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J. E. DUMONT, J. NUNEZ and G. SCHULTZ

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Scientific Committee:

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G. Schultz

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LIST OF PARTICIPANTS

AKTORIES K.
Pharmakologische Inst. der
Univ. Heidelberg,
Im Neuenheimer Feld 366,
D.6900 HEIDELBERG

ALLERA A.
Inst. für klinische Biochemie
der Univ. Bonn,
Sigmund Freud Strasse 25,
D.5300 BONN

BAUER H.
Inst. für Virologie,
Justus Leibig Univ. Giessen,
Frankfurter Strasse 107,
D.6300 GIESSEN

BERTHILLIER G.
Univ. Claude Bernard Lyon 1,
Lab. de Biochimie des Membranes,
43 Bd du 11 Novembre
F.69622 VILLEURBANNE

BIDART M.
INSERM Inst. Gustave Roussy,
Unité de Biologie Clinique,
rue Camille Desmoulins
F.94800 VILLEJUIF

BIRDSALL N.
Nat. Inst. for Med. Res.,
The Ridgeway,
Mill Hill,
LONDON NW7 1AA, UNITED KINGDOM

BIRNBAUMER L.
Baylor College of Medicine,
Dept of Cell Biology,
1200 Moursund ave.,
HOUSTON, TX 77030, U.S.A.

BIRNBAUMER M.
Inst. de Rech. en Biologie
Molèculaire,
Fac. des Sciences,
2 place Jussieu,
F.75005 PARIS

BOCKAERT J.
Lab. de Physiologie Cellulaire,
Collège de France,
Place Marcelin Berthelot,
F.75005 PARIS

BOEYNAEMS J.M.
ULB, Inst. de Rech. Interdisiplinaire,
Fac. de Médecine,
2 rue Evers,
B.1000 BRUXELLES

BOTTARI S.
ULB. Dépt. de Gynécologie,
Obstétrique Hôpital Brugmann,
Place A. Van Gehuchten,
B.1020 BRUXELLES

BRINKMANN A.
Erasmus Univ. Rotterdam,
Dept of Biochemistry II,
P.O. Box 1738,
NL.3000 ROTTERDAM

BROWN B.
Univ. of Sheffield, Medical School,
Dept. Human Metabolism & Clinical Biochem.
Beech Hill Road,
SHEFFIELD, UNITED KINGDOM

BURGER A.
Unité de Thyroïde, Laboratoire 4-467,
Hôpital Cantonal,
CH.1211 GENEVE 4

CANTAU B.
Dépt. de Physiologie Cellulaire,
Collège de France, Place M. Berthelot,
F.75005 PARIS

CATT K.
Nat. Inst. of Health, Nat. Inst. of Child
Health & Human Develop.
BETHESDA, MD 20205, U.S.A.

CHABARDES D.
Lab. de Physiologie Cellulaire,
Collège de France,
Place M. Berthelot,
F.75005 PARIS

CHAMBAZ E.
INSERM U.244, Univ. Scientifique et Médicale de Grenoble (U.S.M.G.), BM53X,
F.38041 GRENOBLE Cedex

CHEVALLIER J.
IBCN. Dépt. Gironde,
1 rue Camille Saint-Saens,
F.33000 BORDEAUX

COOKE B.
Univ. of London, Royal Free Hospital, School of Med., Dept of Biochemistry,
8 Hunter Street,
LONDON WC1N 1BP, UNITED KINGDOM

COURAUD P.O.
IRBM, Paris VII, Dépt. Immunologie Moléculaire,
2 place Jussieu
F.75221 PARIS 05

DANIELSON A.
Univ. of Umea, Int. Medicine, Regionsjukhuset,
S.901.85 UMEA

DAZORD A.
INSERM U.162, Hôpital Debrousse,
29, rue Soeur Bouvier,
F.69322 LYON, Cedex 1

DEMAILLE J.
INSERM U.249, Faculté de Médecine, Inst. de Biologie,
B.P.5051,
F.34033 MONTPELLIER Cedex

