THE CONTENT OF CALMODULIN DURING THE PREREPLICATIVE PERIOD OF ISOPROTERENOL-ACTIVATED RAT PAROTID GLANDS AND ITS RELATION TO DNA SYNTHESIS.

by

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
Calcein	(2',7'-[[bis[carboxymethyl]-amino)-methyl]-fluorescein
CaM	calmodulin
Cell lines:	
C3H/10T½/2	mouse foetal
CHO	chinese hamster ovary
L	mouse connective tissue
NRK	normal rat kidney
3T3	mouse embryo
T51B	rat liver
WI-38	human lung
WISH	human amnion
CNBr	cyanogen bromide
Cyclic AMP	3',5'-adenosine monophosphate
EGTA	ethylene glycol-bis-(beta-aminoethyl ether)N N'-tetra-acetic acid
GnRH	gonadotrophic-releasing hormone
HPX	partial hepatectomy
i.c.	intracardially
i.p.	intraperitoneally
IPR	dl-isoproterenol: 1-(3,4-dihydroxyphenyl)-2-isopropyl-aminoethanol
LSD	least significant difference
MCA	methylcholanthrene
µg/g w wt	micrograms per gram of wet weight

NRCC	National Research Council of Canada
$1,25(OH)_2D_3$	1,25-dihydroxycholecalciferol. 1,25-dihydroxy-vitamin D_3
ODC	ornithine decarboxylase
PAGE	polyacrylamide gel electrophoresis
PCC	plasma calcium concentration
Pluronic F-38	linear polyol. Mol wt 5000 (80% hydrophilic polyoxyethylene groups and 20% hydrophobic polyoxypropylene groups)
PTH	parathyroid hormone
RIA	radioimmunoassay
R24571	1-[bis-(p-chlorophenyl)methyl]-3 [2,4-dichoro beta-(2,4)dichlorobenzyloxy)-phenethyl]-imidazolium chloride
SDS	sodium dodecyl sulfate
TEMED	tetramethylendiamine
TPTX	thyroparathyroidectomy
Tris	tris(hydroxymethyl)amino methane
Viruses:	
ASV	avian sarcoma virus
SV-40	simian virus type 40
V_o	void volume
W5	N-(o-aminohexyl)-1-naphtalenesulfonamide
W7	N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide
W12	N-(4-aminobutyl)-1-naphtalenesulfonamide
W13	N-(4-aminobutyl)-5-chloro-2-naphtalenesulfonamide

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ABSTRACT

In the present investigation, the possible link between CaM content during the prereplicative period and DNA synthesis in rat parotid glands activated by isoproterenol was examined.

A specific radioimmunoassay was developed to detect and measure CaM in rat parotid glands. Calmodulin was purified from rat testis and was iodinated with ^{125}I. Also, purified calmodulin was linked to Sepharose 4B which was used subsequently in the antibody purification. Performic acid-oxidized CaM was used to elicit an immune response in two sheep.

Anti-CaM serum was purified by affinity chromatography in the calmodulin-sepharose gel. Pure CaM was able to displace ^{125}I-CaM in a linear fashion from the purified anti-calmodulin serum. Troponin C and parvalbumin did not compete significantly with calmodulin for the anti-calmodulin serum.

The CaM level in rat parotid glands and liver was found to be different than testis. Most of the rat testis CaM was found in the soluble fraction. Calmodulin in rat parotid glands and rat liver was found equally distributed between soluble and particulate fractions.

DNA synthesis in rat parotid glands was determined by measuring the ^{3}H-thymidine incorporation into DNA. Isoproterenol (i.p.) induced DNA synthesis between 18 and 30 h after the injection with

a maximum activity at 24 h. The DNA synthetic response was dependent on the dose of isoproterenol.

Isoproterenol induced changes in the content of calmodulin during the prereplicative period. Total calmodulin content increased at 3 h after isoproterenol injection. Also, a redistribution of calmodulin occurred at 18 h after isoproterenol injection. This was reflected as a transient increase in the soluble but not total fraction content of calmodulin at 18 h.

Calmodulin increases in the soluble fraction at 18 h were not found with low doses of isoproterenol which induced DNA synthesis. This suggests that calmodulin increases in late G_1 are not an absolute requirement for DNA synthesis.

Nonetheless, experiments conducted with TPTX rats indicated that the calmodulin surge in late G_1 is associated with DNA synthesis in 24 h TPTX rats and is absent as is DNA synthesis in 48 h or 72 h TPTX rats. This suggests a possible relation between the surge in soluble calmodulin at 18 h and subsequent DNA synthesis.

Intraperitoneal injection of calcium did not restore either DNA synthesis or plasma calcium concentration in 48 h TPTX rats. Therefore, a direct cause-effect relationship between the restoration of the 18 h surge of soluble calmodulin and DNA synthesis in 48 h TPTX-isoproterenol activated parotid glands could not be established.

RÉSUMÉ

Dans cette enquête, les glandes parotides activées par l'isoprotérenol chez le rat ont été examinées pour découvrir la posibilité d'un lien entre la teneur en calmoduline presénte durant la période préreplicative et la synthèse de l'ADN.

Une analyze radioinmunologique spécifique a été développé à fin de détecter et mesurer la teneur en calmoduline dans les glandes parotides du rat. La calmoduline testicularie du rat a été purifiée. La protéine calmoduline a été marquée avec l' ^{125}I. De la calmoduline purifieé a aussi été liée a du Sepharose 4B. Cette préparation a été utilisée plus tard pour la purification d'anticorps. De la calmoduline oxidée par l'acide performique a été utilisée pour provoquer l'inmunité chez deux moutons.

Le sérum anticalmoduline a été purifiée par une chromatografie d'affinité en le gel sépharose-calmodulinè. La calmoduline purifiée a causé le déplacement de ^{125}I-CaM lieé à du sérum anticalmoduline purifiée d'une façon linéaire. Troponin C et parvalbumin n'ont pas de maniére significative, réussi a faire concurrence avec la calmoduline pour le sérum anticalmoduline.

Une différence significative entre la teneur en calmoduline des testicules et celle des glandes parotides et du foie du rat a été trouvée. La presque totalité de calmoduline testiculaire du rat des

glandes parotides et du foie du rat se trouvait également distribuée entre les fractions solubles et le précipité.

La mesure de l'incorporation de la ^{3}H-thymidine dans l'ADN a permis de déterminer le taux de synthèse de l'ADN dans les glandes parotides du rat. De 18 à 30 hrs après une injection dans le péritone (du rat) d'isoprotérénol, la synthese de l'ADN a été induite avec un maximum d'activité a 24 hrs. Cette reáction de synthèse de l'ADN était dépendante de la dose d'isoprotérénol.

L'isoprotérenol a provoqué des changements dans la teneur en calmoduline durant la période préréplicative. La teneur totale de calmoduline à augmenté a 3 hrs après l'injection d'isoprotérenol. Une redistribution de la calmoduline a aussi été remarquée a 18 hrs aprés l'injection d'isoprotérénol. Ceci a été traduit par une augmentation transitoire dans seulement la fraction soluble sans changement dans la teneur totale de la calmoduline, à 18 hrs.

De petites doses d'isoprotérenol qui ont occassionné la synthèse de l'ADN n'ont pas causé d'augmentation de la calmoduliné dans la fraction soluble a 18 hrs. Ceci suggére que des augmentations de la calmoduline dans la phase G_1 avancée ne sout pas des prérequis essentiels a la synthèse de l'ADN.

Néanmoins des expériences utilisant des rats TPTX ont démontrés qu'une émergence de calmoduline dans la phase G_1 avancée est associée

avec la synthese d'ADN dans des rats TPTX de 24 hrs. Par contre dans des rats TPTX de 48 on 72 hrs l'émergence de la calmoduline et la synthèse de l'ADN sont absente. Ceci suggère une relation possible entre l'émergence de calmoduline soluble à 18 hrs et des synthese d'ADN subsíquentes.

De injection de calcium intrapéritone n'ont rétabli ni la synthese de l'ADN ni la concentration de calcium plasmatique dans des rats 48 hrs TPTX. Par couséquent une relation cause-effect direct entre le retablissement de l'émergence de la calmoduline soluble a 18 hrs et la synthèse de l'ADN dans les glandes parotides de rats 48 hrs TPTX activées par l'isoprotérenol n'a pu été constatee.

RESUMEN

Durante la presente investigación se estudió la posible relación entre el contenido de calmodulina en el período de pre-replicación y la síntesis del ADN en las glándulas parótidas de rata activadas con isoproterenol.

Se desarrolló un radioinmunoensayo específico para detectar y medir calmodulina en las glándulas parótidas de rata. La calmodulina fué purificada a partir de testículos de rata y una vez purificada fué radio-iodinada con ^{125}I. Posteriormente fué ligada a Sepharosa 4B y dicho gel, CaM-Sepharosa, fué utilizado más tarde en la purificación del anticuerpo contra calmodulina.

Se inmunizaron dos borregos con calmodulina previamente oxidada con ácido perfórmico. El anticuerpo contra calmodulina se purificó por medio de cromatografía de afinidad utilizando el gel de CaM-Sepharosa. La calmodulina purificada fué capaz de competir por el anticuerpo, con la ^{125}I-CaM.

Se encontró que el contenido de calmodulina en testículos de rata, es diferente al detectado en hígado o en glándulas parótidas. Además, la mayor parte de la calmodulina testicular se halla en la fracción soluble después de una centrifugación a 105 000 X g, mientras que la calmodulina de hígado o de glándula parótida está distribuída por igual en la fracción soluble y en el precipitado después de una centrifugación igual a la anterior.

La síntesis del ADN en glándulas parótidas de rata fué cuantificada midiendo la incorporación de timidina tritiada en el material genético.

Se encontró también, que el isoproterenol induce la síntesis del ADN en parótidas de rata, entre 18 y 30 horas después de su administración, detectándose la máxima actividad a las 24 horas aproximadamente. El isoproterenol también indujo cambios en el contenido de calmodulina durante el período pre-replicativo; ésto otra vez, en parótidas de rata. Se detectó un aumento en el contenido total de calmodulina 3 horas después de la inyección de isoproterenol. También se observó que la calmodulina se redistribuye 18 horas después de que las parótidas fueron activadas con isoproterenol, reflejándose ésto último como un aumento temporal de calmodulina en la fracción soluble. Sin embargo, dicho incremento temporal no se observó cuando las parótidas se activaron con dosis bajas de isoproterenol. Lo que sí se encontró fué que tales dosis estimularon la síntesis del ADN, sugiriendo así que un aumento en el contenido de calmodulina en la parte última de la fase G_1, no es un requisito para la síntesis del material genético.

Sin embargo, los experimentos hechos con ratas tiroparatiroidectomizadas 24 horas antes de la administración de isoproterenol, indicaron que el aumento en el contenido de calmodulina medida 18 horas después de la activación, está asociado con la síntesis del ADN. Además, cuando la síntesis fué bloqueada en ratas tiroparati-

roidectomizadas 48 y 72 horas antes de la inyección de isoprotere-
nol, el aumento de calmodulina a las 18 horas después de la acti-
vación, no fué observado. Esta evidencia sugiere una relación
entre el incremento de calmodulina en la fracción soluble a las
18 horas y la síntesis del ADN.

Por último, se encontró que una inyección de calcio adminis-
trada a ratas tiroparatiroidectomizadas 48 horas antes de la ac-
tivación con isoproterenol, no es suficiente para restaurar la
síntesis del ADN, o normalizar la concentración de calcio en el
plasma. Por lo tanto, no fué posible demostrar una relación di-
recta entre la restauración de la síntesis del ADN y el aumento
en el contenido de calmodulina 18 horas después de la activación
con isoproterenol.

INTRODUCTION

Cell Division Cycle

The division cycle of eucaryotic cells is generally (though not necessarily) characterized by four phases. The period of time between cell division and the beginning of chromosome replication is the G_1 (Gap 1) phase. During the G_1 phase, or prereplicative period, the cell gets ready to replicate its chromosomes. Chromosome replication is the next phase, and is generally referred to as the S (DNA-synthetic) phase. After the S phase, the cell enters the G_2 phase (Gap 2) during which it prepares for mitosis and cytokinesis. The process of division is the last phase and is called the M (Mitotic) phase and results in two new individual cells (1-4).

The cells of an animal exist in a variety of states, not all of which involve cell proliferation. For example, hepatocytes in the adult liver or parotid gland acinar cells in adult parotid glands are functionally differentiated cells which under "normal" physiological conditions do not proliferate or enter the cell cycle. Cells such as small peripheral lymphocytes are differentiated cells which are not actively cycling. Other cells, which some refer to as "stem cells", are continuosly proliferating. These stem cells are generally, if not always, localized in specific

regions in the body. For example, the epithelial cells of the intestinal crypt are continuosly proliferating and give rise to cells which again divide or which are probably forced up onto the intestinal villus. Once the cell leaves the crypt, its micro-environment is changed and it no longer proliferates but differentiates and migrates up the intestinal villus, only to eventually slough off and die. This same scenario exists in other continuosly renewing tissues such as skin (5-7).

The differentiated cells, however, under certain circumstances can be induced to divide. For example, adult hepatocytes in the liver will proliferate in response to injury (8), the uterus will proliferate in response to estrogens (9), the fore stomach will proliferate in response to epinephrine (10) and most importantly for the present study parotid gland acinar cells proliferate in response to isoproterenol (11).

Control of Cell Proliferation.

It is known that a tissue contains continuosly dividing cells, non-dividing cells and/or cells capable of cell division after appropiate activation (i.e. activation of the gene or set of genes which coordinate the cell division cycle). Thus, in functionally differentiated cells such as liver hepatocytes or

parotid gland acinar cells, the event(s) which activates cell proliferation is termed "proliferative activation" (12). These proliferatively activated cells now enter the cell cycle and progress through the G_1, S, G_2 and M phases. Obviously a complex set, or series of sequential and/or parallel biochemical events control the progression and successful completion of the cell division cycle. The specific biochemical agents which control specific phases of the cell division cycle are termed "intracycle regulators" as opposed to "proliferative activators" (12).

These intracycle regulators can be simple as ions or complex proteins. A myriad of putative intracycle regulators have been suggested, some of which include Na^{+}, K^{+}, Cd^{+2}, Zn^{+2}, Mg^{+2}, Ca^{+2}, serum growth factors, prostaglandins and thromboxanes, calcium-binding proteins, nucleotides, protein kinases and polyamines (13, 14).

The regulation of cell transit through the division cycle is obviously a complex and multifactor-dependent process, the unraveling of which is only beginning.

Calcium.

The calcium ion, unlike other ions, has some unique characteristics which enable it to act as a cellular regulator. Calcium

has a radius of 0.95 Å, it is less hydrated than, for example Mg^{+2}, which is the most similar cellular cation (15).

Calcium electrostatic interactions are with anionic centers like proteins, phospholipids, fatty acids and small molecules like citrate. There is no constant distance in the interaction which varies from 2.4-3.2 Å and has coordination values between 6-12. By contrast, Mg^{+2} has a coordination value of 8, forms octhaedron salts, and possesses a fixed ionic radius of 2.5 Å. Thus, calcium salt bonds are weaker than magnesium salt bonds giving calcium high mobility and lower relative energy for dissociation compared to Mg^{+2} (15,16).

Because of these properties, calcium is capable of rapid dissociation/association reactions. Calcium forms complexes with multidentate ligands (e.g. proteins) causing a high degree of cross-linking between proteins or protein-small molecules while magnesium does not have these properties (17). In biological systems, calcium interacts with: aspartate, glutamate, gamma-carboxyglutamate, phosphate and uncharged oxygens of hydroxyl or carboxyl groups (15).

Calcium combines easily with some small acidic intracellular proteins (e.g. troponin C, parvalbumins, calmodulin). These calcium-protein complexes are capable of modulating enzyme activities or structural proteins which triggers various physiological responses

(15-17).

Resting mammalian cells maintain a free intracellular calcium concentration of between 10^{-7} to 10^{-8} M.(18). Because of its special "regulator" status, calcium metabolism is under tight control. Cellular calcium availability is regulated mainly by the routes of absorption, resorption and excretion in the whole animal and by the calcium fluxes at the membrane level (19).

Extracellular calcium (total 2.0-2.5 mM; ionic 1.0-1.25 mM) is regulated by three calcium homeostatic hormones: parathyroid hormone which is produced by the parathyroid glands; calcitonin which is synthesized in thyroid C cells in the thyroid gland; and $1,25(OH)_2D_3$ which is produced by the kidneys. These hormones have specific targets in intestinal epithelium, kidney glomerulus, bone and cellular plasma membrane (20). A brief decrease in the extracellular calcium level causes PTH secretion and a decrease in calcitonin secretion, resulting in an immediate calcium absorption in the kidney and an efflux of Ca^{+2} in the bone. If the extracellular calcium rises, the reverse occurs. Also, parathyroid hormone affects the synthesis of $1,25(OH)_2D_3$ which stimulates calcium absorption from the diet and calcium mobilization in the bone (20,21).

The steep inward calcium gradient across the plasma membrane of all cells is maintained by several extrusion and storage

mechanisms (19). Ca^{+2}-transport mechanisms are found in the plasma membrane (22), sarcoplasmic reticulum (23) and mitochondria (24). Furthermore, it is known that some secretory vesicles can accumulate calcium in the secretory product (e.g. amylases in the salivary glands) (25). Finally, the cellular ionic calcium concentration is also regulated by its binding to some specific intracellular proteins like parvalbumins, troponin C, S-100, vitamin D-dependent calcium binding protein and calmodulin (19).

This brief discussion of calcium's unique characteristics make clearer calcium's ability to act as a cellular "master" switch or regulator. Calcium is required for a wide variety of physiological functions such as muscle contraction, neurotransmitter release, egg activation, cell-cell communication and interaction, mineralization of bone, secretion, axoplasmic flow, cation transport, endocytosis, water permeability and membrane stabilization (26).

Calcium and Cell Proliferation.

Calcium was first seriously considered to be involved in cell proliferation in 1958 by Rixon and Whitfield when they noticed that parathyroid hormone (PTH) reduced the mortality of heavily X-irradiated rats (27). It became evident that calcium and PTH stimulated cell multiplication of vital tissues like bone marrow, lymph

nodes and thymus gland (28-33).

Subsequently, it was discovered that decreasing the extracellular calcium concentration in the circulating blood of rats by removing the thyroparathyroid gland complex (TPTX) stops the synthesis of DNA and cell division in hepatocytes after partial hepatectomy (HPX) and in parotid gland acinar cells after proliferative activation by isoproterenol (34-36). However, this block in cell cycle progression of the already activated cells could be reversed by a single injection of $CaCl_2$ if given between 12-15 hours after partial hepatectomy (37), or by restoring the normal calcium concentration by administration of the calcium homeostatic hormones (36-38).

