, however, in spite of extreme precautions, probably because of the delay involved in dissecting out and cleaning the uterus and removing the fetal contents. The densitometry results for 7SL RNA on the slot blots were more uniform than the calpain II mRNA results, perhaps because the very small 7SL RNA was less liable to degradation than the very large calpain II mRNA, and we assume that this was a major factor in the wide variations in the slot-blot data. On each blot, which included multiple samples from every day of the study, the ratios of calpain II 80 kDa subunit mRNA to 7SL RNA had an S.D. of about 40% of the mean (Table 2), but the values did not show any significant trends.

The yield of total RNA/g wet wt. and the level of 7SL RNA/ μ g of total RNA had been shown to be essentially constant (with S.D.s of approx. 25%) throughout pregnancy and involution. The data in Table 2 show that the level of calpain II mRNA did not vary by more than $\pm 40\%$ with respect to 7SL RNA, and are therefore consistent with the hypothesis that the expression of calpain II mRNA is constitutive in the rat uterus during pregnancy and involution. One experiment in which calpain and actin mRNAs were measured also showed no significant or systematic variation in the ratios of the two.

Immunoblotting of calpain

Calpain cannot be measured enzymically in crude tissue extracts with any precision because of the presence of the inhibitor, calpastatin, and in the present case also because of the very small sample size. We had measured calpain previously in the uterus by means of a sandwich e.l.i.s.a. with 125 I-labelled anti-calpain IgG, and reported a marked increase in calpain in pregnancy with respect to wet weight [13]. Further work led us to doubt these results, and we devoted much effort to development of a classical radioimmunoassay, and of a competitive e.l.i.s.a. for calpain [31] similar to one recently described [32]. Although these assays were highly accurate with pure standard calpain, we have been unable, without introducing extensive purification steps, to resolve problems of tissue interference in these immunoassay procedures for calpain. We conclude that our earlier results on uterine calpain were distorted by tissue in-

terference and were not correct. In our hands, the only reliable assay for calpain in crude extracts is by means of quantitative immunoblotting.

Since there are practical limits on the number of samples that can be assayed at one time by immunoblotting, uterine extracts covering the period of pregnancy and involution were therefore assayed repeatedly using different sets of replicates. It can be seen from Table 3 that no statistically significant variations or trends in calpain II 80 kDa subunit content/g wet wt. occurred over this period.

Other direct studies of the regulation of calpain synthesis have not been reported, but the 5'-region of the human calpain II 80 kDa subunit gene has been sequenced. This region contained 'features common to many genes of housekeeping proteins' as well as 'multiple negative enhancer-like elements' [9]. In accordance with this, the measurements reported here show that the uterine content of calpain II 80 kDa subunit protein, and of its specific mRNA, when expressed in units/g wet wt. of tissue, did not vary significantly throughout the roughly 10-fold growth of the uterus during pregnancy, and its subsequent 10-fold involution. It may be concluded that expression of calpain II in the pregnant and involuting uterus is constitutive.

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