DE MEYTS P.
Int. Inst. of Cellular and Molecular Pathology, PATG 7529, Ave Hippocrate 75,
B.1000 BRUXELLES

DENTON R.
Dept. Biochemistry, Med. School,
Univ. of Bristol,
University Walk,
BRISTOL BS8 1TD, UNITED KINGDOM

DE WULF H.
Afd. Biochemie, Fac. der Geneeskunde,
Campus Gasthuisberg, K.U. Leuven,
B.3000 LEUVEN

DONNER M.
INSERM U.95, Centre de Recherches de l'INSERM, Plateau de Brabois,
F.54500 VANDOEUVRE les NANCY, Cedex

DOUCET A.
Lab. Physiologie Cellulaire,
Collège de France, Place M. Berthelot,
F.75005 PARIS

DUMONT J.E.
ULB. Inst. de Rech. Interdisciplinaire,
Ecole de Médicine,
2 rue Evers, B.1000 BRUXELLES

DUSTIN P.
ULB. Lab. d'Anatomie Pathologique & Microscopie Electronique,
Rue Aux Laines, 97,
B.1000 BRUXELLES

EIK-NES K.B.
Dépt. Biophysics, Sem Sealands vei 9,
N.7034 TRONDHEIM-NTH

FELLOUS A.
INSERM U.96, Hôpital de Bicêtre,
78, avenue du Général Leclerc,
F.94270 LE KREMLIN -BICETRE

FELTZ P.
Univ. L. Pasteur, Inst. de Physiologie et Chimie Biologique,
21 rue R. Descartes,
F.67000 STRASBOURG

FISCHER S.
Inst. de Pathologie Moléculaire,
24 rue du Fg. Saint Jacques,
F.75014 PARIS

FLOCKERZI V.
Pharmacologisches Inst. der Univ. Heidelberg,
Im Neuenheimer Feld 366,
D.6900 HEIDELBERG

FRANCON J.
INSERM U.96, Hôpital de Bicêtre,
78, avenue du Général Leclerc,
F.94270, LE KREMLIN-BICETRE

FULPIUS B.
Univ. de Genève, Dépt. Biochimie,
30 quai E. Ansermet,
CH.1211 GENEVE

GABEN A.M.
INSERM U.29, Hôpital Port-Royal,
123 bd de Port Royal,
F.75674 PARIS, Cedex 14

GARNIER J.
INRA, Biochimie Physique,
Univ. Paris-Sud,
F.91405 ORSAY

GAVARET J.M.
INSERM U.96, Hôpital de Bicêtre
78, rue du Général Leclerc
F.94270 LE KREMLIN-BICETRE

GENTY N.
Inst. de Microbiologie,
Univ. Paris-Sud,
F.91405 ORSAY

GILL G.
Div. Endocrinology, Dept. Med.,
School of Medicine, San Diego,
LA JOLLA CA.92093, U.S.A.

GIUDICELLI Y.
Centre Hospitalier de Poissy,
Dépt. Biochimie,
Rue du Champ Gaillard,
F.78303 POISSY

GJORSTRUP P.
Inst. of Physiology,
Sövegatan 19, S.22362 LUND

GLOSSMANN H.
Justus Liebig-Univ.,
Inst. für Pharmakologie,
Frankfurter Strasse 107,
D.6300 GIESSEN

GOLDENBERG H.
Med. Chemisches Inst. der Univ.
Wien, Währingerstrasse 10,
A.1090 WIEN

GOODHARDT M.
INSERM U.99, Hôpital Henri Mondor,
F.94010 CRETEIL

GRONER B.
Kernforschunszentrum Karlsruhe,
Inst. für Genetik und Toxicologie
von Spaltstoffen, Postfach 3640,
D.7500 KARLSRUHE

GUENET L.
Fac. de Médecine, Biochimie Med.,
Ave Prof. L Bernard,
F.35043 RENNES

GUILLON G.
Lab. de Physiologie Cellulaire,
Collège de France,
Place M. Berthelot,
F.75005 PARIS

HADJIAN A.
INSERM U.244, Univ. Scientifique et
Médicale de Grenoble (U.S.M.G.), BM53X,
38041 GRENOBLE, Cedex