The DNA synthetic block caused by TPTX does not impair some prereplicative events elicited after the activation (39). For example, the prereplicative period of TPTX-HPX rats still exhibits the normal increases of cAMP (40), mRNA and rRNA (41), ODC and putrescine (42,43), membrane autophosphorylation (44), accumulation of calcium in endoplasmic reticulum (45) and prostaglandin synthesis (46). The only events which do not take place in the low-calcium condition are the activation of cAMP-dependent protein kinases and a nuclear Ca^{+2}-calmodulin stimulable protein kinase (47), the activation or synthesis of thymidylate synthetase (48), the

activation of synthesis of ribonucleotide reductase (49) and the synthesis of the four deoxyribonucleotides (42). Similarly, the rat thymic lymphoblast arrested in the late prereplicative period with a low-extracellular calcium concentration can not activate thymidylate synthetase (48). However, in both of these examples, the restoration of a normal extracellular calcium concentration leads to DNA synthesis and mitosis (46). This evidence strongly suggests that calcium might be involved in the "on/off" of some reactions necessary for DNA synthesis.

A substantial amount of evidence using in vitro systems also supports the idea of a calcium requirement for G_1/S transition. For example, calcium stimulates the entry of thymic lymphocytes into S phase (50). Moreover, a critical level of extracellular calcium is essential for the proliferative activities of several cell lines (46).

It should be noted that chemically-induced pre-neoplastic, neoplastic and virus-transformed cells are capable of cell proliferation and colony formation in very low concentrations (less than 0.05 mM) of extracellular calcium which do not support proliferation of their non-neoplastic counterparts (51). Adenovirus type 12-infected hamster cells (52), Rous sarcoma virus-infected chicken fibroblasts as NRK cells (53,54), spontaneously-transformed BALB/3T3

mouse cells (55), methylcholanthrene (MCA) transformed C3H10T1/2 type III mouse cells (56) and SV-40-W1-38 human lung fibroblasts (57) are a few examples of transformed cell lines that proliferate in a low calcium medium.

Furthermore, cells in culture which are normally arrested in their G_1 phase with a low extracellular calcium concentration, can be stimulated to proliferate by the addition of some tumor promoters such as 12-O-tetradecanoyl phorbol-13-acetate and saccharin (58, 59).

Taken together, this evidence points to the fact that the calcium ion is a requisite for G_1/S transition of non-neoplastic cells, both in vivo and in vitro and that a disruption or bypass of this mechanism may occur during the neoplastic process. Calcium may be involved somehow in nucleotide metabolism by acting through the calcium-dependent regulator, calmodulin.

Calmodulin.

Calmodulin is the name given (CaM) to a calcium-binding protein discovered as a phosphodiesterase activator thirteen years ago (60). This protein has been localized in a wide variety of eucaryotic cells ranging from protozoans to mammals and several plants. The protein has been suggested to be present in all mammalian tissues (61). It is now known that CaM is distributed between soluble and

particulate fractions including the plasma membrane, mitochondria, microsomal fraction, nuclear membrane and secretory vesicles. Studies carried out with EGTA buffers in the calmodulin extraction procedure suggest that the calmodulin association to membranes is calcium-dependent (62).

Figure 1 illustrates the primary structure of vertebrate calmodulin. It exhibits an internal homology between domains I (residues 8-40) and III (residues 81-113) and between domains II (residues 44-76) and IV (residues 117-148). Each domain (I-IV) binds one mol of calcium, accounting for the four calcium ions per molecule of calmodulin. Metal binding studies (63) indicate that CaM binds Ca^{+2} with a high affinity (Kd = 2.4 X 10^{-6}M).

Table 1 lists some of the properties of rat testis calmodulin (64). Among others, calmodulin is heat resistant ($T_{1/2}$ = 7 min at 100°C); has a molecular weight of 16 700 in SDS-PAGE; contains 148 amino acids with the amino terminal end acetylated, and has 30% acidic amino acid residues accounting for its pI of 4.0. The protein does not contain cysteine, tryptophan or hydroxyproline. It has a high Phe/Tyr ratio (4/1) which is responsible for the characteristic ultraviolet spectrum showing vibrations between 250 and 282 nm and it has a very low extinction coefficient ($E_{276-278}$ = 1.9). It has 50% direct homology and 70% conservative homology

Figure 1. The amino acid sequence of mammalian calmodulin.

The primary structure of calmodulin is represented with the one-letter code. The domains were aligned to facilitate comparisons. The postulated calcium-binding sites are indicated (62).

1 7
AcHN-A-D-Q-L-T-E-E-

DOMAIN I:

8 10 20 30 40
-Q-I-A-E-F-K-E-A-F-S-L-F-D-K-D-G-D-G-T-I-T-T-K-E-L-G-T-V-M-R-S-L-G-

CALCIUM BINDING SITE

41 43
-Q-N-P-

DOMAIN II:

44 50 60 70 76
-T-E-A-E-L-Q-D-M-I-N-E-V-D-A-D-G-N-G-T-I-D-F-P-E-F-L-T-M-M-A-R-K-M-

CALCIUM BINDING SITE

77 80
-K-D-T-D-

DOMAIN III:

81 90 100 110 113
-S-E-E-E-I-R-E-A-F-R-V-F-D-K-D-G-N-G-Y-I-S-A-A-E-L-R-H-V-M-T-N-L-G-

CALCIUM BINDING SITE

$(Me)_3$
N
-E-K-L-

DOMAIN IV:

120 130 140 148
-T-D-E-E-V-D-E-M-I-R-E-A-N-I-D-G-D-G-Q-V-N-Y-E-E-F-V-Q-M-M-T-A-K-COOH

CALCIUM BINDING SITE

TABLE I. Physical properties of rat testis calmodulin.

PROPERTY	SPECIFIC VALUE
Aminoacid number	148
Molecular weight	16 700
% acidic aminoacids	33
N-terminus	N-acetylated
Phe/Tyr ratio	4/1
Trimethyllysine/mol	1
Calcium binding	4 mol/mol; Kd= 2.4×10^{-6} M
$E^{1\%}_{276}$	1.8-2.0
Svedberg units (S_{20w})	1.83-2.0
Isoelectric pH	3.9-4.0
Conformation	40-55% alpha helix
Drug binding	phenothiazines and naphthalenesulfonamides

References: 61 and 64.

with troponin C. In all known calmodulin sequences there are a maximum of seven conservative substitutions (65). Some calmodulins contain an epsilon-N-trimethyllysine residue at the 115 position which appears to be a post-translational modification of unknown function.

Calmodulin has the ability to interact with various pharmacological agents. The phenothiazines (66) and naphthalenesulfonamides (67) are examples. These drugs are used as research tools, because during the interaction CaM losses its biological activity.

It is postulated that the mechanism of action of calmodulin begins with the calcium-calmodulin complex formation. This interaction increases the hydrophobicity of the protein molecule and the exposed hydrophobic part of calmodulin is responsible for the binding with several CaM-dependent enzymes (62,68,69). However, it is suspected that calcium-free CaM is able to modify the activity of some enzymes (70,71). Ca^{+2}-calmodulin itself has no enzymatic properties but does modulate the activity of a long list of calcium-dependent enzymes and processes (Table II).

Calmodulin and Cell Proliferation.

In recent years, several studies have suggested that calcium-calmodulin may be involved in control of cellular proliferation. The intracellular concentration of calmodulin increases during the

TABLE II. Calmodulin-regulated processes or enzymes.

ENZYME OR PROCESS	SOURCE
Cyclic Nucleotide Metabolism	
Cyclic nucleotide phosphodiesterase	Several tissues
Adenylate cyclase	Several tissues
Guanylate cyclase	*Tetrahymena pyriformis*
Calcium Transport Systems	
Plasma membrane Ca^{2+}-pump ATPases	Red blood cells Adypocytes
Sarcoplasmic reticulum Ca^{2+}-ATPases	Cardiac muscle
Cellular Motility	
Myosin light chain kinase	Smooth muscle Skeletal muscle Cardiac muscle
Dynein ATPase	*Tetrahymena pyriformis*
Glycogen Metabolism	
Phosphorylase kinase	Skeletal muscle Cardiac muscle Platelets
Glycogen synthase kinase	Liver
Biosynthetic Metabolism	
NAD kinase	Pea seedlings Sea urchin
Cytoskeletal and Membrane Components	
MAP_2	Brain
Caldesmon	Chicken gizzard

TABLE.II (continued)

ENZYME OR PROCESS	SOURCE
Spectrin	Erythrocytes
Mitotic apparatus	Cells in culture
Protein Phosphorylation/Dephosphorylation	
Vimentin	Sertoli cells
Phospholamban	Cardiac muscle
Several proteins	Brain
Tyrosine hydroxylase	Brain
Tryptophan hydroxylase	Brain
Myelin	Brain
Histones	Calf thymus
Globin	Erythrocytes

References: 60-62, 69 and 72.

prereplicative period of regenerating liver *in vivo* (73). Calmodulin does increase its cellular content during G_1 phase of T51B cells (12,74) and BALB/c 3T3 cells *in vitro* (75). In all cases mentioned above, calmodulin returns to basal levels before the initiation of DNA synthesis. Exogenous added calmodulin can stimulate DNA synthesis of calcium deprived T51B cells (76) and planaria (*Dugesia lugubris*) cells (77). This stimulatory activity of calmodulin was blocked by low concentrations of trifluoperazine and chlorpromazine. Prereplicative increases of CaM are also described in synchronized CHO-K1 cells (78-80), pig lymphocytes activated with lectins (81) and synchronized human WISH cells (82).

An increase in CaM may also occur during the prereplicative period of *Tetrahymena pyriformis* (83) and rat thymocytes (84). The authors report a stimulation of the activity of calmodulin-dependent enzymes (guanylate cyclase for *T. pyriformis* and cyclic nucleotide phosphodiesterase for rat thymocytes) from the total homogenates of the cells during the G_1 phase of both cell types.

Recent studies indicate that increases in the calmodulin content during the G_1 phase are not needed for the subsequent DNA synthesis in some *in vitro* systems. Thus, NRK cells infected with the avian sarcoma virus *ts*LA23, progress through the G_1 and S phases without a rise in the total content of calmodulin (85). Also,

serum stimulated T51 B cells still exhibit the G_1 CaM surge even without progress into S phase (86).

Nevertheless, in all cells tested to date (in vitro), the transition of the cells from G_1 to S phase is totally blocked by the calmodulin antagonists W-7, R24571 or W-13 but not by the analogs W-5 or W-12 which do not interfere with CaM action. These results indicate that even if CaM content does not increase during the prereplicative period, CaM function is still required for normal G_1/S transition of the cells.

Evidence provided by virus-transformed cells and solid tumors, also suggests a possible involvement of CaM in cell proliferation. Increased amounts of calmodulin have been observed in several cell lines compared with their normal counterparts. For example, chicken embryo fibroblasts infected with Rous sarcoma virus (87-90), SV-40-transformed 3T3 cells and NRK cells transformed by Rous sarcoma virus (91). This increased calmodulin content is probably due to an increase in the synthesis of CaM, rather than inhibition of the degradative processes. Similarly, transplantable Morris hepatomas contain more calmodulin than normal or fetal liver (73,92). Also, direct correlation exists between the CaM content and growth rates in rat hepatomas (93).

Cyclic AMP and Protein Kinases in Cell Proliferation.

Cyclic AMP was discovered in 1956 as the mediator of epinephrine and glucagon-induced glycogenolysis (94). Since its discovery, it has become clear that cyclic AMP is also involved in many other cellular functions such as lipolysis, steroidogenesis, etc. (94).

In 1969, cyclic AMP (cAMP) was proposed to be a positive effector on the initiation of DNA synthesis of freshly isolated rat thymic lymphoblasts (95). Since this initial observation, cyclic AMP has been shown to stimulate the initiation of DNA synthesis and cell proliferation of a wide variety of cell types *in vitro* (12). Moreover, an apparently ubiquitous surge of cyclic AMP occurs during the G_1 phase of most cell types tested to date, both *in vivo* and *in vitro* (75). The delay or inhibition of this surge is always associated with a delay or inhibition of DNA synthesis. Recent evidence indicates that cAMP stimulates the activity of DNA polymerase of mouse liver and tumor cells (90).

Cyclic AMP is known to function through activation of two protein kinases. These two cyclic AMP-dependent protein kinases (type I and type II) exist as tetramers (two regulatory subunits and two catalytic subunits). The catalytic subunits of both type I and II are apparently identical while the regulatory subunits are different. Cyclic AMP binds to the regulatory subunit of either type I or type

II protein kinase which results in release of the active catalytic subunits (97).

Recent evidence suggests that cyclic AMP-dependent protein kinases are involved in the onset of DNA synthesis. Type I and type II isoenzymes change their activities during the G_1 phase of CHO-cells (98, 99) and rat fibroblasts (100). Stimulation of DNA synthesis in T51B cells by calcium addition, is preceeded by an increase in the activity of cAMP-dependent protein kinases (101-103). Both, DNA synthesis and the kinases activity are inhibited by a specific protein kinase inhibitor of the catalytic subunit of cAMP-dependent kinases.

In addition, rat hepatocytes activated by partial hepatectomy undergo surges in the activities of type I and type II cyclic AMP-dependent protein kinases and also an increase in a nuclear Ca^{+2}-CaM dependent protein kinase (47). TPTX, which prevents DNA synthesis of HPX activated rat hepatocytes, modifies or eliminates the pre-replicative surges of the three protein kinases. The impairment of type I activity induced by TPTX can be reversed by $1,25(OH)_2D_3$, administered immediately after HPX. It is known that $1,25(OH)_2D_3$ in combination with PTH or calcitonin can restore DNA synthesis of liver cells in TPTX-HPX rats (38).

Rodent Salivary Gland Cells.

General description.

Mammalian salivary glands have great morphological diversity. However, they are basically composed of excretory ducts, intralocular ducts and secretory endpieces. Gross anatomical classification divides salivary glands in two groups. The major group includes parotid, submandibular (submaxillary) and sublingual glands while the minor group includes bucal, infraorbital, labial, lingual and some others distributed in the oral cavity. Salivary glands are involved in the secretion of some substances directly into the oral cavity; the nature of the secretory product depends on the specific gland and the species involved. The substances secreted are mainly glycoproteins, sulfated-glycoproteins, sialylated proteins, water and ions (104,105). The importance of the secretory product is enormous because they are directly involved in the first steps in the metabolism of exogenous nutrients.

It is proposed that secretory products synthesized in membrane-bound ribosomes are maturated in golgi apparatus and then carried within secretory vesicles which fuse with the basal membrane releasing their product (106-110).

Rodent salivary gland cells contain several of the best characterized hormone-receptor systems. It is possible to study hormone

action and receptor relationship in the same cell. Activation of alfa-adrenergic receptors stimulate saliva production and calcium influx (111); the activation of the beta-adrenergic receptors results in secretion and synthesis of alfa-amylase and other proteins (112-114). These cells also have cholinergic receptors (109), a sensitivity to substance P (111) and $1,25(OH)_2D_3$ receptors (115).

Among salivary glands, parotids have been the most extensively studied because they are easily activated and blocked by a wide variety of compounds. Furthermore, parotids contain a relatively pure population of acinar (endpieces) secretory cells. Rat parotid glands contain an unusually high calcium concentration and 80% of it is associated with alfa-amylase in the secretory granules (116-118). Acinar cell membrane has all of the receptors mentioned above, as well as adenylate cyclase (118) and guanylate cyclase (112) activities. Membrane-bound ribosomes account for 86% of total ribosomes, indicating a high synthesis of exportable proteins (119). Parotid gland cells have the two types of cAMP-dependent protein kinases (120) with the type II isoenzyme accounting for 90% of the total kinase activity (121), several cyclic nucleotide phosphodiesterases and a high ratio of adenylate cyclase/phosphodiesterase (120).

The synthetic catecholamine isoproterenol (IPR) interacts with the beta-adrenergic receptor of parotid gland cells and triggers

several biochemical processes and physiological responses. Among these, are the activation of adenylate cyclase and cAMP production (118), phosphorylation of nuclear acidic proteins (122), carboxymethylation of proteins (123), synthesis and exocytosis of exportable proteins (124), phosphatidylethanolamine methylation (125), activation of protein kinases and specific cytoplasmic protein phosphorylations (126-129).

Cell proliferation.

The major salivary glands have been extensively used in the study of cell proliferation. Adult rodent salivary gland cells are not engaged in proliferation, however, injection of (dl)-isoproterenol proliferatively activates these cells which synthesize DNA 20-30 hours later. This is followed by a wave of mitosis resulting in an increase in cell number and enlargement of the glands (11,130,131).

The salivary gland model possesses several advantages over other systems. For example, the prereplicative period can be followed in vivo under physiological conditions; the cells are activated by a single injection of (dl)-isoproterenol (130).

The activation process is not blocked by hypophysectomy, adrenalectomy or extirpation of the superior cervical ganglion (132). Isoproterenol activates 60-80% of the acinar cells but not duct cells or interstitial cells (133). The activation process seems to be

mediated directly by isoproterenol because inhibition of its intracellular metabolism enhaces the DNA synthetic response (134) and the response to isoproterenol is dose-dependent (130). Table III lists the known prereplicative events which occur in rodent salivary glands in response to (dl)-isoproterenol.

Hypothesis.

The aim of this research was to amplify the role of CaM in cell proliferation. The model investigated was isoproterenol-activated rat parotid glands.

It has been shown that "normal" cells *in vivo* depend on a physiological calcium concentration for the initiation of DNA synthesis. This critical extracellular calcium requirement is regulated by the calcium-homeostatic hormones.

Calcium, parathyroid hormone, $1,25(OH)_2D_3$ and calcitonin maintain the proliferative capability of several organs and tissues (e.g. liver, parotid glands, bone marrow and thymus). Furthermore, calcium modulates the progression of the cell division cycle through the activity of cAMP, protein kinases, thymidylate synthetase, ribonucleotide reductase, etc.

Several authors suggest that an increase in the intracellular calmodulin concentration during the late G_1 phase is a requirement for the progression into S phase (73-84). Other lines of evidence

TABLE III. Time course of events occurring in rodent salivary glands after an injection of (dl)-isoproterenol.

ENZYME OR PROCESS	TIME (h) AFTER IPR	REFERENCES
Adenylate cyclase	.04	135
cAMP and cGMP	.25	136,137
Chromatin template activity	1-18	138
RNA	1-24	139-142
Alfa-amylase secretion	2	143
Carbamyl-P-synthetase and aspartate transcarbamylase	4-6	144
Ornithine decarboxylase, putrescine and S-adenosyl-Met decarboxylase	8	145,146
Glycogen synthesis	8-36	147
^{3}H-Leu into non-histone nuclear proteins	12	148
cAMP	12	149
Thymidine kinase and DNA polymerase	18-48	130,150
$PO_4^{=}$ into non-histone nuclear proteins	20	122
Deoxythymidylate kinase and deoxythymidylate synthetase	20-36	151

support the hypothesis that calmodulin function(s) during the late G_1 phase, rather than an increase in calmodulin concentration, are related to the initiation of DNA synthesis (85,86).