HAMPRECHT B.
Physiologisch-Chemisches Inst. der Univ.,
Koellikerstrasse 2,
D.8700 WURZBURG

HANOUNE J.
INSERM U.99, Hôpital Henri Mondor,
F.94010 CRETEIL

HARDEVELD C. van
Univ. Hosp., Dept. of Chemical Pathology,
Rijnsburgerweg 10,
NL.2333AA LEIDEN

HEDIN L.
Göteborg Univ., Fysiologiska Inst.
P.O. Box 33031, S.40033 GOTEBORG

HEIDMANN T.
Institut Pasteur, 28 rue du Dr Roux,
F.75724 PARIS

HENRIKSSON R.
Univ. of Umea, Inst. of Histology and
Cell Biology, S.90187 UMEA

HERS H.G.
Univ. Catholique de Louvain,
Lab. de Chimie Physiologique,
ave Hippocrate 75,
B.1200 BRUXELLES

HOFMANN F.
Pharmakologisches Inst. der Univ.
Heidelberg, Im Neuenheimer Feld 366,
D.6900 HEIDELBERG

HOLDER F.
Lab. de Physiologie Comparée des
Régulations, 23 rue de Loess, B.P. 20CR
F.67037 STRASBOURG

IMBS I.L.
Dépt. de Pharmacologie, 11 rue Human,
F.67000 STRASBOURG

INSEL P.
Univ. of California, San Diego,
Div. Pharmacology M.013H, School of Med.
LA JOLLA CA.92093, U.S.A.

JACQUEMIN C.
Faculté des Sciences, Dept. de
Biochimie, B.P.347, F.51062 REIMS

JAHN R.
Abt. klin. Biochemie, Med. Univ.
Kliniken, Humboldtallee 1,
D.3400 GÖTTINGEN

JAKOBS K.H.
Pharmakologisches Inst. der Univ.
Heidelberg, Im Neuenheimer Feld 366,
D.6900 HEIDELBERG

JOHNSON R.A.
Dept. of Physiology, Vanderbilt
Univ., School of Medicine,
NASHVILLE, TN 37232, U.S.A.

KEEFER L.M.
Int. Inst. of Cellular &
Molecular Pathology, Patg 7529, Ave
Hippocrate 75, B.1200 BRUXELLES

KERR I.
Imp. Cancer Res. Fund Lab.,
Lincoln's Inn Field,
LONDON WC2A 3PX, UNITED KINGDOM

KRAWIETZ W.
Med. Klinik I der Univ.,
Klinikum Grobhadern, Postfach 701260
D.8000 MUNCHEN 70

KOHL P.
Univ. Basel, Biozentrum, Klingel-
bergstrasse 70, CH.4056 BASEL

KUNAU W.H.
Inst. für Physiologische Chemie,
Arbeitsgruppe für Bioorganische
Chemie, Gebäude MA, D.4630 BOCHUM

LADURON P.
Janssen Pharmaceutica, Res. Labs.,
B.2340 BEERSE

LAMBERT M.
Univ. Libre de Bruxelles,
Dept. Biochimie, 2 rue Evers,
B.1000 BRUXELLES

LAZDUNSKI M.
Univ. de Nice, Centre de Biochimie,
Parc. Valrose,
F.06034 NICE

LENNON A.M.
INSERM U.96, Hôpital de Bicêtre,
78 avenue du Général Leclerc,
F.94270 LE KREMLIN-BICETRE

LUCAS M.
INSERM Lab. Physiologie Cellulaire,
Collège de France, Place M. Berthelot,
F.75005 PARIS

LUBBECKE F.
Justus Liebig-Univ., Pharmakolog. Inst.,
Frankfurter Strasse 107,
D.6300 GIESSEN

MANDEL J.L.
Lab. de Génétique Moléculaire des
Eucaryotes, 11 rue Human,
F.67000 STRASBOURG

MARVALDI J.
Inst. de Chimie Biologique, Dept Bioch.,
Place Victor Hugo, F.13331 MARSEILLE

MAUGER J.P.
Lab. de Physiologie Cellulaire,
Collège de France, Place M. Berthelot,
F.75005 PARIS

MONNERON A.
Institut Pasteur, Biologie Moléculaire,
25 rue du Dr Roux, F.75015 PARIS

MUNARI-SILEM Y.
INSERM U.197, Lab. de Méd. Expérimentale,
Fac. de Med. Alexis-Carrel,
Rue Guillaume Paradin,
F.69372 LYON Cedex

MUTT V.
Karolinska Inst., Dept. Biochemistry II,
Medical Nobel Institute, PO Box 60400,
S.10401 STOCKHOLM

NUNEZ J.
INSERM U.96, Hôpital de Bicêtre,
78 avenue du Général Leclerc,
F.94270 LE KREMLIN-BICETRE

NORJAVAARA E.
Göteborg Univ., Fysiologiska Inst.,
P.O. Box 33031, S.400 33 GOTEBORG

OSBORN M.
Max-Plank-Inst. für Biophysikalische Chemie, D.3400 GOTTINGEN

OWEN W.G.
Int. Inst. Cellular and Molecular Pathology, General Pathology,
Ave Hippocrate 75,
B.1200 BRUXELLES

PAVLOVIC-HOURNAC M.
INSERM U.96, Hôpital de Bicêtre,
78 avenue de Général Leclerc,
F.94270 LE KREMLIN-BICETRE

PFEIFFER E.F.
Univ. Ulm, Medizinische Klinik,
Steinhövelstrasse 9, D.7900 ULM

PIANTANELLI L.
INRACA Gerontology Res. Dept.
Via Birarelli 8, I.60100 ANCONA

POMMIER J.
INSERM U.96, Hôpital de Bicêtre,
78, avenue du Général Leclerc,
F.94270 LE KREMLIN-BICETRE

PORZIG H.
Univ. Bern, Pharmakologisches Inst.
Friedbuhlstrasse 49,
CH.3010 BERN

PRESEK P.
Rudolf Buscheim Inst. für Pharmakologie der Justus-Liebig-Univ., Frankfurtstrasse 107,
D.6300 GIESSEN

RAO G.
Inst. für klinische Biochemie der Univ. Bonn, Sigmund Freud Strasse 25,
D.5300 BONN-VENUSBERG

RAO M.L.
Universitätsnervenklinik, Abt. Psychiatrie Venusberg,
D.5300 BONN

RODBELL M.
Nat. Inst. of Arthritis , Metabolism and Digestive Diseases,
BETHESDA, MD.20205, U.S.A.

ROUOT B.
INSERM U.206, Inst. de Pharmacologie et de Méd. expérim., 11 rue Humann,
F.67000 STRASBOURG

ROUSSEAU G.
General Pathology, Ave Hippocrate 75,
B.1200 BRUXELLES

ROUSSET B.
INSERM U.197, Fac. de Médecine A. Carrel,
Rue G. Paradin, F.69372 LYON

ROSSIER J.
INSERM, Lab. de Physiologie Nerveuse,
CNRS, F.91190 GIF SUR YVETTE

SALTARELLI D.
CNRS, Lab. d'Enzymologie,
F.91190 GIF SUR YVETTE

SAMMECK R.
Inst. Für Histologie und Neuroanatomie,
Univ. Göttingen, Kreuzbergring 36,
D.3400 GOTTINGEN

SCHATZMANN H.J.
Univ. Bern, Veterinar-Pharmakologisches Inst., Langgass-strasse 124,
CH 3000 BERN

SCHULSTER D.
Hormones Division, Nat. Inst. Biol. St.
Holly Hill Hamstead,
LONDON NW3 6RB, UNITED KINGDOM

SCHULTZ G.
Pharmakologisches Inst. der Univ.
Heidelberg, Im Neuenheimer Feld 366,
D.6900 HEIDELBERG

SCHWOCH G.
Univ. Hamburg, Inst. für Physiologische Chemie, Martini-strasse 52, D.2000 HAMBURG 20

SHEPPARD J.R.
Dight Inst. for Human Genetics,
Univ of Minnesota,
MINNEAPOLIS, MN.55455, U.S.A.