If a calmodulin increase during the late G_1 phase is an absolute requirement for chromosome replication, IPR should induce this change in rat parotid tissue. To establish a close relation between CaM content in late G_1 phase and DNA synthesis, both responses must exhibit the same sensitivity to IPR doses. Moreover, the calmodulin increase in late G_1 phase must be directly related to TPTX-induced inhibition of DNA synthesis.

MATERIALS AND METHODS

Reagents.

The chemicals used were reagent grade. The water utilized was distilled and deionized. dl-isoproterenol: 1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol, Trizma: tris(hydroxymethyl)aminomethane, EGTA: ethylene glycol-bis-(beta-aminoethyl ether)N N'-tetracetic acid, albumin: bovine Fraction V, calcein: (2',7'-[(bis [carboxymethyl]-amino)-methyl]-fluorescein and calf thymus DNA, were all obtained from Sigma Chemical Co. (St. Louis, MO.). Sephadex G-25 (medium) columns PM-10, DEAE-Sephacel and CNBr activated Sepharose 4B were obtained from Pharmacia Fine Chemicals (Montréal, Que.); AFFIGEL Phenothiazine, DEAE-AFFIGEL BLUE, acrylamide, N,N'-methylene-bis(acrylamide), ammonium persulfate, tetramethylendiamine (TEMED), sodium dodecyl sulfate (SDS) and Bradford Reagent were all obtained from Bio-Rad Laboratories (Missisagua, Ont.). Pluronic F-38 was obtained from BASF-Canada (Rexdale, Ont.). ^{3}H-methyl-thymidine (sp. act. 20 Ci/mmol, conc. : 5.0 mCi; 0.060 mg in 5.0 ml) and Bolton-Hunter reagent: ^{125}I-N-succinimidyl-3-(4-dihydroxyphenol) propionate (sp. act. 2000 Ci/mmol, conc.: 1 mCi) were obtained from New England Nuclear (Boston, MA.). Halothane was purchased from Ayerst (Montréal, Que.). Calcium standard (2.0 millimoles/l) was

obtained from Orion Res. Inc. (Cambridge, MA.). Incomplete Freund's adjuvant was obtained from Difco (Detroit, MI.). ACS scintillation cocktail was obtained from Amersham (Arlington Heights, IL.). Rabbit skeletal muscle troponin C and rat skeletal muscle parvalbumin were gifts from Dr. J.P. MacManus (Biol. Sci. NRCC, Ottawa).

Animals.

Rats and two sheep were maintained in the Animal Facility of the Biological Sciences Division of the National Research Council of Canada. The rats used were males of a Sprague-Dawley specific pathogen-free strain which weighed between 190-210 g and were 7-8 weeks old. These animals were maintained on a light schedule of 12 h light/12 h dark and fed Purina chow and water ad libitum.

Calmodulin Purification.

Calmodulin was purified from rat testis using affinity chromatography procedures according to Jamieson and Vanaman (152), followed by anion-exchange chromatography according to MacManus (153). Testicles were obtained from overweight rats by standard surgical procedures, immediately frozen in liquid nitrogen and stored at $-80^{\circ}C$. Testis were weighed and thawed before homogenization. Tissue was homogenized in a commercial blender for 1 min in 3 volumes of cold buffer: 20 mM imidazole, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM beta-

mercaptoethanol, 2 mM EGTA (adjusted to pH 6.5 with HCl). The homogenate was heated during 5 min at 80°C, cooled immediately and centrifuged at 10 400 X g for 30 min at 4°C. The pellet was discarded and ammonium sulfate (313 g/l) added to the supernatant. The pH was adjusted to 7.0 with 1N ammonium hydroxide and stirred at 4°C for 1 h. The mixture was centrifuged at 10 400 X g for 30 min at 4°C. The supernatant was collected and adjusted to pH 4.0 with 1N sulfuric acid in 50% ammonium sulfate, then stirred for 1 h at 4°C. The mixture was again centrifuged at 10 400 X g for 30 min at 4°C and the supernatant was discarded. The pellet was resuspended in 50 ml of buffer consisting of 50 mM Tris, 0.10 mM $CaCl_2$, 1 mM beta-mercaptoethanol and the pH adjusted to 7.5 with HCl, and finally dialyzed overnight at 4°C against 4 l of the same buffer.

AFFIGEL Phenothiazine Chromatography.

Calmodulin binds phenothiazines in presence of calcium. The use of such drugs facilitates the purification of CaM.

The dialyzed material was then poured onto a 1.5 X 30 cm column of AFFIGEL Phenothiazine with a flow rate of 20 ml/h. The effluent was recorded with a single path monitor unit, an optical unit at 280 nm and a recorder with a paper velocity of 0.5 mm/min. The effluent was collected with a Gilson microfractionator in 5.0 ml/ fractions. Calmodulin bound to the column was eluted with a buffer

consisting of: 50 mM Tris, 5 mM EGTA, 1 mM beta-mercaptoethanol and pH adjusted to 7.5 with HCl. Fractions containing calmodulin were pooled and dialyzed against 4 l of water overnight. All the operations were carried out at 4°C. Calmodulin was freeze-dried, weighed and stored at -20°C.

DEAE-Sephacel Chromatography.

Calmodulin obtained from the AFFIGEL Phenothiazine chromatography step was dissolved in 50 ml of a buffer containing 20 mM imidazole, 20 mM NaCl, 5 mM $MgCl_2$ and 5 mM beta-mercaptoethanol and the pH adjusted to 6.5 with HCl. The sample was poured onto a DEAE-Sephacel column (1.5 X 30 cm) with a flow rate of 8 ml/h and 5 ml fractions were collected. The column was washed with 150 ml of the same buffer, followed by 100 ml of the loading buffer containing 100 mM NaCl. Calmodulin was eluted with an increasing gradient of 100-600 mM NaCl. The fractions were recorded with a single path monitor unit, an optical unit at 280 nm and a recorder with a paper velocity of 2 mm/min. Calmodulin containing fractions were pooled and dialyzed against 4 l of water overnight. All procedures were carried out at 4°C. Calmodulin was freeze-dried, weighed and stored at -20°C.

Ultraviolet Scan.

One milliliter of calmodulin containing fractions obtained from

DEAE-Sephacel chromatography was scanned using a Beckman DU-8 spectrophotometer provided with a wavelenght scan program from 350 nm to 220 nm.

Electrophoresis.

Polyacrylamide gel electrophoresis was carried out according to the system described by Laemmli (154) with minor modifications. The acrylamide concentration was 4% in 0.125 M Tris and 0.1% SDS (pH 6.8) for the stacking gel. The separatory gel (0.5 X 5.5 cm long) contained 10% of the monomer in 0.375 M Tris and 0.1% SDS (pH 8.8). The gels were polymerized by adding 25 µl of 0.1% ammonium persulfate and 25 µl of TEMED. The electrode buffer was 0.025 M Tris, 0.19 M glycine and 0.1% SDS (pH 8.8). Samples were prepared in 50 µl of electrode buffer containing 1 mM EGTA, 5% beta-mercaptoethanol, 5 µl of bromophenol blue and 3 to 4 sucrose grains. Before loading, the samples were immersed in boiling water for 2.5 min. The gels were run at 1.0 mA/gel for 30 min and at 1.5 mA until the bromophenol blue dye reached 0.5 cm from the bottom of the tube. The gels were stained overnight with 0.04% Coomassie blue and destained with 10% acetic acid by repetitive washings.

Calmodulin Oxidation.

The oxidation of calmodulin was performed according to the

method described by Hirs (155). Five vol. of 30% H_2O_2 were mixed with 95 vol. of formic acid (performic acid) and allowed to stand for 2 h at room temperature and 1/2 h at 0°C. The calmodulin sample (25 mg in 2.5 ml of formic acid + 0.5 ml of methanol) was allowed to stand at 0°C for 30 min. Five milliliters of performic acid were added to the calmodulin sample and allowed to stand for 2.5 h at 0°C and then diluted with 400 ml of water and freeze-dried.

Calmodulin Iodination.

The iodination of calmodulin was conducted according to the Bolton and Hunter procedure (156). One mCi of ^{125}I-Bolton-Hunter reagent was evaporated under a stream of nitrogen. Five µg of CaM in 10 µl of 0.125 M borate buffer (pH 8.4) were added to the ^{125}I-Bolton-Hunter reagent and the mixture incubated for 30 min at 4°C. The reaction was stopped by adding 150 µl of 0.2 M glycine in borate buffer (pH 8.4) and allowed to stand for 15 min at 4°C. The mixture was poured onto a column (0.7 X 20 cm) of Sephadex G-25 M and washed with 0.05 M NaH_2PO_4, 0.05% NaN_3, 0.25% (w/v) gelatin, 0.1 M NaCl, pH 7.5. Fractions of 1.5 ml were collected and 10 µl of each fraction were added to a scintillation vial plus 10 ml of scintillation cocktail and counted in a Beckman LS-255 liquid scintillation counter. Fractions containing ^{125}I-CaM were pooled, cpm/100 µl determined and then stored in small aliquots at -20°C.

Calmodulin-Sepharose Coupling.

Calmodulin was coupled to cyanogen bromide activated Sepharose 4B according to Klee and Krinks (157). Forty five mg of calmodulin were dissolved in 50 ml of 0.1 M sodium borate, 1.0 M $MgCl_2$, 0.02 M $CaCl_2$, pH 8.2 and mixed with 10 g of CNBr activated Sepharose 4B previously swollen in 15 ml of 1 mM HCl (washed with 1 liter of 1 mM of HCl and then equilibrated with 500 ml of borate buffer). The mixture was agitated for 18 h at 4°C. The resin was then washed with 500 ml of borate buffer in a glass funnel connected to a vacuum source. The resin was resuspended in 50 ml of 0.5 M aminoethanol, adjusted to pH 8.0 with HCl and mixed for 24 h at 4°C. Finally, the resin was washed with 500 ml of 20 mM Tris, 1.0 mM $MgCl_2$, 0.02 mM $CaCl_2$, 0.02% (w/v) NaN_3, packed into two columns (1.0 x 10 cm), and stored at 4°C. The amount of calmodulin bound to Sepharose was determined by radioimmunoassay of the buffer used for washing the resin after incubation with calmodulin.

Immunization.

The immunization procedure was conducted according to Van Eldik and Watterson (158) with some modifications.

Sheep A and sheep B were shaved around their necks. Five or two mg of oxidized calmodulin were dissolved in 1.0 ml of water and then emulsified with 1.0 ml of incomplete Freund's adjuvant. The

emulsion was injected intradermally in four different places on the back of each sheep's neck.

Antibody Purification.

The immunoglobulin fraction of serum was obtained according to the procedure of Harboe and Ingild (159) and subsequently, the specific antibody which recognizes CaM was isolated according to the method described by Dedman et al. (160).

Two hundred ml of blood were obtained from the jugular vein of the sheep in each bleed. The blood was allowed to clot for 2 h at room temperature, followed by 18 h at 4°C. Serum was obtained by centrifuging the clot at 2 520 X g for 10 min at 4°C. The crude immunoglobulin fraction was precipitated by adding solid ammonium sulfate to the serum (25 g/100 ml), stirring the mixture slowly and then allowing it to stand for 18 h at 4°C. The mixture was centrifuged at 10 300 X g for 10 min at 4°C. The precipitated proteins were washed twice with 1.75 M ammonium sulfate and centrifuged at 10 300 X g for 10 min at 4°C each time. The final pellet was suspended in 50 ml of water and dialyzed overnight against 4 l of water and at 4°C. The dialysis bags were changed into a beaker with 50 mM sodium acetate (pH 5.0 adjusted with acetic acid) and dialyzed overnight against 4 l of the same buffer followed by an additional overnight dialysis against water. All dialyses were carried out at 4°C.

DYE-Ligand Chromatography.

The immunoglobulins from the last step were centrifuged at 16 300 X g for 15 min at 4°C. The supernatant, containing the immunoglobulins was dialyzed overnight at 4°C against 4 l of 20 mM KH_2PO_4 adjusted to pH 8.0 with KOH. The immunoglobulins were poured onto a DEAE-AFFIGEL BLUE column (1.5 X 30 cm) previously equilibrated with phosphate buffer with a flow rate of 15 ml/h. The effluent was collected in 10 ml fractions and recorded with a single path optical unit at 280 nm and registered on paper with a recorder velocity of 0.2 mm/min.

After loading the sample, the column was extensively washed with the same phosphate buffer until the pen of the recorder reached the basal line. The immunoglobulins are not retained in this column, however, some proteins like albumins, proteases, clotting factors are adsorbed by the DEAE groups and the dye ligand. The unwanted proteins were washed off the column with o.4 urea followed by repetitive washings with water. The resin was stored at 4°C in phosphate buffer plus 0.02% NaN_3 until next use. The non-adsorbed fractions containing immunoglobulins were pooled and dialyzed overnight against 100 mM boric acid, 25 mM sodium borate, 75 mM NaCl (pH 8.4). All procedures were conducted at 4°C.

Calmodulin-Sepharose Chromatography.

The immunoglobulins obtained from the DEAE-AFFIGEL BLUE column were poured onto the (1.0 X 16 cm) calmodulin-Sepharose column previously equilibrated with: 100 mM boric acid, 25 mM sodium borate, 75 mM NaCl (pH 8.4). The sample was loaded at a flow rate of 15 ml/h. Fractions of 10 ml were collected and the absorbence was recorded with a dual path monitor and an optical unit at 280 nm and registered on paper with a velocity of 0.2 mm/min.

The immunoglobulins which recognize CaM as antigen were eluted with a solution consisting of 6 M urea, 10 mM EGTA, 0.8 M NaCl and adjusted to pH 4.5 with HCl. The flow rate was changed to approximately 5 ml/h and the velocity of paper of the recorder to 2 mm/min. One milliliter fractions were collected and neutralized immediately with 50 µl of 3 M Tris. Samples containing anti-CaM were desalted using the PM-10 columns in the following way: every 2 fractions eluted from the CaM-Sepharose column were mixed and poured onto a PM-10 column, washed with 0.5 ml of the borate buffer and eluted with 3.0 ml of the same buffer. After desalting, the samples were dialyzed overnight against a buffer consisting of 10 mM imidazole, 100 mM NaCl and adjusted to pH 7.4 with HCl. All procedures were conducted at 4 °C. After dialysis, the antibody concentration was estimated by measurement of the protein concentration and stored

at -20°C in 1.0 ml aliquots. The columns were extensively washed with the loading buffer plus 0.02% Na_2N_3 (w/v) and stored at 4°C until next use.

Radioimmunoassay.

The radioimmunoassay was conducted according to the procedure described by MacManus et al. (73). The assay was performed using a RIA buffer of 125 mM Tris, 75 mM NaCl, 1 mM EGTA, 20 µg/l of albumin and adjusted to pH 8.4 with HCl. The assay was performed in plastic tubes and the final volume of the reaction was 500 µl. The reaction mixture contained the following: RIA buffer, 40 000 cpm of ^{125}I-CaM (previously diluted in RIA buffer), standard amounts of calmodulin or tissue extracts (diluted with RIA buffer) and 100 µl of the appropriate amount of anti-CaM (diluted in RIA buffer). The tubes were incubated for 2 h at room temperature, and then for 18 h at 4°C. The antigen-antibody complex was precipitated by adding 1.0 ml of 21% Pluronic F-38 and allowed to stand for 1 h at room temperature. The tubes were centrifuged at 600 X g for 25 min. The supernatant was then aspirated and the precipitate counted using a Beckman Biogamma counter.

Sample Preparation.

Tissues to be tested for CaM were removed from rats under

halothane anaesthesia by standard surgical procedures. The samples were cleaned of lymph nodes and adipose tissue and immediately frozen in liquid nitrogen and stored at -80°C until assayed. All samples were removed between 08:00 and 17:00 h. Samples were thawed at 0°C and homogenized 1:10 (w/v) with the isolation medium consisting of 10 mM imidazole, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM beta-mercaptoethanol and the pH adjusted to 7.5 with HCl. The homogenization was conducted using a teflon pestle-glass TRI-R homogenizer at 3000 r.p.m. and 10 up-down strokes. The samples were homogenized in an ice-water bath at 4°C.

Supernatant or pellet fractions were obtained by centrifugation of the total homogenate at 105 000 X g for 30 min at 4°C in a Beckman L2 65B ultracentrifuge. The supernatant and the particulate pellet were diluted to the original volume with isolation medium. Twenty five microliters of the total, supernatant or pellet fractions were taken and used for protein determination. Total, supernatant and pellet fractions were heated at 80°C for 5 min and cooled immediately. The three fractions were re-centrifuged at 105 000 X g for 30 min at 4°C and the resulting supernatant stored in 100 µl aliquots at -80°C until assay.

DNA Synthesis.

The determination of DNA synthesis in rat parotid gland acinar

cells was conducted according to the procedure described by Tsang et al. (149). Parotid glands were removed by surgery from light ether anaesthetized rats previously injected with (dl)-isoproterenol or saline solution. One hour before sacrifice, rats were administered intracardially with 1.25 μCi/g body weight of ^{3}H-methyl-thymidine while under light ether anaesthetic. Parotid glands were cleaned of lymph nodes and adipose tissue. The glands were immediately frozen in liquid nitrogen and stored at -80°C for no more than 48 h before assay. All samples were removed between 09:00 and 17:00 h because of circadian variations in DNA synthesis (161).

DNA was extracted according to the method described by Hopkins et al. (162). Samples were thawed at 0°C and homogenized with 4 ml of cold 10% TCA per 100 mg of parotid tissue using a teflon pestle-glass homogenizer TRI-R at 3000 r.p.m. and 10 up-down strokes. The samples were homogenized in an ice-water bath at 4°C. The homogenate was centrifuged at 700 X g for 10 min at 4°C. The supernatant was discarded and 4.0 ml of 1 N NaOH added to the pellet followed by mixing until it was broken. Samples were tightly capped and allowed to stand overnight at room temperature. The next day, samples were chilled for 20 min followed by the addition of 2.0 ml of 2 N HCl in 15% TCA, mixed and centrifuged at 700 X g for 10 min at 4°C. The supernatant was discarded and 4.0 ml of cold 10% TCA added to the pellet, mixed until completely broken and centrifuged at

700 X g for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 4.0 ml of 5% TCA. The samples were heated at 85°C for 20 min, cooled and centrifuged at 700 X g for 10 min at 4°C. One milliliter of the supernatant was added to 10 ml of ACS scintillation cocktail. The radioactivity from ^{3}H-labelled DNA was measured with a L2-255 liquid scintillation counter. The concentration of DNA extracted was measured according to the method of Burton (163) as modified by Hopkins et al. (162). Five hundred µl of the DNA extract were mixed with 0.5 ml of 5% TCA and the mixture was agitated and then 10 µl of 5% acetaldehyde were added and the mixture was mixed once again. Finally, 2.0 ml of diphenylamine reagent (20.0 g diphenylamine, 56 ml of 70% perchloric acid, 1000 ml of acetic acid, prepared just before the assay) were added, 1.0 ml at a time with mixing between additions. The samples were tightly capped and allowed to stand in the dark overnight at room temperature. The samples were then returned to room light for 30 min and absorbance determined at 600 nm in a SP 30 Pye Unicam spectrophotometer. The samples were measured against a standard curve constructed with increasing amounts of calf thymus DNA between 10-150 µg and treated in the same manner as the sample. The results were expressed as cpm/µg of DNA extracted.

Calcium Determination.

The determination of plasma calcium concentration was conducted

according to Rixon and Whitfield (35) using the fluorometric method of Borle and Briggs (164). Five hundred µl of blood were taken by cardiac puncture. Blood was collected in syringes previously moistened with a saline solution of heparin (15 mg heparin/5 ml 0.9% NaCl). The blood samples were centrifuged at 700 X g for 10 min at 4 °C and the plasma fraction was stored at 4 °C for no more than 24 h before assay. The plasma calcium concentration was determined using a Fiske automatic fluorometric titrator (Fiske Assoc. Inc., Uxbridge, MA.). One hundred µl of plasma were added into a mixture of 30 mg calcein in 12 ml of 1N KOH. The resulting fluorescence was quenched by titration with 0.25 M EGTA. The values were read against a standard curve which was linear between 2-12 mg of calcium/100 ml.

Thyroparathyroidectomy.

TPTX was carried out according to Ingle and Griffith (165) on halothane anaesthetized rats. A small incision was made in the skin on the larynx. The sublingual glands were pulled apart and another small longitudinal incision was made in the musculi covering the trachea. The isthmus was broken and all the thyroparathyroid tissue visible with the naked eye pulled out. The tissues were rejoined and the incision on the skin closed with surgical staples. The SHAM operated animals were surgically treated in the same way, except

the thyroparathyroid complex was not removed.

TPTX halved the plasma calcium concentration. However, the presence of extra parathyroid tissue in some rats minimizes the TPTX effect (165). If the plasma calcium concentration after TPTX was above 1.50 mM (6 mg of calcium/100 ml of plasma), the TPTX surgery was considered not successful and the results obtained were not used.

Protein Determination.

The method of Bradford (166) was used to determine the concentration of protein in a specific sample. One hundred µl of sample properly diluted with water were mixed with 5 ml of Bradford's reagent previously diluted 1:5 with water and mixed. The absorbance of the mixture at 595 nm was measured within the next 10 min using a SP 30 Pye Unicam spectrophotometer. The samples were compared against a standard curve constructed using IgG as reference which gave a linear relation between 10-125 µg.

Statistical Analysis.

Statistical analysis was performed by appropiate analysis of variance (167). Since irregular replicates were occurring within the assays, the least significant difference (LSD) was used to determine differences between groups.

RESULTS

Calmodulin Purification.

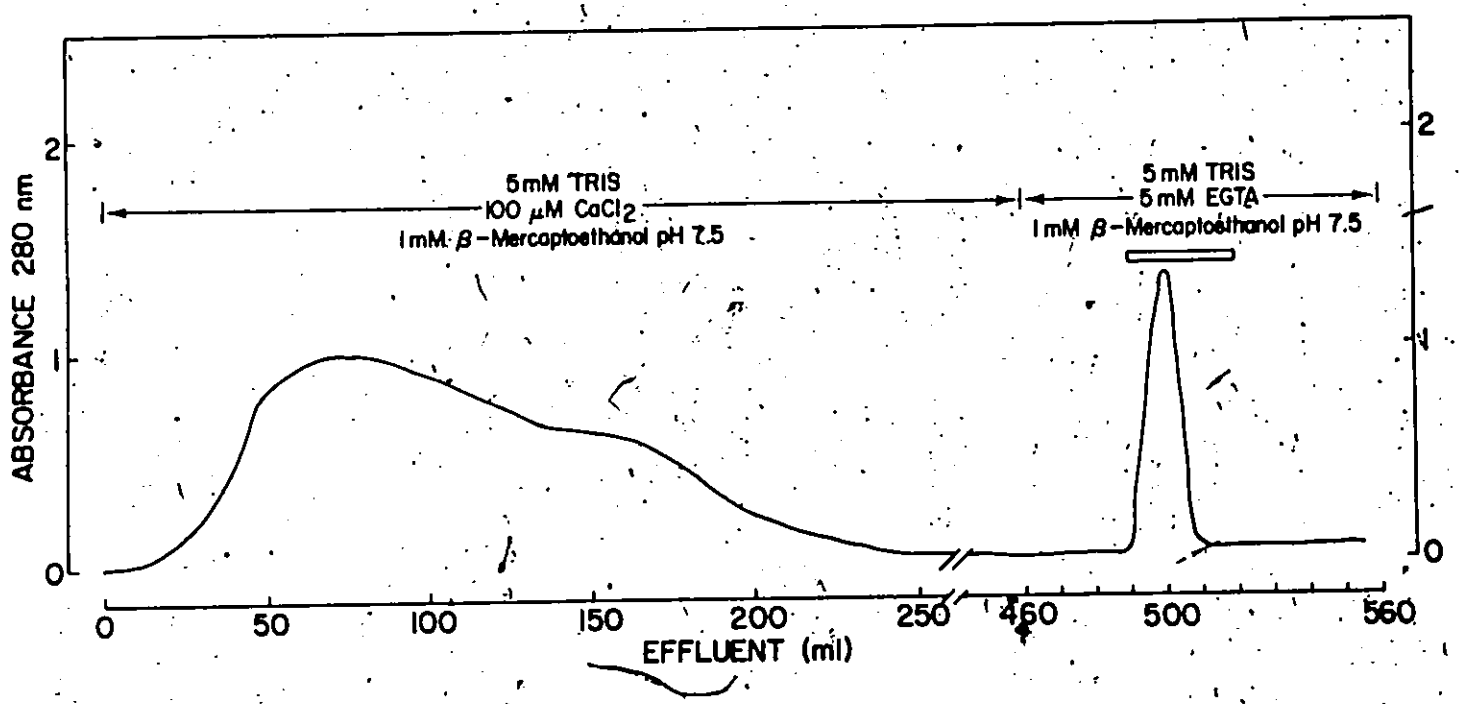
Calmodulin was purified from 300 g of rat testis. The pellet obtained after isoelectric precipitation at pH 4.0 was resuspended in 50 ml of 50 mM Tris, 300 mM NaCl, 100 μM $CaCl_2$, 1 mM beta-mercaptoethanol with the pH adjusted to 7.5 with HCl. The dissolved pellet was poured onto an AFFIGEL Phenothiazine column. Most of the non-calmodulin proteins were eluted by extensively washing the column with the loading buffer (Fig. 2). Calmodulin was eluted with a buffer of 50 mM Tris, 5 mM EGTA, 1 mM beta-mercaptoethanol at pH 7.5. Calmodulin containing fractions were collected, pooled and dialyzed against 4 l of water. The yield of CaM (weight of lyophilized protein) was 82 mg or 273 mg/Kg of testis.

Calmodulin was further purified by anion-exchange chromatography using DEAE-Sephacel. This step was done to eliminate minor contaminants which may co-elute with calmodulin during the affinity chromatography step.

Calmodulin was dissolved in 50 ml of a buffer containing 20 mM imidazole, 20 mM NaCl, 5 mM $MgCl_2$ and 5 mM beta-mercaptoethanol, adjusted to pH 6.5 with HCl. The sample was poured onto a DEAE-Sephacel column and washed with 300 ml of the loading buffer plus

Figure 2. The purification of calmodulin by phenothiazine affinity chromatography.

The proteins obtained from isoelectric precipitation of the crude extract were poured onto an AFFIGEL phenothiazine column (see Materials and Methods). Non-calmodulin proteins were washed with 5mM Tris, 100μM $CaCl_2$, 1mM beta-mercaptoethanol (pH 7.5). Calmodulin was eluted with the same buffer except $CaCl_2$ was substituted by 5mM EGTA. The bar indicates the effluent pooled and dialyzed extensively against water.


5mM TRIS
100 μM CaCl2
1mM β-Mercaptoethanol pH 7.5
5mM TRIS
5mM EGTA
1mM β-Mercaptoethanol pH 7.5
ABSORBANCE 280 nm
2
1
0
0
50
100
150
200
250
460
500
560
EFFLUENT (ml)

100-ml of the same buffer containing 100 mM NaCl. Calmodulin was eluted with a linear gradient of 100-600 mM NaCl (Fig. 3). It was released at a salt concentration of about 250 mM.

Ultraviolet scan of the fractions eluted by 250 mM NaCl confirmed the characteristic absorption spectra of calmodulin (Fig. 4). Protein was measured by weight after dialysis against water and lyophilization and was 37 mg or 123 mg/kg wet tissue. Calmodulin purity was also determined by SDS-PAGE using 10% gels and 50 µg of CaM/gel (Fig. 5).

Iodination of Calmodulin.

Calmodulin was iodinated by the Bolton-Hunter procedure and separated from the Bolton-Hunter reagent residues and ^{125}I-glycine Sephadex G-25 M chromatography (Fig. 6). By using this procedure, ^{125}I-CaM was eluted in the Vo of the column. Fractions 10 to 18, which contained ^{125}I-CaM were pooled and stored at -20°C in aliquots containing 1 X 10^6 cpm. Radioiodination of calmodulin was performed routinely every three weeks yielding similar results.

Calmodulin-Sepharose Coupling.

Calmodulin was coupled to Sepharose for use as a chromatographic matrix for antibody purification. Forty five mg of CaM were mixed with ten mg of CNBr-activated Sepharose 4B under the conditions

Figure 3. The purification of calmodulin by anion-exchange chromatography.

The calmodulin from AFFIGEL-phenothiazine chromatography was poured onto the DEAE-Sephacel column, as described in Materials and Methods. The column was washed with 150 ml of a buffer consisting of 20 mM NaCl, 20 mM imidazole, 1 mM $MgCl_2$, 1 mM beta-mercaptoethanol (pH 6.5), followed by 100 ml of the same buffer containing 100 mM NaCl. Calmodulin was eluted by increasing gradient from 100 mM to 600 mM of NaCl. The bar indicates the effluent containing calmodulin, pooled and dialyzed against water.

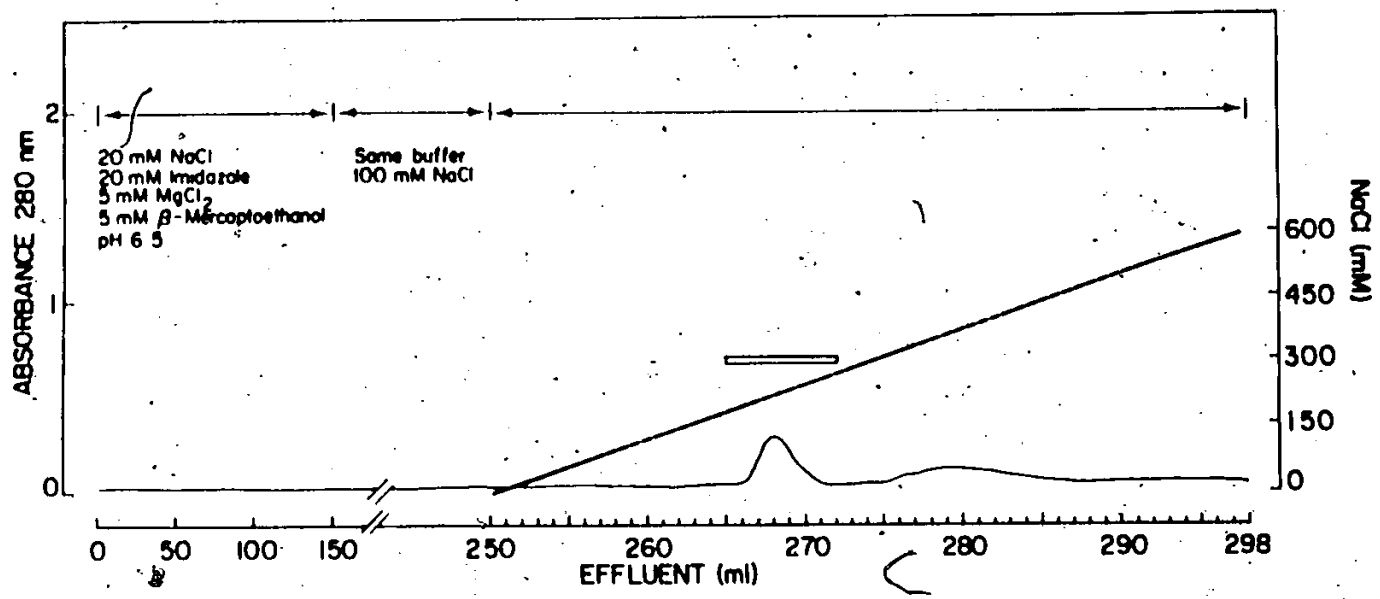

ABSORBANCE 280 nm
2
1
0
20 mM NaCl
20 mM Imidazole
5 mM MgCl2
5 mM β-Mercaptoethanol
pH 6.5
Same buffer
100 mM NaCl
NaCl (mM)
600
450
300
150
0
0
50
100
150
250
260
270
280
290
298
EFFLUENT (ml)

Figure 4. The absorption spectrum of calmodulin.

One milliliter of the protein eluted from the DEAE-Sephacel column at 250mM NaCl was scanned from 350 to 220nm on a Beckman DU-8 spectrophotometer. All the values of absorbance assigned to the peaks correspond to those previously reported for CaM by other authors (62-64).

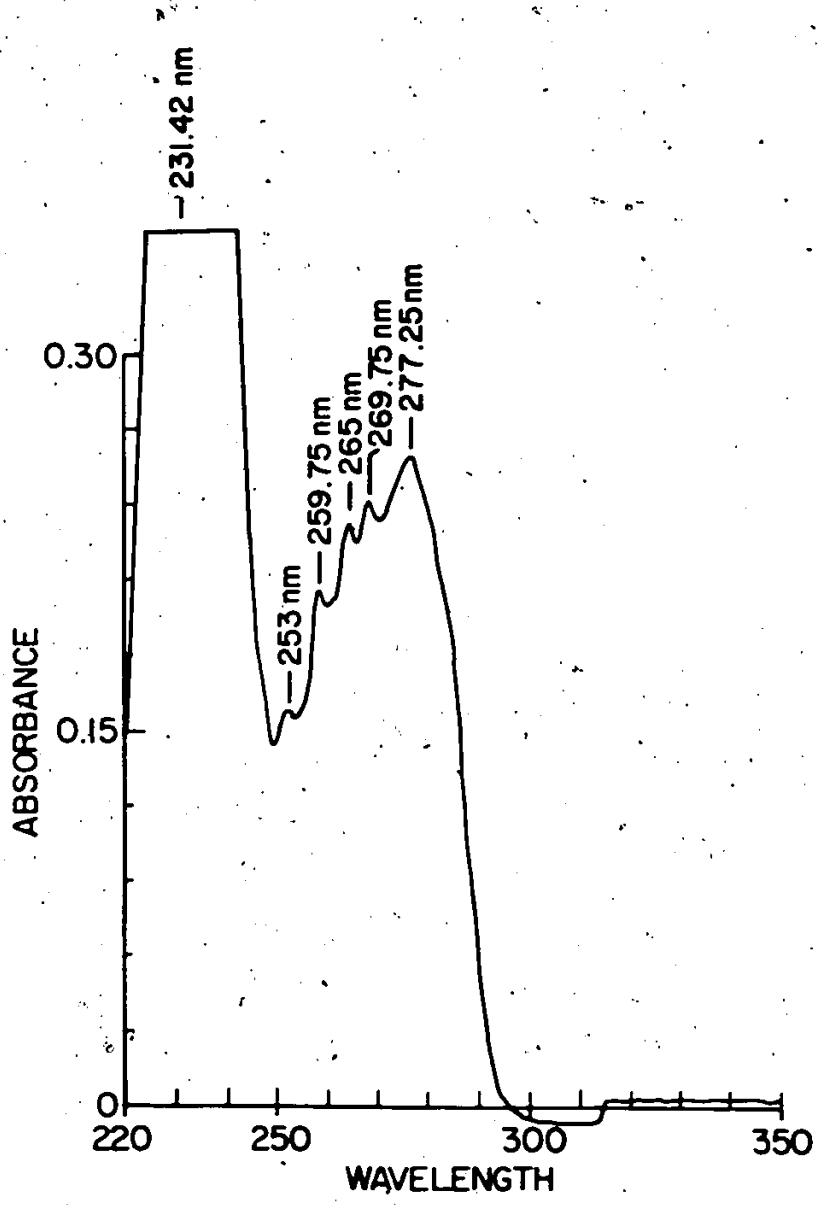

231.42 nm
253 nm
259.75 nm
265 nm
269.75 nm
277.25 nm
0.30
0.15
0
ABSORBANCE
220
250
300
350
WAVELENGTH

Figure 5. The electrophoresis of calmodulin in SDS-polyacrylamide.

Polyacrylamide gel electrophoresis was performed using 10% acrylamide and 0.2% sodium dodecyl sulfate. The samples were heat-treated (100°C) for 2.5 min in the sample buffer before electrophoresis. Fifty micrograms of calmodulin were loaded per gel. The gels were electrophoresed at 1.5 mA/gel for 30 min and continued at 1.5 mA/gel until the marker dye was at 0.5cm from the bottom of the gel. The gels were stained with 0.04% Coomassie Blue and destained with 10% acetic acid.