SLADECSEZK F.
Lab. de Physiologie Cellulaire,
Collège de France, 11 Place M
Berthelot, F.75005 PARIS

SÖLING H.D.
Med. Universitätsklinik, Abt. fur
Klin. Biochemie, Humboldtallee 1,
D.3400 GOTTINGEN

STENGEL D.
INSERM U.99, Hôpital Henri Mondor,
F.94010 CRETEIL

STOCLET J.C.
Univ. L. Pasteur, Fac. de
Pharmacie, B.P.10,
F.67048 STRASBOURG

STRELLER I.
C.H. Boehringer, Dept of
Pharmacology, Binder Strasse,
D.6507 INGELHEIM

STROSBERG A.D.
Inst. de Rech. en Biologie
Moléculaire, Im. Moléculaire,
2 place Jussieu, F.75005 PARIS

SVOBODA M.
Faculté de Méd., Dept. de
Biochimie, 2 rue Evers,
B.1000 BRUXELLES

SWIERCZEWSKI E.
Biologie du Développement Foetal
et Néonatal, Hôpital Port Royal,
123 Bd de Port Royal,
F.75014 PARIS

TERPSTRA P.
Erasmus University,
Dept of Biochemistry
P.O. Box 1738,
NL.3000 DR ROTTERDAM

TIXIER-VIDAL A.
Neuroendocrinol. Cell., Coll.
de France, Pl. M.Berthelot, PARIS

TOMLINSON S.
Univ. of Sheffield, Human Metab.&
Clin. Biochem., Beech Hill Road,
SHEFFIELD S10 2RX, United Kingdom

TORU-DELBAUFFE D.
INSERM U.96,Hôp. de Bicêtre, Ave.
du Général Leclerc,F.94270 BICETRE

UZAN A.
Pharmuka Lab. Biochimie et Pharmacologie,
35 quai du Moulin de Cage,
F.92231 GENNEVILLIERS

VAN DER MOLEN H.
Erasmus Univ. Rotterdam, Dept of Biochistry, P.O. Box 1738,
NL3000 DR ROTTERDAM

VAN OBBERGHEN E.
INSERM U.145, Faculté de Médecine,
Chemin de Vallombrose,
F.06034 NICE Cedex

VAN SANDE J.
ULB, Inst. de Rech. Interdisciplinaire,
2 rue Evers, B.1000 BRUXELLES

VAYSSE N.
INSERM U.151, CHU de Rangueil, Bat L.3,
F.31052 TOULOUSE

VIETH U.L.
Rudolf Buchheim Inst. für Pharmakologie
der Univ. Giessen, Frankfurtstrasse 107,
D.6300 GIESSEN

VOKAER A.
ULB, Dept. de Gynécologie-Obstétrique,
4 place van Gehuchten, B.1020 BRUXELLES

WAELBROECK M.
ULB, Dépt. Biochimie, Bd de Waterloo,
B.1000 BRUXELLES

WEBER U.
Physiologisch-Chemisches Inst., Hoppe-
Seyler-Str. 1, D.7400 TUBINGEN

WEBER W.
Inst. für Phys. Chemie, Universitäts-
krankenhaus Hamburg Eppendorf,
Martinstrasse 52, D.2000 HAMBURG 20

WIERINGA T.
Univ. Hosp. Leiden, Dept of Endocrinology,
Rijsburgerweg 10, NL.2333 AA LEIDEN

ZWILLER J.
CNRS, Centre de Neurochimie,
5 rue Pascal, F.67084 STRASBOURG

CONTENTS

Hormones and Cell Regulation, Volume 6
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CALCIUM BINDING PROTEINS ARE INVOLVED BOTH IN CALCIUM EFFECTS AND IN THEIR SUPPRESSION

JACQUES HAIECH , CHRISTIAN J. LE PEUCH , MURIELLE L. RINALDI AND JACQUES G. DEMAILLE
Unité 249 "Biochimie des Régulations des Systèmes contractiles" de l'INSERM, Centre de Recherches de Biochimie macromoléculaire du CNRS, and Faculté de Médecine, BP 5015, 34033-Montpellier Cedex (France)

INTRODUCTION

The unstimulated eukaryotic cell is characterized in metazoans by a low free Ca^{2+} level (ca 0.1 µM). Upon excitation leading to contraction, secretion or other physiological effects, the free Ca^{2+} concentration rises to approx. 10 µM (1). Then Ca^{2+} level returns to its initial low value. Thereby a calcium wave is formed, by which the system departs from its stationary non-equilibrium state. Prigogine (2) wrote in his "Introduction to thermodynamics of irreversible processes" : "When a system is in a state of minimum entropy production then .... it cannot leave this state by a spontaneous irreversible change. If, as a result of some fluctuation, it deviates slightly from this state, internal changes will take place and bring back the system to its initial state, which may be referred to as a stable state".

It is our purpose in this paper to describe in molecular terms the role of the intracellular calcium-binding proteins both in the translation of the calcium wave into physiological effects and in the return to the stable state.

THE STABLE STATE

In the stationary state, cell membranes must be considered as permeable to calcium ions only through pumps (Ca^{2+}-stimulated Mg^{2+}- dependent ATPases) and exchange proteins (antiporter). The Ca^{2+} pumps are able to transport two Ca^{2+} ions per mol ATP hydrolyzed, while the Na^+/Ca^{2+} exchange protein, taking advantage of the Na^+ gradient maintained by the Na^+, K^+-ATPase, exchanges three Na^+ ions against one Ca^{2+} ion (figure 1). In both cases, the ATP-dependent Ca^{2+}- efflux from the cytosol results in the separation of three compartments. The cytosol is characterized by a low Ca^{2+} concentration (pCa $\geq$7) while much higher levels are reached in the extracellular space (pCa $\sim$ 3) or intracellular stores (pCa $\sim$ 2) such as the sarcoplasmic or endoplasmic reticulum. In the stable state, the rate of transport of Ca^{2+} out of the cytosol is minimal (3), and the intracellular low molecular weight calcium-binding proteins are free of Ca^{2+}. They form a family of evolutionarily related proteins that are all derived from a common four-domain ancestor. The ancestor protein was formed

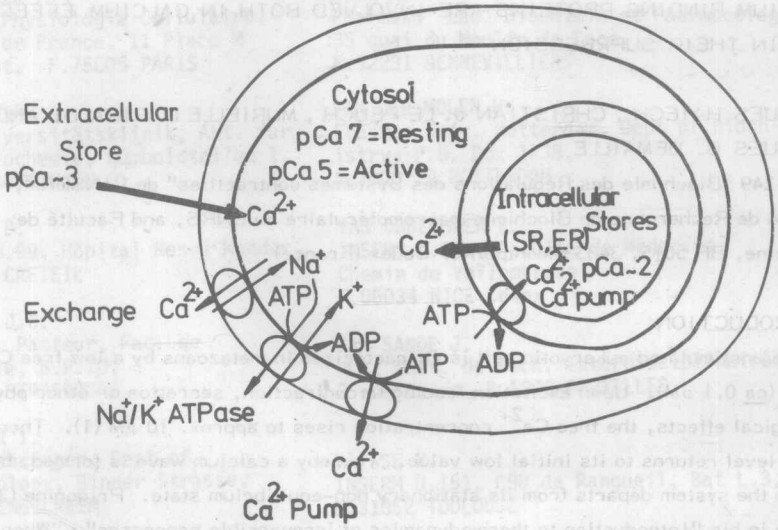

Fig. 1. A schematic representation of Ca^{2+} fluxes. Thick arrows represent Ca^{2+} channels. SR and ER stand for sarcoplasmic and endoplasmic reticulum, respectively.

by doubling and then quadrupling of a primordial gene that coded for a one-domain primordial peptide, made of a central 12-residue ion-binding loop flanked on each side by a 12-residue α-helix (4, 5). The ancestor evolved into three subfamilies. One of them includes the brain S-100 protein and the intestinal calcium-binding protein. Another contains the myosin regulatory light chains and parvalbumins, the soluble relaxing factor of fast skeletal muscle. The third subfamily is the most interesting in that it includes, besides the myosin alkali light chains, troponin C and calmodulin, that convey the calcium message to the calcium-activated enzymes (6).