SDS-PAGE of CaM
(50μg / gel)

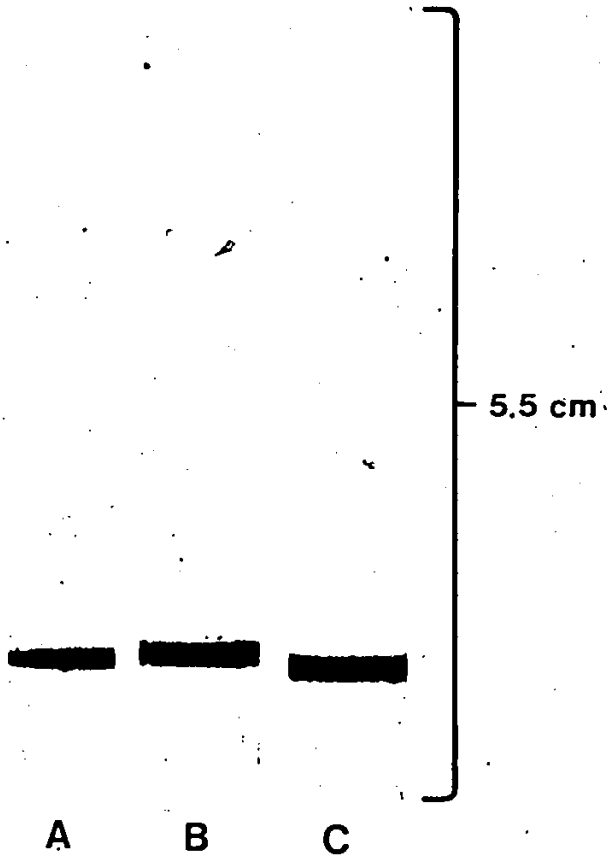
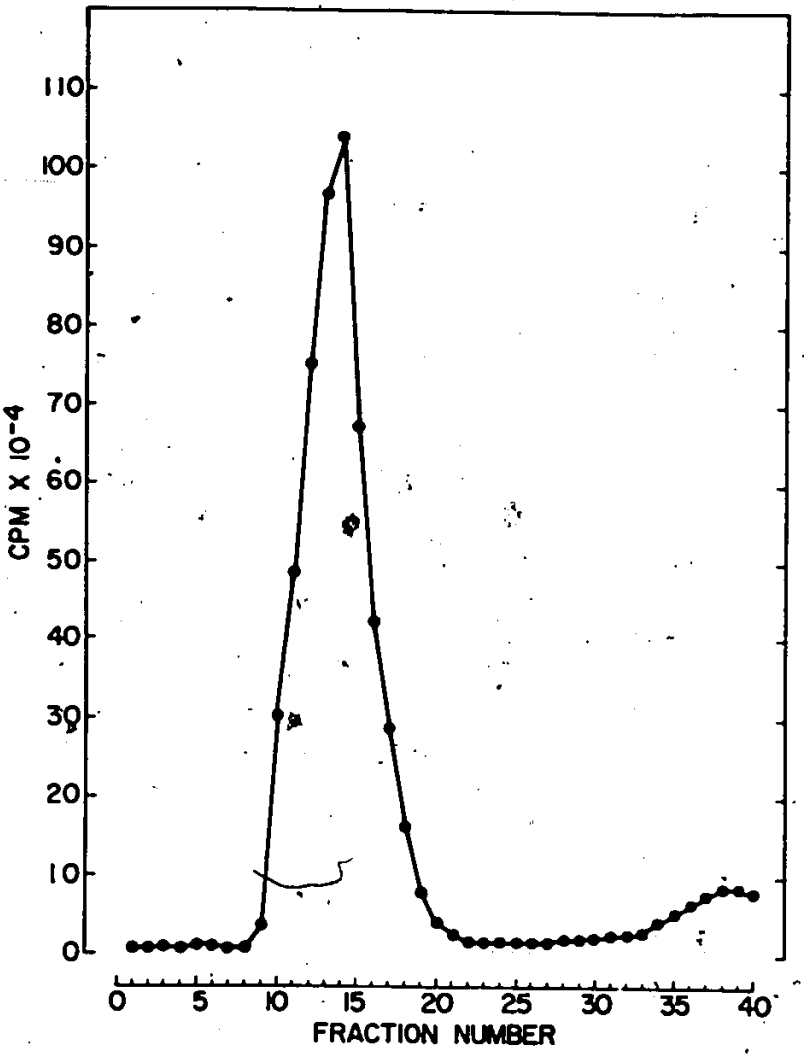


Figure 6. The purification of ^{125}I-labelled CaM by Sephadex G-25 chromatography.

Five micrograms of calmodulin were iodinated by the procedure described in Materials and Methods. The reaction was stopped by adding an excess of glycine. The mixture was poured onto a Sephadex G-25 column, and washed with a buffer containing 0.05% NaH_2PO_4, 0.05% NaN_3 (w/v), 0.25% gelatin and 0.1M NaCl (pH 7.5). Fractions of 1.5 ml were collected. The radioactivity of each fraction was tested. Fractions containing CaM (^{125}I-labelled CaM) were pooled and stored at -20°C.


CPM X 10⁻⁴
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
FRACTION NUMBER

described previously in Materials and Methods. The yield of the conjugated CaM-Sepharose was 2.5mg CaM/g gel. The CaM-Sepharose matrix was packed into columns to be used in anti-calmodulin purification.

Antibody Purification.

Serum from each sheep was processed separately. The immunoglobulins were precipitated with ammonium sulfate and partially purified using DEAE-AFFIGEL BLUE chromatography as described in Materials and Methods; the anti-CaM fraction was obtained by passing the IgG's from the Dye-ligand chromatography step on the CaM-Sepharose columns previously prepared. The column was washed overnight with the loading buffer and the anti-CaM fraction was eluted with 6 M urea, 0.8 M NaCl, 10 mM EGTA, pH 4.5 (Fig. 7). Two ml fractions were collected and rapidly neutralized with 50 µl of 3 M Tris and desalted in pre-packed PM-10 columns. Anti-CaM fractions were pooled and dialyzed against a buffer consisting of 200 mM imidazole, 100 mM NaCl, adjusted to pH 7.4 with HCl. The amount of anti-CaM obtained was measured according to the procedure described in Materials and Methods, and stored at -20°C in small aliquots.

Radioimmunoassay.

The ability of the anti-CaM IgG's to precipitate a known amount of ^{125}I-CaM was tested next (Fig. 8). The reaction mixture contained

Figure 7. The purification of anti-CaM by affinity chromatography in CaM-Sepharose.

The IgG fraction from sheep's serum was poured onto the CaM-Sepharose column. Non-bound IgG was washed with the loading buffer consisting of 100mM boric acid, 25mM sodium borate, 75mM NaCl (pH 8.4). Anti-CaM was eluted with a solution consisting of 8M urea, 10mM EGTA, 0.8mM NaCl and adjusted to pH 4.5 with HCl. The bar indicates the fractions collected, desalted in PD-10 columns and subsequently used in the radioimmunoassay.

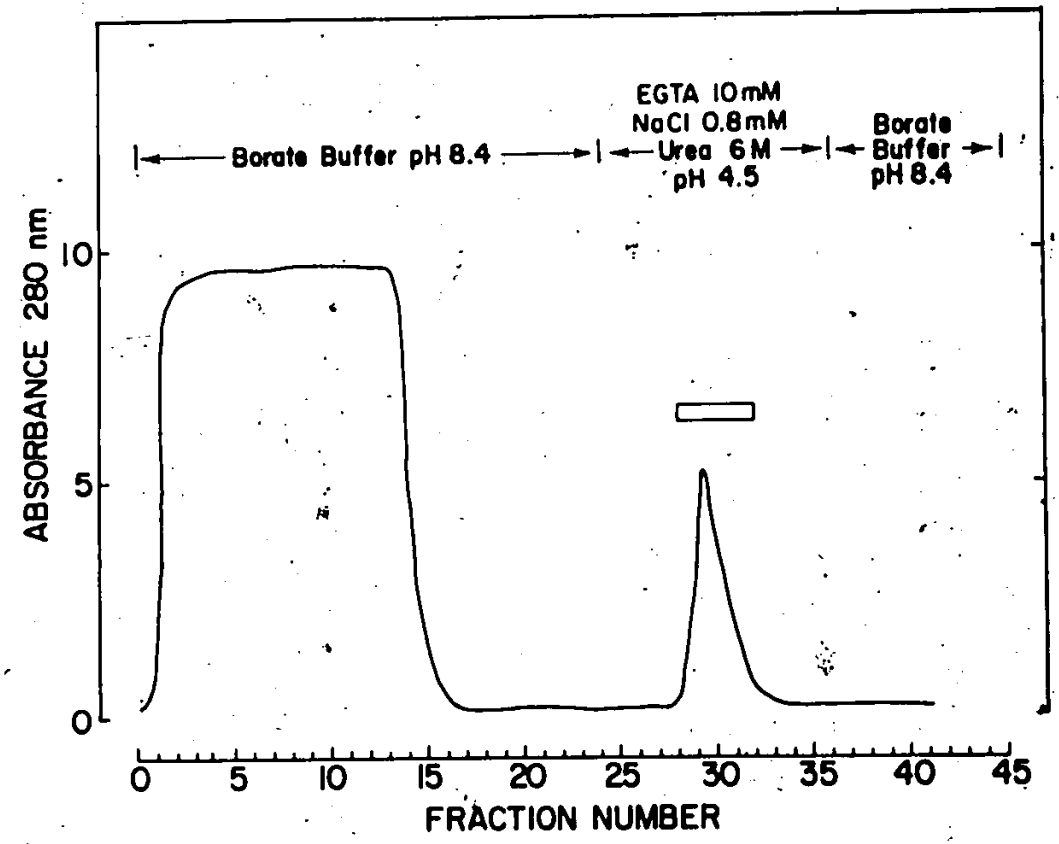
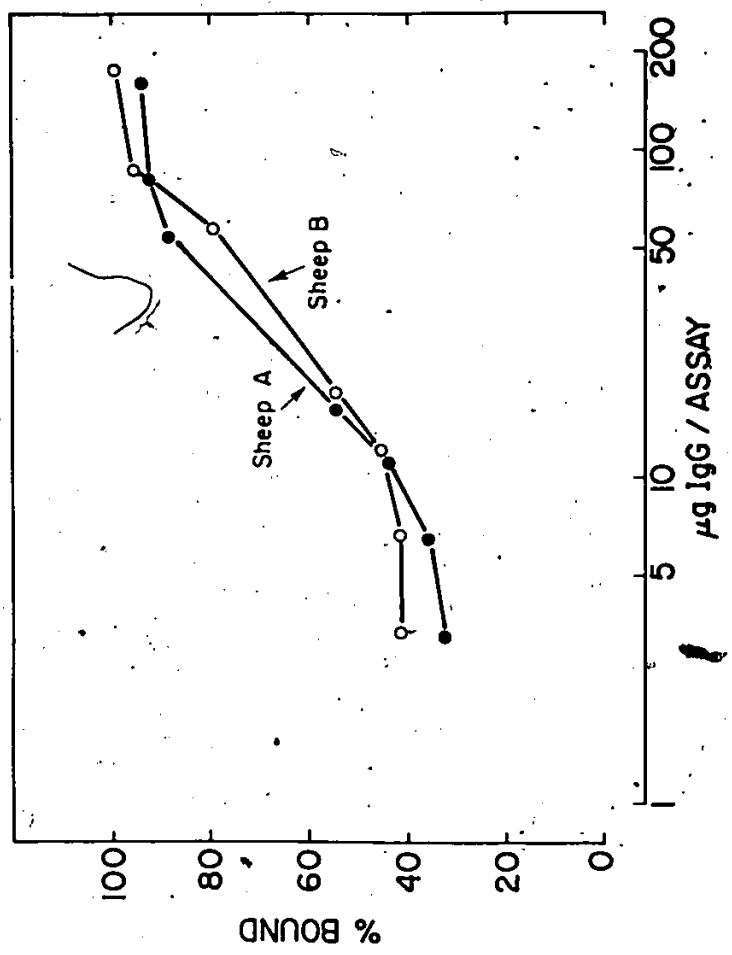

EGTA 10mM
NaCl 0.8mM
Urea 6M
pH 4.5
Borate Buffer pH 8.4
Borate Buffer pH 8.4
ABSORBANCE 280 nm
10
5
0
0
5
10
15
20
25
30
35
40
45
FRACTION NUMBER

Figure 3. The binding of anti-CaM to ^{125}I-CaM.

Various amounts of anti-calmodulin of sheep A or B were added to 40000 cpm of ^{125}I-CaM and processed as described in Materials and Methods. The ordinate represents the percentage of cpm bound to the anti-CaM. The non-specific counts per min bound were subtracted. The points are the means of triplicate values.


% BOUND
100
80
60
40
20
0
Sheep A
Sheep B
1
5
10
50
100
200
µg IgG / ASSAY

^{125}I-CaM 40 000 cpm in RIA buffer, plus increasing amounts of anti-calmodulin diluted with RIA buffer (see Materials and Methods). Each sample was assayed in triplicate and the non-specific binding was subtracted from each sample (no more than 800 cpm). The anti-calmodulin used in the assays of figure 8 was obtained from the first bleed of sheep A and B. In all subsequent assays enough anti-CaM was used to precipitate 35% of the ^{125}I-CaM (40 000 cpm) used per assay. It should be noted that the IgG fraction which did not bind to the CaM-Sepharose did not precipitate ^{125}I-CaM.

Figure 9 illustrates the immunization times and the time course of the anti-CaM production in both sheep. The results are expressed as the amount of anti-CaM necessary to precipitate 35% of the total cpm of ^{125}I-CaM present per assay.

Figure 10 illustrates a typical standard curve constructed with increasing amounts of CaM, 40 000 cpm of ^{125}I-CaM and enough IgG to precipitate 35% of the label. Each point is the mean of three separate determinations with non-specific binding subtracted. Using the described conditions, it was possible to detect CaM between 10 and 2000 ng/assay.

Standard dilution curves for CaM, troponin C and parvalbumin were generated by mixing 100 µl of the appropriate dilution of each protein into the mixture of buffer and 40 000 cpm of ^{125}I-CaM. The reaction was started by adding the anti-CaM. Rabbit skeletal muscle

Figure 9. The time-course of immunizations and antibody production.

Sheep A and B were immunized with 5.0mg of oxidized CaM at time zero. In subsequent injections were used 2.0mg of oxidized CaM/sheep. Normally, the bled was conducted 7-9 days after a CaM injection. Two hundred ml of blood were obtained at each bled. The antibody production was scored as the amount of anti-CaM needed to precipitate 25% of 40000 cpm of ^{125}I-CaM in a total binding assay, (Figure 8).

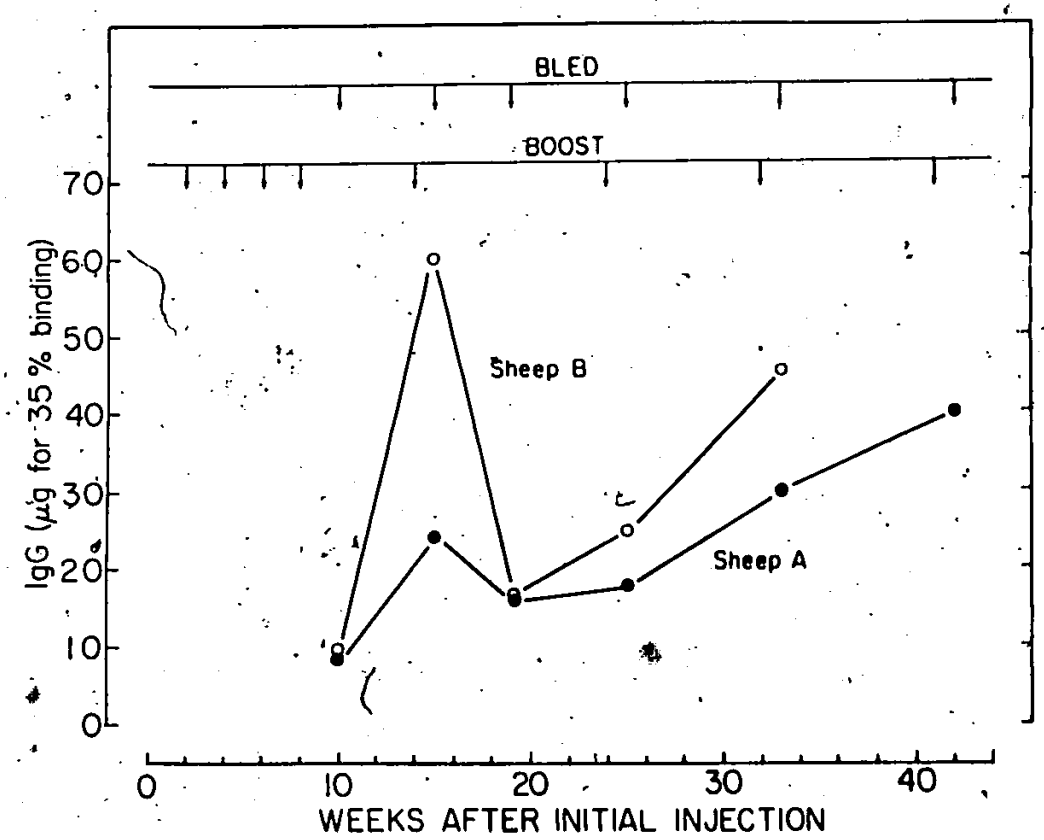
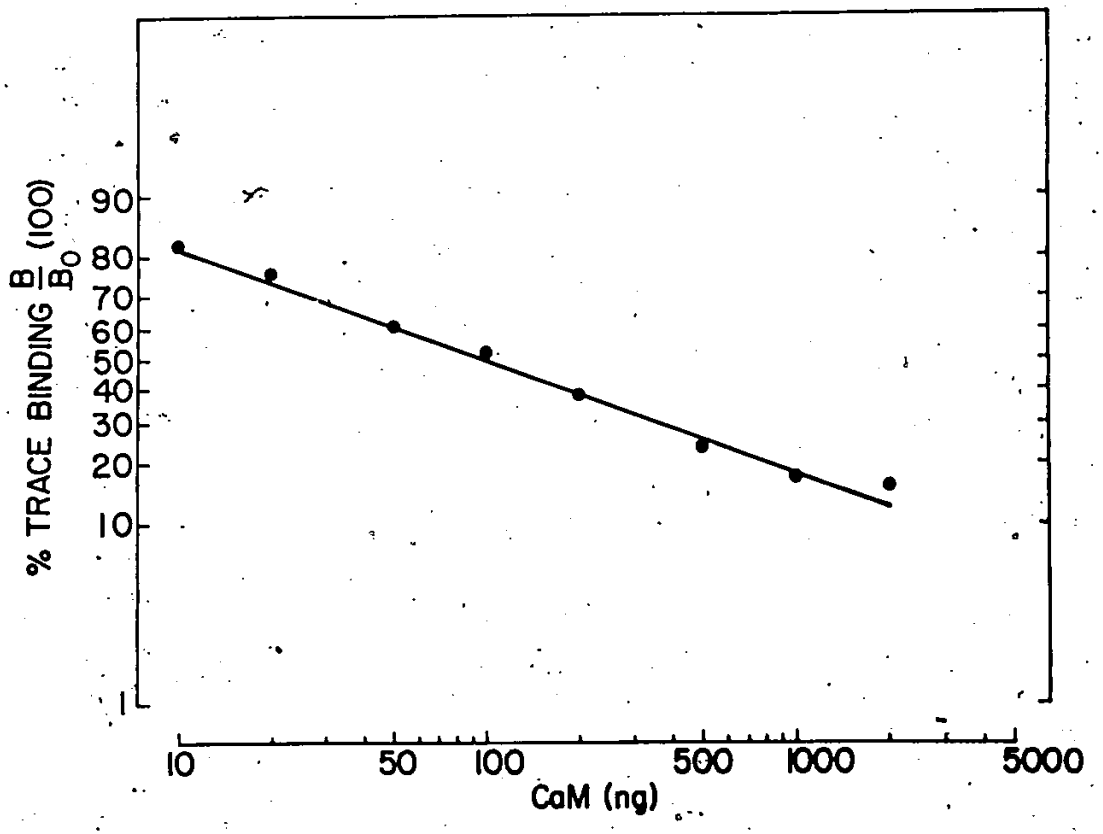

BLED
BOOST
70
60
50
40
30
20
10
0
IgG (µg for 35 % binding)
Sheep B
Sheep A
0
10
20
30
40
WEEKS AFTER INITIAL INJECTION

Figure 13. The radioimmunoassay typical standard curve.