Most remarkable in the story of the family was the divergent evolution of calcium-binding sites toward either the loss of divalent metal-binding (in myosin alkali light chains or in parvalbumin domain II) or the acquisition of different Ca^{2+}- and Mg^{2+}-binding properties (5). Some sites, e.g. domains III and IV of parvalbumins and

troponin C evolved to high affinity Ca^{2+}-Mg^{2+} sites with affinities for Ca^{2+} in the nanomolar range and $pK_{d\ Mg^{2+}} \sim 5$ (7). Those sites are saturated by Mg^{2+} in the resting cell where free Mg^{2+} concentration is ca 0.6 mM (8). Other sites, such as skeletal troponin C domains I and II or calmodulin domains, are considered as low affinity Ca^{2+}-specific sites because their affinity for Mg^{2+} ions is too low for the sites to be saturated by Mg^{2+} in the stable state. For instance, calmodulin is best described as

$$CaM \cdot Ca_0^{2+} \cdot Mg_{0.7}^{2+} \cdot K_{3.2}^{+}$$

in the presence of 150 mM KCl, 0.6 mM Mg^{2+} and 0.1 µM Ca^{2+} (9). Under these conditions, all Ca^{2+}-binding proteins are either free (parvalbumins, calmodulin) or, when they are bound to other macromolecules (troponin C to troponin I and T, the tightly bound calmodulin, or δ subunit, to the other subunits of phosphorylase kinase (10)), they exhibit the "Ca^{2+}-free" conformation that leaves the particular target in an inhibited state.

THE EXTERNAL PERTURBATIONS

The stable state is altered when primary signals from other cells, such as hormones or neuromediators, reach the cell membrane and interact with specific receptors. A physical discontinuity in the cell membrane permeability barrier to Ca^{2+} ions is then created by the opening of Ca^{2+} channels. Almost nothing is known about the proteins the channels are built from. It seems, at least from observations on the slow Ca^{2+} channel of the cardiac muscle, that the increase in the cytosolic Ca^{2+} level resulting from the voltage-dependent Ca^{2+} uptake is moderate and at any rate unable to trigger and sustain contraction. There is however evidence that the spatial and temporal distribution of Ca^{2+} ions within the cell is not uniform (11,12). Much of the Ca^{2+} increase appears to be restricted to the vicinity of the plasma membrane, where it can already activate Ca^{2+} pumps which exhibit highly cooperative activation curves in the range 0.1 to 1 µM (3). More important, Ca^{2+} entry is able to trigger the "Ca^{2+}-induced Ca^{2+}-release" from internal stores (13), through undefined channels. Such autocatalytic release is massive enough to increase Ca^{2+} level up to 10 µM (1) within a few milliseconds. What is more puzzling is the reason why Ca^{2+} efflux from e.g. sarcoplasmic reticulum stops while the concentration gradient is still quite steep, unless we assume that Ca^{2+} activates the reticular channels at low concentrations and blocks them at higher levels. An interesting feature of the slow Ca^{2+} channel in cardiac muscle is its modulation by the other second messenger, namely cAMP. Upon epinephrine stimulation, the contractile force increases as a result of a better Ca^{2+} load of the cell. This positive inotropic effect is mediated at the plasma membrane level by the dissociation of type II cAMP-

-dependent protein kinase and the subsequent phosphorylation of calciductin, a 23 000-dalton proteolipid that is either the Ca^{2+} channel or its activator. Such phosphorylation results in a ca 2-fold increase in the voltage-dependent Ca^{2+} uptake, measured in purified right-side-out sarcolemmal vesicles (14).