The radioimmunoassay was conducted according to the method described in Materials and Methods. The assay was performed in a final volume of 500 µl and using a buffer consisting of 125mM Tris, 75mM NaCl, 1mM EGTA, 20 µg albumin 1 adjusted to pH 8.4 with HCl. The reaction mixture contained RIA buffer, 40000 cpm of ^{125}I-CaM standard amounts of pure calmodulin or tissue extracts and the appropiate amount of anti-CaM. The assays were processed as previously described in Materials and Methods. Non-specific binding was subtracted. The results were plotted in logit-log paper. Each point represents the mean of triplicate assays.


% TRACE BINDING $\frac{B}{B_0}$ (100)
90
80
70
60
50
40
30
20
10
1
10
50
100
500
1000
5000
CaM (ng)

troponin C showed some crossreactivity with anti-CaM IgG (Fig. 11) but approximately 500 times more troponin C was required for 30% binding. Rat skeletal muscle parvalbumin was not recognized by anti-CaM when present at a 1000 fold excess.

Determination of Calmodulin in Rat Tissue Extracts by Radioimmunoassay.

The usual sample to be assayed for calmodulin content was a parotid gland extract. To demonstrate the specificity of the radioimmunoassay, it was necessary to show that fractionation of a tissue extract and subsequent assay of the fractions resulted in a high recovery of the total CaM value. Samples were assayed in three different dilutions and by addition of known amounts of calmodulin into the samples to be assayed.

Table IV illustrates the calmodulin content of various tissues obtained from intact rats. The recoveries of calmodulin from the supernatant and pellet fractions in rat testis, parotid glands and liver were 92%, 89.24% and 88.44% respectively. The difference in calmodulin content of testis with liver or parotid glands was significant ($p < .05$). The distribution of calmodulin in rat liver and parotid glands is almost equal between supernatant and pellet of a 105 000 X g centrifugation. By contrast, rat testis calmodulin is predominantly found in the supernatant fraction (76.6%) compared

Figure 11. The specificity of anti-CaM in a competition radioimmunoassay.

Various amounts of calmodulin, troponin C and parvalbumin, were mixed with 40000 cpm of ^{125}I-CaM and enough anti-CaM to precipitate 35% of the label in a final volume of 500 µl/assay. All the assays were processed as described previously in Materials and Methods. The non-specific binding was subtracted and the results plotted on logit-log paper. Each point represents the mean of triplicate assays.

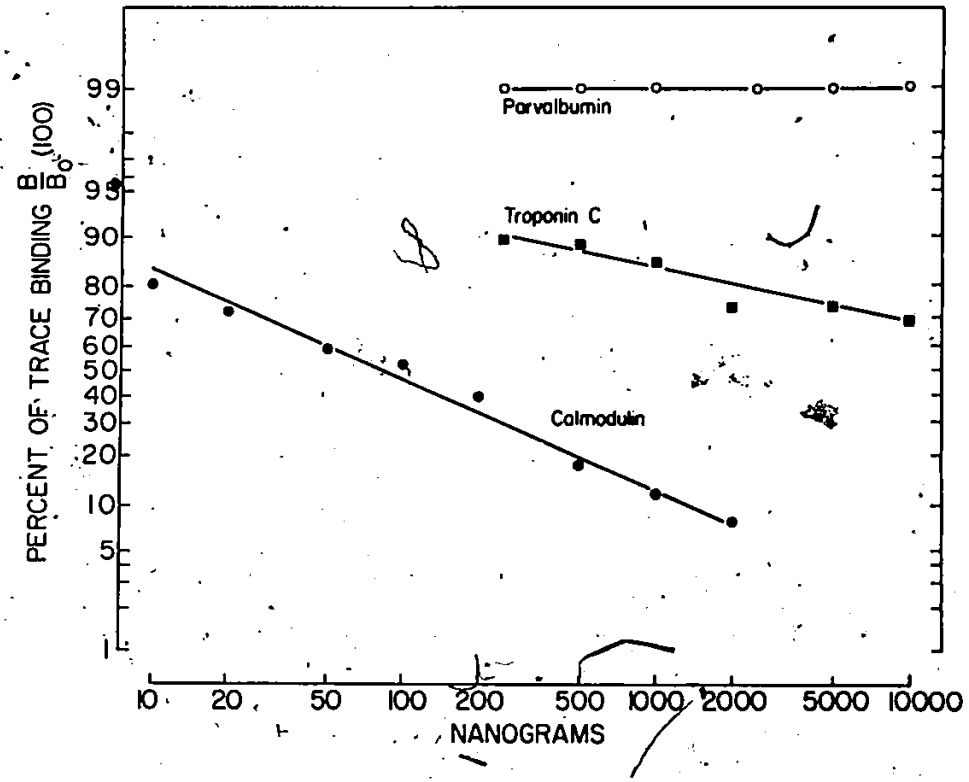

PERCENT OF TRACE BINDING $\frac{B}{B_0}$ (100)
99
95
90
80
70
60
50
40
30
20
10
5
1
Parvalbumin
Troponin C
Calmodulin
10
20
50
100
200
500
1000
2000
5000
10000
NANOGRAMS

TABLE IV. Calmodulin content in different rat tissues.

TISSUE	CALMODULIN CONTENT		
	FRACTION		
	TOTAL	SUPERNATANT 105 000 X g	PELLET 105 000 X g
TESTIS			
µg/g w wt	1206±49.89	924±90.24	196±30.58
LIVER			
µg/g w wt	495±49.20	251±32.05	155±34.50
µg/mg prot.	0.72±0.11	0.71±0.19	0.62±0.09
PAROTID GLANDS			
µg/g w wt	548±02.15	233±27.64	256±29.88
µg/mg prot.	1.13±0.15	1.15±0.16	1.53±0.26

The radioimmunoassay procedure was utilized to determine the CaM content in rat testis, liver and parotid glands. Total homogenate, high speed supernatant and pellet were assayed. The values are expressed as µg of CaM/g w wt or µg of CaM/mg protein. Values are means ± standard error of ten different determinations and some determinations assayed at three different dilutions.

with the pellet fraction (16.26%).

Effects of Isoproterenol on DNA Synthesis.

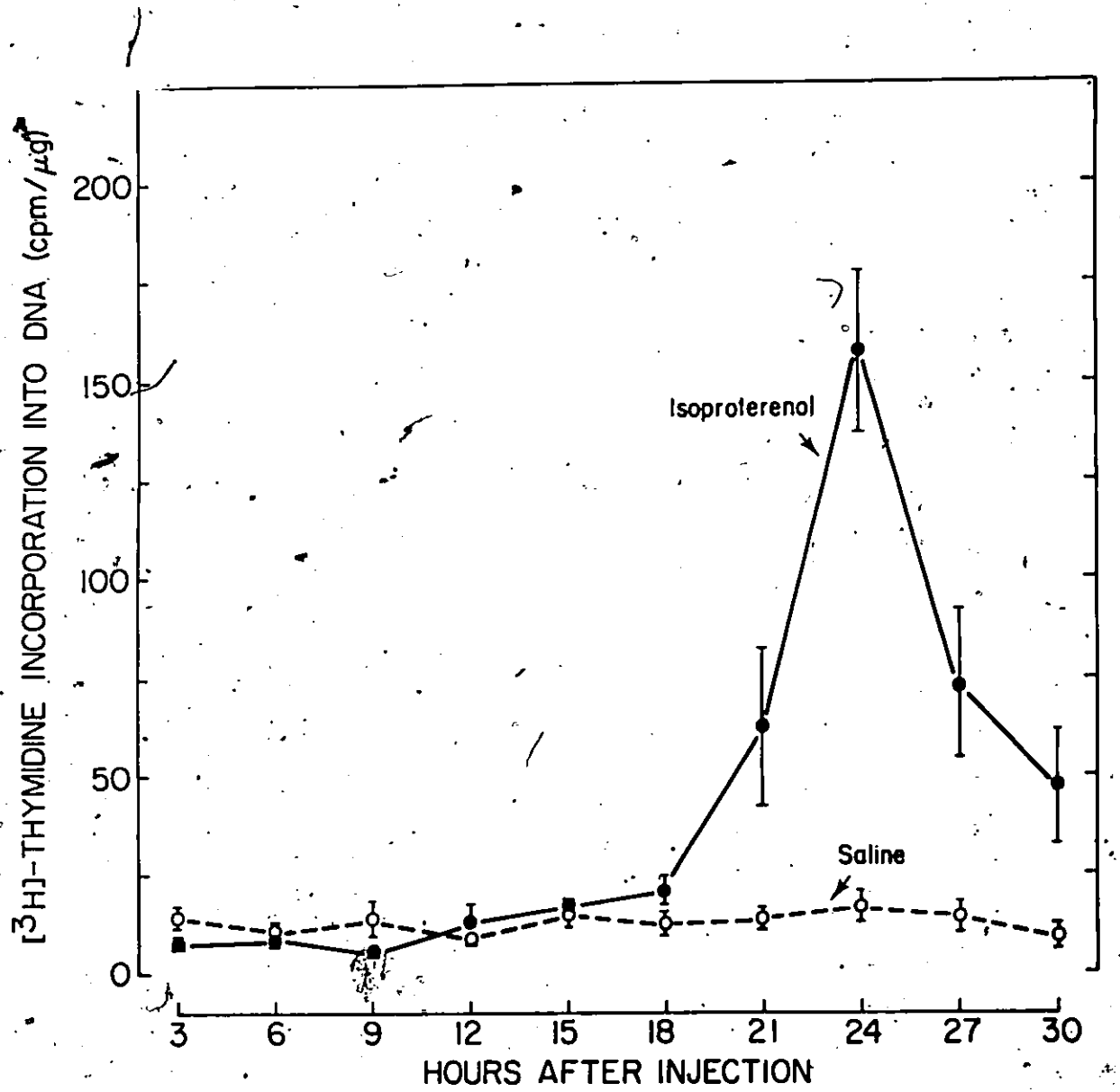
Before investigating the effects of isoproterenol on the CaM content of rat parotid glands, it was necessary to confirm that isoproterenol stimulated DNA synthesis of rat parotid glands and to determine the time course of the event.

In a series of experiments, two groups of animals were considered; one group was injected intraperitoneally with 150 mg of IPR/Kg body weight dissolved in 0.2 ml of 0.9% NaCl; the second group was injected i.p. with 0.2 ml of 0.9% NaCl. Parotid glands were removed at different times after injection of IPR or saline. DNA synthetic activity was determined by measuring the incorporation of ^{3}H-thymidine into the DNA extracted from parotid glands (see Materials and Methods).

The results presented in figure 12 indicate that DNA synthesis in rat parotid glands was stimulated by IPR injection. The initiation of DNA synthesis began at 18 h after IPR administration and the maximum activity was at 24 h (157.92 $\pm$ 20.16 cpm/µg of DNA) which represented a 6-fold increase over the control value (26.00 $\pm$ 4.18 cpm/µg of DNA). The difference between IPR-treated animals and saline treated animals was highly significant ($p < 0.001$).

Figure 12. The ability of isoproterenol to stimulate DNA synthesis of rat parotid glands.

Different groups of animals received an i.p. injection of 150 mg IPR/Kg body weight in 0.2 ml of 0.9% NaCl or 0.2 ml of 0.9% NaCl only. Parotid glands were removed at different times after the initial injection. One hour before the removal of parotid glands, all animals received an i.c. injection of 1.25 µCi of ^{3}H-thymidine per gram of body weight. DNA synthesis was determined as described in Materials and Methods. Points are the means ± standard error of the mean of values from 10-20 rats and from two different experiments.


[3H]-THYMIDINE INCORPORATION INTO DNA (cpm/μg)
200
150
100
50
0
Isoproterenol
Saline
3
6
9
12
15
18
21
24
27
30
HOURS AFTER INJECTION

Effect of IPR Dose on DNA Synthesis.

In order to determine the effects of different doses of IPR on DNA synthesis, the next experiment was performed. Seven groups of animals were injected with the following doses of IPR: 5, 10, 25, 50, 75, 100 and 150 mg/Kg body weight dissolved in 0.2 ml of 0.9% NaCl. Parotid glands were assayed for ^{3}H-thymidine incorporation into DNA (see Materials and Methods). The results illustrated in figure 13 indicate that DNA synthesis was stimulated in a dose-dependent fashion. The maximal response observed was with doses between 25-150 mg/Kg body weight.

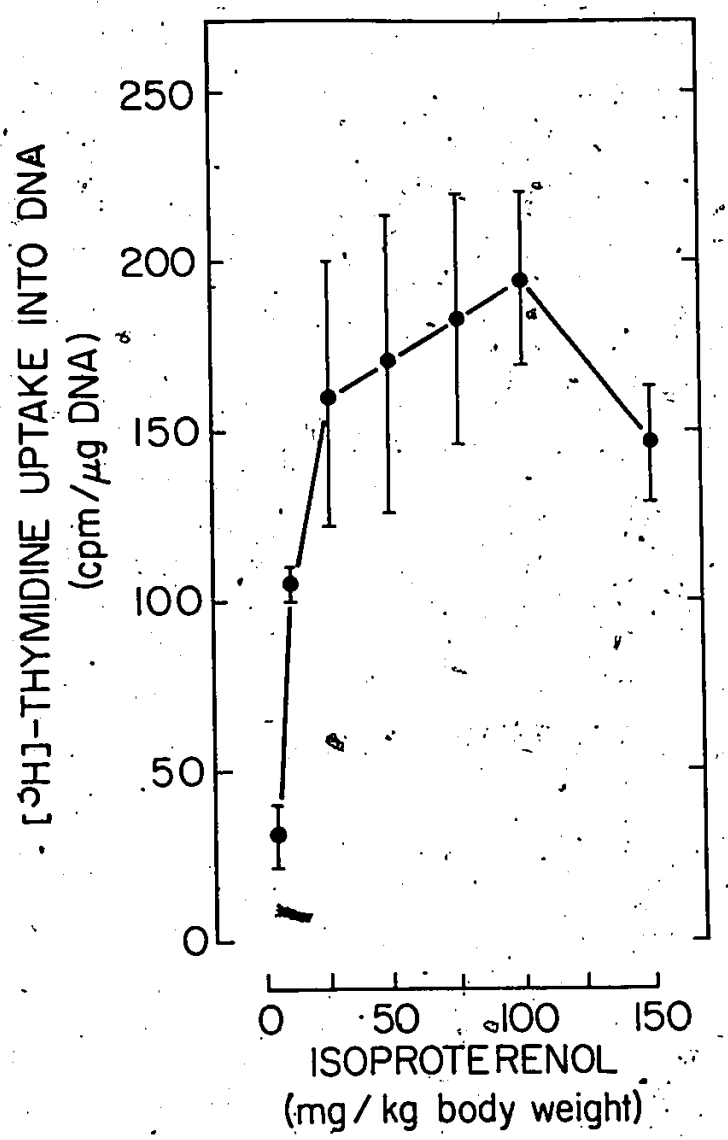
Effect of IPR on Calmodulin Content of 105 000 X-g Supernatant.

A series of experiments was conducted to determine the time course of changes in calmodulin content induced by a single injection of isoproterenol.

One group of animals was injected i.p. with 150 mg/Kg body weight of IPR in 0.2 ml of 0.9% NaCl, a second group of animals was injected with 0.2 ml of 0.9% NaCl and a third group of animals was simply manipulated but not injected. Parotid glands were removed immediately after injection or handling for "zero" values or at different times after the initial treatment. Calmodulin content in the 105 000

Figure 13. Effect of dose of isoproterenol on DNA synthesis of rat parotid glands.

Separate groups of rats were injected with 5,10,25,50,75,100 or 150 mg of IPR/Kg body weight dissolved in 0.9% NaCl. Twenty three hours after the initial injection, all animals were injected i.c. with 1.25 μCi ^{3}H-thymidine/g body weight and the parotid glands removed one hour later. DNA synthesis was measured as described previously in Materials and Methods, and expressed as cpm of ^{3}H-thymidine incorporated into DNA per μg of DNA. Points are means ± standard error of the mean of values from 10-14 rats and three separate experiments.


[3H]-THYMIDINE UPTAKE INTO DNA
(cpm/μg DNA)
250
200
150
100
50
0
0
50
100
150
ISOPROTERENOL
(mg / kg body weight)

X g supernatant was determined by the previously described radioimmunoassay procedure. Samples were assayed twice and in triplicate within each assay. Results presented in figure 14 indicate that IPR causes two significant ($p < 0.02$) transient increases in the CaM content of 105 000 X g supernatant of rat parotid tissue. The first surge occurred at 3 h after IPR injection and increased to a value of 864 $\pm$ 138 µg/g w wt compared to 248 $\pm$ 64.31 µg/g w wt for saline treated animals and 267.33 $\pm$ 36.88 µg/g w wt for manipulated animals. The second increase occurred between 15 and 21 h after proliferative activation and peaked at 18 h at a value of 887.00 $\pm$ 174 µg/g w wt compared to 315.27 $\pm$ 74.51 µg/g w wt in controls.

The two-fold increase in CaM content at 18 h was still evident when the values were expressed as µg CaM/mg protein. Isoprpterenol treated animals had 2.0 µg CaM/mg protein while controls had only 0.98 µg CaM/mg protein. The difference between two groups was significant ($p < 0.02$).

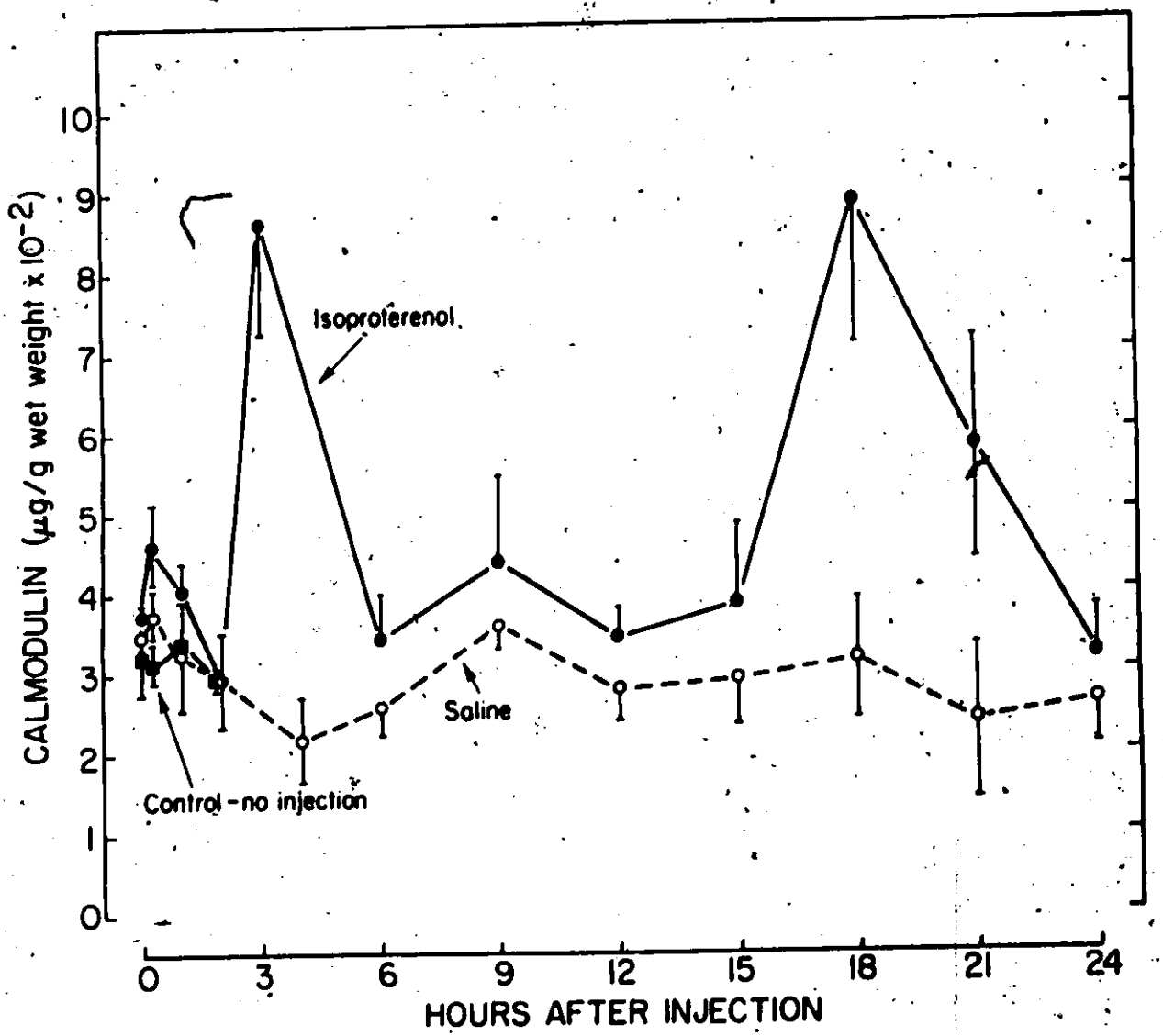
The small changes in the CaM content in the 105 000 X g supernatant between 0 and 2 h after IPR or saline injection were not significant ($p > 0.05$) compared with controls without injection and were probably due to stress elicited by the procedure of injection.

Effect of Isoproterenol on Calmodulin Content in Total Homogenate.

In order to determine whether the calmodulin increase in the

Figure 14. The ability of isoproterenol to stimulate calmodulin accumulation in the 105 000 X g supernatant of rat parotid glands.

Different groups of animals were injected i.p. with 150 mg of IPR/Kg body weight dissolved in 0.2 ml of NaCl or an injection of 0.2ml of 0.9% NaCl only. Another control group did not receive any injection but was manipulated identical to the isoproterenol or saline groups. Parotid glands were removed immediately for "zero" values or at different times after the initial treatment. Calmodulin content in the high-speed supernatant was measured by radioimmunoassay as described in Materials and Methods. Points are the means ± standard error of the mean of values from 5 to 7 rats per group and from two different experiments.


CALMODULIN (μg/g wet weight x 10⁻²)
0
1
2
3
4
5
6
7
8
9
10
Isoproterenol
Saline
Control - no injection
0
3
6
9
12
15
18
21
24
HOURS AFTER INJECTION

105 000 X g fraction caused by IPR injection was due to a subcellular redistribution of calmodulin or to an increase in the total calmodulin content the next series of experiments were performed.

One group of animals was injected intraperitoneally with 150 mg of IPR/Kg body weight dissolved in 0.2 ml of 0.9% NaCl and a control group was injected i.p. with 0.2 ml of 0.9% NaCl. Parotid glands were removed at 3, 18 and 24 h after IPR or saline injection and the calmodulin content in the total homogenate was determined by radioimmunoassay. Values presented in table V were obtained from two different experiments with each sample assayed twice in triplicate.

The results indicate that the total calmodulin content was increased significantly ($p < 0.05$) by IPR at 3 h after injection (704 ± 47.38 µg CaM/g w wt). No significant difference was found between IPR and saline treated groups at 18 or 24 h after injection.

Effects of Different IPR Doses on the Calmodulin Surge at 18h After Injection.

It was demonstrated that an injection of 150 mg IPR/Kg body weight caused an increase in calmodulin content in the 105 000 X g supernatant at 18 h after injection.

A series of doses (5, 10, 25, 50, 75, 100, 150 mg/Kg body weight)

TABLE V. Effects of IPR on the calmodulin content of total homogenate.

	CALMODULIN IN TOTAL HOMOGENATE µg CaM/g w wt		
	TIME AFTER INJECTION HOURS		
	3	18	24
TREATMENT			
ISOPROTERENOL	704±47.38	516±48.78	529±45.44
SALINE	462±51.44	585±48.08	564±80.63

Separate groups of animals were injected i.p. with 150 mg of IPR in 0.2 ml of 0.9% NaCl or 0.2 ml of 0.9% NaCl. Parotid glands were removed at 3,18 or 24h after the initial injection. Calmodulin was measured in the total homogenate by the radioimmunoassay procedure described in Materials and Methods. Values are expressed as µg of CaM/g w wt. The values are means ± standard error of the mean of two different experiments and four determinations. The number of rats per group in each experiment was between 7 to 9.

of IPR dissolved in 0.2 ml of 0.9% NaCl were administered by i.p. injection into different groups of animals. Parotid glands were removed at 18 h after injection and calmodulin content in the 105 000 X g supernatant determined by radioimmunoassay.

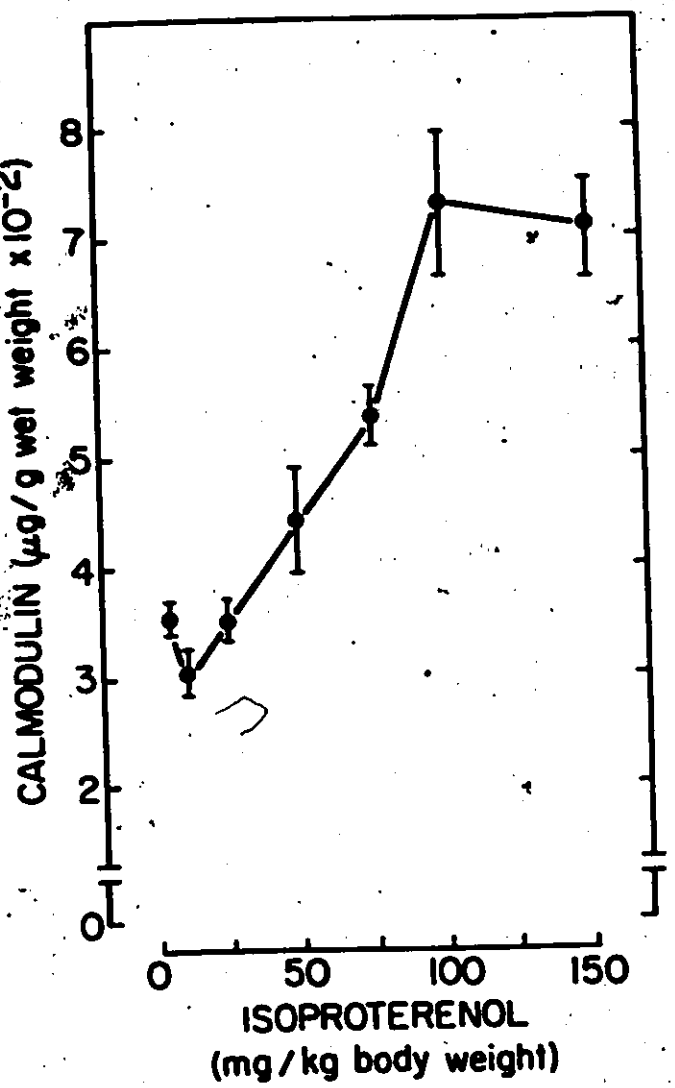
The results presented in figure 15 indicated that the CaM increase at 18 h after IPR injection depended on the amount of IPR injected. The maximum response was obtained with 100 mg of IPR which resulted in 732 ± 64.93 µg CaM/g w wt. This value represented a 2-fold increase over the lowest dose injected (5 mg resulted in 345 µg CaM/g w wt). The difference between maximal and minimal doses was significant ($p < 0.02$). Doses greater than 100 mg/Kg body weight did not cause a further increase.

Effect of TPTX on DNA Synthesis.

Three different protocols were used to determine the effect of TPTX on activation of DNA synthesis by injection of IPR. The first protocol consisted of two groups of TPTX animals and two SHAM-TPTX groups. Twenty four hours after TPTX one group was injected i.p. with 150 mg IPR/Kg body weight dissolved in 0.2 ml of 0.9% NaCl and another group with saline only. One SHAM-TPTX group was injected with the same dose of IPR and the second SHAM-TPTX group was injected with 0.2 ml of 0.9% NaCl. The second protocol consisted of two groups of TPTX animals and two SHAM-TPTX groups. Forty eight hours

Figure 15. The ability of different concentrations of isoproterenol to stimulate calmodulin accumulation in the 105 000 X g supernatant of rat parotid glands.

Separate groups of animals were injected i.p. with 5, 10, 25, 50, 75, 100 or 150 mg of IPR/100 g body weight dissolved in 0.2 ml of 0.9% NaCl. Eighteen hours after the initial treatment, the rat parotid glands were removed. Calmodulin was measured in the supernatant of 105 000 X g centrifugation by radioimmunoassay as described in Materials and Methods. Values are the means ± standard error of the mean from 5 to 9 rats.


CALMODULIN (μg/g wet weight x10⁻²)
0
2
3
4
5
6
7
8
0
50
100
150
ISOPROTERENOL
(mg / kg body weight)

after surgery, one TPTX group and one SHAM-TPTX group was injected with 150 mg IPR/Kg body weight in 0.2 ml of 0.9% NaCl. The second SHAM-TPTX group was injected with 0.2 ml of 0.9% NaCl. In the third protocol the same procedure was used except IPR or saline was injected 72 h after TPTX or SHAM-TPTX treatment.

Twenty four hours after the initial injection, blood samples were taken by cardiac puncture for determination of the plasma calcium concentration and the parotid glands removed. DNA syntnesis was assessed by measuring the incorporation of ^{3}H-thymidine into the DNA extracted from parotid glands (see Materials and Methods).

The results presented in table VI indicate that the plasma calcium concentration was significantly reduced ($p < 0.001$) from 2.14 mM (avg) in SHAM-TPTX groups to 1.16 mM (avg) in TPTX groups. Twenty four hour TPTX-IPR treated rats were stimulated to synthesize DNA, however 48 or 72 h TPTX-IPR treated rats showed DNA synthetic values similar to those of normal-saline treated rats.

The difference between the ^{3}H-thymidine uptake into DNA of the 24 h TPTX-IPR treated rats with 48 or 72 h TPTX-IPR treated rats was significant ($p < 0.01$).

Effect of TPTX on the IPR-Induced CaM Surge at 18 h in 105 000 X g Supernatant.

Because IPR did not induce DNA synthesis in 48 and 72 h TPTX rats, an opportunity existed to find out whether the CaM surge was

TABLE VI. Effect of TPTX on DNA synthesis.

	^{3}H-THYMIDINE INCORPORATION INTO DNA AND THE PLASMA CALCIUM CONCENTRATION					
	TIME AFTER TPTX (HOURS)					
	24		48		72	
TREATMENT	cpm / µg DNA	PCC mM	cpm / µg DNA	PCC mM	cpm / µg DNA	PCC mM
TPTX + IPR	182.30 ±30.14	1.28 ±0.70	20.46 ±8.61	1.35 ±0.146	16.65 ±4.07	0.99 ±0.06
TPTX + SALINE	17.68 ±2.21	1.15 ±0.80	17.24 ±2.37	1.16 ±0.121	14.49 ±3.25	1.05 ±0.11
SHAM-TPTX + IPR	173.41 ±85.60	2.19 ±0.80	186.88 ±68.93	2.16 ±0.023	129.75 ±22.26	2.20 ±0.06
SHAM-TPTX + SALINE	28.36 ±2.95	2.10 ±0.92	34.14 ±0.32	2.10 ±0.118	27.95 ±3.52	2.10 ±0.09

Different groups of animals were subjected to TPTX or SHAM-TPTX. The rats were injected with 150 µg of IPR in 0.2 ml of 0.9% NaCl or 0.2 ml of 0.9% NaCl at 24, 48 or 72h after the initial surgery. All groups were injected i.c. with 1.25 µCi of ^{3}H-thymidine at 23h after IPR or saline injection. One hour later, blood samples were taken and parotid glands removed. Plasma calcium concentration was determined as described in Materials and Methods and expressed as mmoles/l. DNA synthesis measured as described in Materials and Methods and expressed as cpm/µg DNA. The values are means ± standard error of the mean of 4-7 rats.

also inhibited. In these experiments, 24, 48 and 72 h TPTX and the corresponding SHAM-TPTX animals were injected with 150 mg IPR/Kg body weight in 0.2 ml of 0.9% NaCl. Normal animals were also injected with IPR or saline. Eighteen hours after injection of IPR or saline, blood samples were collected by cardiac puncture and the parotid glands removed. The calcium concentration in the plasma was determined and the CaM content in the parotid glands measured by radioimmunoassay.

The results presented in table VII indicate that plasma calcium concentration was 1.18 mM (avg) for TPTX rats. The plasma calcium concentration was 2.22 mM (avg) for SHAM-TPTX rats. The differences between the plasma calcium concentration in SHAM-TPTX and TPTX groups were significant ($p < 0.001$).

Table VII shows the effect of IPR on the CaM content at 18 h after IPR injection in TPTX, SHAM-TPTX and normal rats. The surge of CaM at 18 h was still present in the 24 h TPTX-IPR treated animals (785 ± 38.42 µg CaM/g w wt); but the surge was not present in 48 h TPTX-IPR treated animals (321 ± 43.01 µg CaM/g w wt) or the 72 h TPTX-IPR treated animals (312 ± 46.81 µg CaM/g w wt). A significant difference was found between the CaM content in the parotid glands of 24 h TPTX-IPR treated animals and 48 or 72 h TPTX-IPR treated rats ($p < 0.02$).

TABLE VII. Effect of TPTX on calmodulin content of the 105 000 X g supernatant.

	CALMODULIN CONTENT AND THE PLASMA CALCIUM CONCENTRATION					
	TIME AFTER TPTX (HOURS)					
	24		48		72	
TREATMENT	µg/g w wt	PCC mM	µg/g w wt	PCC mM	µg/g w wt	PCC mM
TPTX + IPR	785 ±38.42	1.23 ±0.06	321 ±43.01	1.04 ±0.09	312 ±46.81	1.28 ±0.08
SHAM-TPTX + IPR	603 ±27.10	21.7 ±0.06	728 ±117.49	2.34 ±0.05	709 ±103.36	2.17 ±0.06
NORMAL + IPR	630 ±54.09	- -	707 ±89.79	- -	836 ±116.93	- -
NORMAL + SALINE	373 ±41.09	- -	366 ±25.59	- -	434 ±66.18	- -

Different groups of animals were subjected to TPTX or SHAM-TPTX. Operated rats and normal control rats were injected i.p. with 150 mg of IPR in 0.2 ml of 0.9% NaCl at 24, 48 or 72h after the initial surgical treatment. Blood samples were taken 18h after the IPR or saline injection and the parotid glands removed. CaM content expressed as µg/g w wt was determined in the 105 000 X g supernatant by radioimmunoassay. Plasma calcium concentration is expressed in mmoles/l. The values are means ± standard error of the mean of two separated experiments. The number of rats/group in each experiment was between 5 to 9.

Effect of Injection of $CaCl_2$ on DNA Synthesis of TPTX Animals Treated with IPR.

If the 48 h TPTX-induced block of IPR-induced DNA synthesis was due to hypocalcemia and a failure to elicit a late G_1 CaM surge, it follows that restoration of the plasma calcium level to the physiological range should evoke a calmodulin surge and subsequent DNA synthesis. To test this hypothesis the following experiments were performed.

Forty eight hour TPTX animals were divided into two different groups and both were injected with 150 mg IPR/Kg body weight dissolved in 0.2 ml of 0.9% NaCl. Calcium (0.5 mg/100 g body weight) as $CaCl_2$ dissolved in 0.2 ml of 0.9% NaCl was administered i.p. into one group while 0.2 ml of 0.9% NaCl was injected i.p. into the second group. $CaCl_2$ or saline was injected after the initial IPR administration at either 0, 6, 12, 16, 20, 24 or 28 h. ^{3}H-thymidine at a dose of 1.25 µCi/g body weight was injected i.c. at 23 h after the IPR injection for the rats injected with $CaCl_2$ or saline at 0, 6, 12, 16, 20 and 24 h. ^{3}H-thymidine 1.25 µCi/g body weight was injected i.c. at 27 h after IPR for the rats injected with $CaCl_2$ or saline at 28 h after IPR. Blood samples and parotid glands were removed one hour after the ^{3}H-thymidine injection. The control group consisted of normal rats and no injected with $CaCl_2$ or NaCl. However, this con-

trol group was injected with the same dose of IPR and 23 h later with ^{3}H-thymidine (1.25 µCi/g body weight) and their parotid glands removed 1 hour after that.

The results illustrated in figure 10 indicate that $CaCl_2$ (2.5 mg/100 g body weight) injected at the times described after the initial activation by IPR did not restore the normal DNA synthesis exhibited by the control group of normal rats (140 ± 26.8 cpm/µg DNA).

Effect of Injection of $CaCl_2$ on Plasma Calcium Concentration.

The results from the last experiment demonstrated the failure of a $CaCl_2$ injection to restore DNA synthesis of 48 h TPTX + IPR treated rats. The next experiment explored the possibility that the $CaCl_2$ injection did not restore the plasma calcium concentration.

Two groups of animals were subjected to TPTX. The plasma calcium concentration was measured at 10 h after for one group and at 48 h after for the second group. $CaCl_2$ (2.5 mg/100 g body weight) was injected 15 min later, and the plasma calcium concentration measured at 0, 5, 10, 20 and 45 min after the calcium injection. The values for plasma calcium concentration are illustrated in table VIII. It is evident that the plasma calcium concentration after a calcium injection in 48 h TPTX rats did not differ from the values before

Figure 16. The effects of $CaCl_2$ injection on DNA synthesis of parotid glands in hypocalcemic-isoproterenol treated rats.

Forty eight hours after TPTX different groups of rats were injected with 150 mg of IPR/Kg body weight dissolved in 0.2 ml of 0.9% NaCl. Experimental groups were injected i.p. with 0.5 mg of calcium as $CaCl_2$ of IPR/100 g body weight dissolved in 0.2 ml of 0.9% NaCl, along with the IPR injection or at 6, 12, 16, 20 or 28 h after the initial IPR injection. Control groups were injected i.p. with 0.2 ml of 0.9% NaCl along with the IPR injection or at 6, 12, 16, 20 or 28 h after the IPR injection. Another control group of normal rats was injected with 150 mg of IPR/Kg body weight dissolved in 0.2 ml of 0.9% NaCl. All groups were injected i.c. with ^{3}H-thymidine (1.25 μCi/g body weight) at 23 or 27 h after the initial IPR injection. One hour after the ^{3}H-thymidine injection, blood samples were taken and parotid glands removed. Plasma calcium concentration and DNA synthesis were measured as described in Materials and Methods. Values are the means ± the standard error of the mean from 3 to 5 rats. The shaded area represents the mean ± the standard error of the mean of the values obtained from the control group of normal animals.

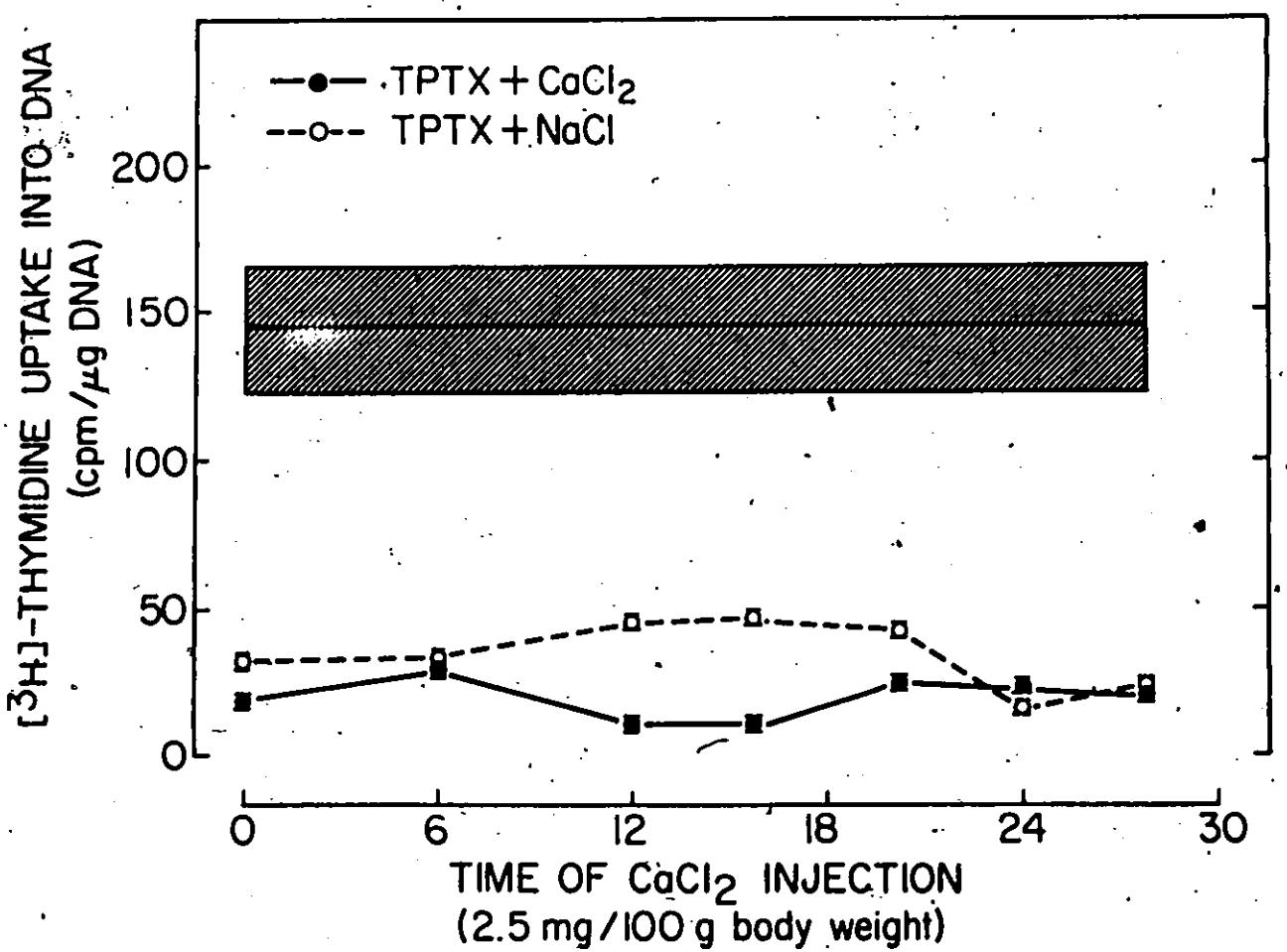

—●— TPTX + CaCl2
--o-- TPTX + NaCl
[3H]-THYMIDINE UPTAKE INTO DNA
(cpm/μg DNA)
200
150
100
50
0
0
6
12
18
24
30
TIME OF CaCl2 INJECTION
(2.5 mg/100 g body weight)

TABLE VIII. Effects of $CaCl_2$ injection in hypocalcemic rats on plasma calcium concentration.

	PLASMA CALCIUM mM	
	TPTX	
TIME FROM $CaCl_2$ INJECTION (MINUTES)	16h	48h
-15	1.31±0.03	1.23±0.07
0	1.40±0.053	1.23±0.05
+5	1.53±0.146	1.49±0.06
+10	1.88±0.015	1.42±0.01
+15	1.64±0.23	1.40±0.02
+20	-	1.40±0.07
+30	1.43±0.12	1.40±0.03
+45	-	1.32±0.085

Two groups of rats were subjected to TPTX. Blood samples were taken at 16 h after TPTX for one group and at 48 h after TPTX for the second group. Both groups were injected i.p. with 2.5 mg of calcium as $CaCl_2$/100g body weight. All animals were fasted 24 h before the calcium injection. Blood samples were taken from both groups after the calcium injection. The plasma calcium concentration is expressed as mmoles/l. The values are means ± standard error of the mean of 3-5 rats.

the injection. However, the plasma calcium concentration of 16 h TPTX rats was increased significatively ($p < 0.02$) between 10 and 15 min after the calcium injection.

DISCUSSION

The aim of this research was to investigate the possible relation between the calmodulin content during the prereplicative period and DNA synthesis in rat parotid glands activated by isoproterenol.

Cell reproduction is a vital process in every organism (e.g. growth, development, regeneration after injury, defense against foreign substances or other organisms) which is under balanced control (168,169). Several lines of evidence suggest that resting cells decide to enter the S phase and proliferate during the G_1 phase of the growth-division cycle. However, the nature of the master mechanism(s) is still unknown.

The presence of extracellular calcium ions during the G_1/S transition period is necessary for a succesful completion of the cell cycle. Other evidence suggests that calcium fluxes during the early prereplicative period initiate the progression through the cell cycle (170). However, the requirement of calcium fluxes during the early part of the cell division cycle is still controversial (46). For example, alfa-adrenergic agonists which promote calcium influx in rat parotid gland acinar cells do not stimulate DNA synthesis (112).

Other ions have been postulated as key factors in the control of cell proliferation. Nonetheless, these ions do not have the unique characteristics of calcium which enable it to be used as a cellular

regulator in a variety of well defined processes (171).

It is known that the calcium ion executes its tasks through several anionic centers, one of which is calmodulin. It must be pointed out that the regulation of calmodulin dependent enzymes can be modified either, by increasing the calmodulin concentration without altering the amount of calcium or by increasing the calcium concentration while the calmodulin concentration remains constant (68-71).

Calmodulin links the two major intracellular messengers: calcium and cAMP. Calmodulin affects the metabolism of cAMP by activation of adenylate cyclase and cyclic nucleotide phosphodiesterase. Both regulators (cAMP and calmodulin) act through the activation of several protein kinases. The substrates affected by the calmodulin dependent protein kinases are enzyme-specific, nevertheless, those substrates modified by the cAMP dependent protein kinases are not enzyme-specific and sometimes can be altered by several protein kinases (172).

It has been noticed that increases of CaM content precedes DNA synthesis in some cells but not others (73-86). When such an increase occurs it suggests a possible involvement of Ca-calmodulin in the events leading to the initiation of DNA synthesis. NRK cells infected with ASV tsLA23 do not require an increase in CaM to enter S phase (85), and T51B cells exhibit a CaM surge before DNA synthesis when stimulated with serum + calcium (74). When these cells are only

treated with serum, they still exhibit a CaM surge but do not synthesize DNA (86). It should be noted that only total CaM measurements were considered. This does not exclude the possibility of intracellular redistribution of calmodulin occurring before S phase.

In both studies described above, cell progression from G_1 to S phase was blocked by CaM antagonists indicating a positive requirement of Ca-CaM function(s) for S phase entry.

At the beginning of the present study it was considered that calmodulin might be involved in the initiation of DNA synthesis of rat parotid glands activated by isoproterenol. The relation between CaM and DNA synthesis could be reflected by changes in the cellular content of CaM during late G_1 phase of the activated glands.

One of the difficulties of this type of study is the detection of calmodulin. Most studies use the activation of a CaM dependent enzyme (e.g. cyclic nucleotide phosphodiesterase) by a CaM containing extract as a reflection of the CaM content in the extract. Enzymatic assays only provide information on the CaM available to the particular enzyme. It is assumed that there is no interference of some other molecules capable of altering the enzyme activity or CaM binding.

Other procedures require the production of antibodies against calmodulin and their utilization in a radioimmunoassay. Since CaM is a very conservative protein and shares some homology with other Ca-binding proteins, radioimmunoassay development is a difficult

task.

The radioimmunoassay detects immunogenic molecules present in a sample, without interference from non-related proteins. In spite of the advantages, the radioimmunoassay must satisfy at least two requirements: 1) utilization of a nearly pure antigen for immunization and labelling; 2) production and purification of a specific antibody directed against the antigen.

During the present study CaM was purified to homogeneity from rat testis. Affinity chromatography in AFFIGEL Phenothiazine followed by anion-exchange chromatography in DEAE-Sephacel were used for purification. Ultraviolet scan of the purified CaM demonstrated the abscence of nucleic acids or other protein contaminants in the preparation. Also, SDS-PAGE of CaM demonstrated the absence of minor protein contaminants coeluting with calmodulin along the purification procedure. This CaM of high-purity grade was subsequently utilized for iodination, coupling to CNBr-activated Sepharose and immunization of two sheep.

Since CaM contains only two tyrosines, it is advisable to use a labelling method which does not have Tyr as the iodine target, such as the Bolton-Hunter method (151). This method labels proteins with ^{125}I in the epsilon-amino of Lys residues.

Calmodulin was utilized for the construction of an affinity chromatography matrix which was used during the antibody purification.

This CaM-Sepharose matrix was utilized in order to separate immunoglobulins which did not recognize CaM as antigen from the specific anti-CaM fractions.

Since CaM is a very conservative protein, it is difficult to elicit an immune response against it. Several methods have been reported in the literature to overcome this problem resulting in a variety of responses and specificities of the antibody obtained (73, 150,160,173,174). It was decided to use the method described by Van Eldik and Watterson (158), because it is possible to elicit an immune response using small amounts of CaM. This method utilizes native CaM or oxidized CaM as antigen.

The oxidation of calmodulin was performed using performic acid. Since the molecule does not contain Cys residues, the only amino acids susceptible to oxidation are the nine methionine residues which are converted to Met-sulfone.

The immunoglobulin fraction was purified from the serum of immunized sheep. The anti-CaM fraction was obtained by affinity chromatography on the CaM-Sepharose column previously prepared. This step increases the titre of the antibody. It also eliminates the immunoglobulins which do not recognize CaM as ligand thus, increasing the specificity of the antibody to be used in the radioimmunoassay.

It was possible to elicit an immune response in both sheep; however, only the antibodies from sheep A and bleeds 3-6 were used

during this investigation. This was done in order to minimize heterogeneity on the avidity of the different antibodies obtained.

In order to evaluate the specificity of the antibody obtained, a competition assay was performed (Fig. 11). It is clear that rat parvalbumin did not crossreact with the antibody. Troponin C shows some crossreactivity, however, 500-fold more Troponin C was required for 30% competition with CaM. This crossreactivity was seen in all antibodies obtained against CaM using different methods (73,158,173, 174), the reason being that Troponin C and CaM share 50% direct homology and 70% conservative homologies.

Table IV describes the differences in CaM content of three different tissues of rat. According to these values, testis contains a significantly higher concentration of CaM than parotid glands or liver. It is noticeable that testis contains 76% of CaM in the soluble compartment and 16% in the particulate fraction.

The recovery of CaM from both soluble and particulate fractions was nearly 90% for the three tissues which indicates that the antibody was detecting the same antigen in total, soluble and particulate fractions.

The presence of CaM in rat parotid glands was first suspected by Ku and Butcher (175). During the fractionation of a parotid gland extract using DEAE-cellulose chromatography, fractions were found which stimulated cyclic nucleotide phosphodiesterase. However, there

was no data about the amount of calmodulin present in the parotid gland extract.

When IPR (150 mg/Kg body weight) was injected into the rats, their parotid glands responded by incorporating ^{3}H-thymidine into DNA between 18 and 30 h after IPR injection. ^{3}H-thymidine incorporation is a commonly accepted measurement of DNA synthesis. This represents a six-fold increase over the control values injected with saline solution. The prereplicative period of rat parotid gland cells activated by isoproterenol was shown to be between 0-18 h after IPR injection. The increase in DNA synthesis at 24 h after the activation was dependent on the dose of IPR injected. The maximum DNA synthetic activity was achieved with 25 mg IPR/Kg body weight.

The examination of the CaM content in rat parotid gland acinar cells during their prereplicative period, indicates that CaM in the soluble fractions increases significantly at 3 and 18 h after IPR. The second surge in CaM subsides to the normal levels at 24 h after IPR (Fig. 14). Table V illustrates that the increase of CaM at 3 h is due to an increase on the synthesis or inhibition of degradation of CaM, whereas the 18 h increase is a redistribution effect. This profile of CaM during the prereplicative period suggests that CaM may be involved in the onset of DNA synthesis occurring approximately at 18 h after IPR. Nevertheless, it is clear that increases in the total content of CaM during late G_1 phase are not an absolute requirement for DNA syn-

thesis.

The increase in CaM at 3 h could be a response to an early depletion of cytoplasmic proteins during secretion which occurs between 0-2 h after IPR injection (124).

The role of CaM in rat parotid gland acinar cells is completely unknown. This is not the only system in which CaM is mobilized after a specific stimulus. ACTH induces CaM translocation from the cytosol to the nuclear membrane in adrenal cortex (176); dopamine releases CaM from striatal membranes to cytosol (177); GnRH promotes a redistribution of calmodulin in membranes (microsomes and periplasmic membrane) of pituitary cells (178). Further experiments are still necessary to determine the exact subcellular components involved in the mobilization of CaM in rat parotid gland acinar cells activated by IPR.

The next step was the examination of the surge of CaM content at 18 h in the soluble fraction after increasing doses of IPR. Figure 15 demonstrates that this prereplicative surge can be induced with doses from 50 to 150 mg of IPR/Kg body weight. It was shown previously that DNA synthesis occurs even with doses of 10 mg IPR/Kg body weight, but the surge of CaM in the soluble fraction at 18 h is not evident with doses between 5-25 mg IPR/Kg body weight (Figures 13 and 15). This suggests that the increase of CaM in the soluble fraction at 18 h after IPR is not required for subsequent chromosome replication. However, it can not be excluded that small changes in CaM content at 18 h

occurred with IPR doses between 5-25 mg/Kg body weight which might not be detected by the assay. Probably, the development of an *in vitro* system for rat parotid gland acinar cells, and the exact knowledge of the number of cells induced to divide by the different doses of IPR would provide valuable information to exclude this later possibility.

The low-calcium condition induced by removal of the thyroparathyroid gland complex does not stop DNA synthesis induced by 150 mg of IPR in 24 h TPTX rats (Table VI). The same dose of IPR did not stimulate DNA synthesis in 48 and 72 h TPTX rats.

The results presented in table VII indicate that similar to the DNA synthetic response (Table VI), a low calcium condition caused by 24 h TPTX does not block the surge of soluble CaM at 18 h in IPR-stimulated parotid glands. Also, the soluble content of CaM at 18 h did not increase in 48 or 72 h TPTX rats. This suggests a direct co-relation between the calmodulin surge at 18 h after IPR and the DNA synthetic response which is also initiated at 18 h after IPR. However, it is also possible that long-term TPTX (48-72 h) affects an early stage of prereplicative development on which the CaM surge at 18 h and DNA synthesis are dependent.

This evidence suggested that calcium homeostatic hormones and/or extracellular calcium might modulate the CaM surge in the soluble fraction at 18 h to the same extent as DNA synthesis. These hormones (PTH and $1,25(OH)_2D_3$) may modulate cell division by maintaining an

optimal extracellular calcium concentration and/or by affecting the activity of other intracycle regulators such as calmodulin and protein kinases (47). Unfortunately the effects of PTH and $1,25(OH)_2D_3$ on the CaM content in late G_1 could not be examined.

The next step was the examination of the role of extracellular calcium in the initiation of DNA synthesis. It was found that calcium injection into 48 h TPTX rats during the prereplicative period did not restore DNA synthesis at 24 h after IPR activation. This failure to restore DNA synthesis in 48 h TPTX rat parotid glands could be due to a failure to restore the plasma calcium concentration. This may have been the case because a single injection of calcium as $CaCl_2$ did not significantly affect the normal plasma calcium concentration within 45 min. However, the same dose of calcium induced a transient increase in the plasma calcium concentration of 10 h TPTX rats. This could be explained by the fact that $1,25(OH)_2D_3$ is absent from plasma at around 48 h after TPTX (179). It is thought that $1,25(OH)_2D_3$ stimulates intestinal calcium absortion by inducing the appearance of several proteins during the calcium absortive process of intestine (180). Thus, there exists a possibility of some $1,25(OH)_2D_3$ dependent process or molecule which is responsible for the transport of calcium through the blood vessel epithelium, which might be absent in 48 h TPTX rats.

In summary, during the present investigation the relation of CaM to DNA synthesis in rat parotid glands was examined. Calmodulin in

rat parotid tissue was detected by using a specific radioimmunoassay. IPR induced DNA synthesis between 18 and 30 h, with a maximum at 24 h. The DNA synthetic response to IPR was dose-dependent. Furthermore, IPR induced an increase in the total concentration of CaM after 3 h and a transient redistribution after 18 h after activation. This redistribution was reflected as an increase of CaM in the soluble fraction. Calmodulin increases during late G_1 were not required for DNA synthesis. However, the concomitant dissapearance of the CaM surge at 18 h and DNA synthesis at 24 h in 48 or 72 h TPTX rats suggested a possible relation between both responses. Calcium injection could not restore DNA synthesis because of the failure to restore the normal plasma calcium concentration in 48 h TPTX rats. This indicates the need for calcium homeostatic hormones to probe a direct relationship between CaM in late G_1 phase, and DNA synthesis in long-term TPTX rats.

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