CALMODULIN AND CALCIUM-DEPENDENT ENZYMES

When the Ca^{2+} level increases in the cytosol from pCa 7 to pCa 5, the Ca^{2+}-specific sites of calmodulin and troponin C are first saturated with diffusion-limited kinetics. Calmodulin primary structure (15) points to four calcium-binding sites, as confirmed by most ion-binding studies (see ref. 16 for review). It was proposed in one report that all four sites are equivalent and saturated at random (17). More recent studies (16) show however that Ca^{2+}, Mg^{2+} and K^{+} compete for each site (Table I) and that Ca^{2+} binding is sequential and ordered with a unique binding sequence.

TABLE 1

ION BINDING TO CALMODULIN [a]

Domain [b]	II	I	III	IV
Ca^{2+} (nM)	67	170	600	900
Mg^{2+} (μM)	70	270	100	90
K^{+} (mM)	3.7	10.6	8.7	1.5

[a] Intrinsic dissociation constants

[b] Labeled from their order in the primary structure from the NH_2- to the COOH-terminus.

Assuming thermodynamic equilibrium is reached, free calmodulin would be under the form :

$$CaM \cdot Ca^{2+}_{2.8} \cdot Mg^{2+}_{0.1} \cdot K^{+}_{1.2}$$

in the presence of 150 mM KCl, 0.6 mM Mg^{2+} and 10 μM Ca^{2+} (9). This indicates that the fourth site is probably saturated only in the enzyme-calmodulin complex. The calcium-binding sequence of free calmodulin was determined by using the luminescent properties of Tb^{3+} ions when bound to a protein close to a tyrosyl phenol group (18). No enhancement of Tb^{3+} luminescence was observed up to two mol Tb^{3+} per mol, that were therefore bound to higher affinity domains I and II that lack tyrosyl residues. Domain III is next in the saturation sequence, as shown by similar experiments performed on octopus calmodulin which contains only tyrosine 138 in domain IV (19). Finally, the ion-binding sequence is II → I → III → IV. Calcium binding to calmodulin induces conformational changes that were studied by a number of techniques, including NMR, CD and UV differential spectroscopy and tyrosine

fluorescence (see ref. 20 for a review). Most spectral changes are complete after binding of only two mol Ca^{2+} per mol, indicating that Ca^{2+} binding occurs in at least two steps, corresponding to binding of a first pair of Ca^{2+} ions to domains II and I, followed by saturation of domain III and eventually IV (20) that leads to activation of calmodulin-dependent enzymes.

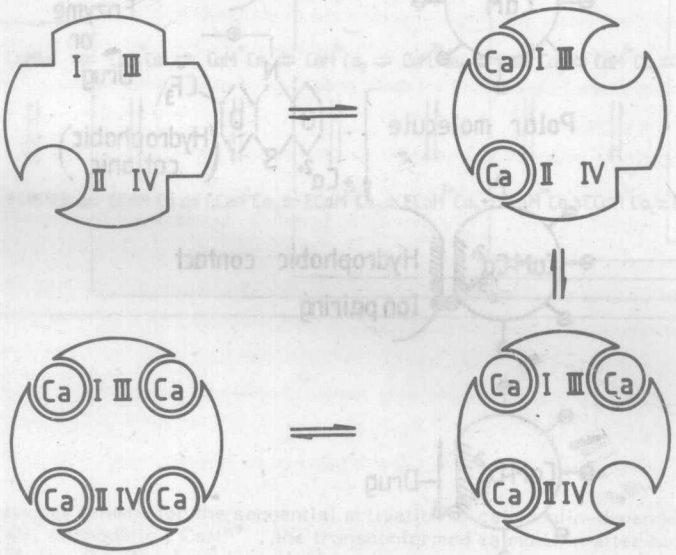

Fig. 2. A pictorial representation of the sequence of ion-binding to calmodulin and of the resulting conformational changes. The domains of the primary structure are numbered from the NH_2- to the COOH-terminus.

It has indeed been shown that myosin light chain kinase or cyclic nucleotide phosphodiesterase are activated only by CaM . Ca^{2+}_{3-4} and not by CaM . Ca^{2+}_{2}, even though the subtle conformational changes that occur in the transition from the latter to the former calmodulin form are as yet unknown (21-23). In any case, the final activating calmodulin exhibits a hydrophobic patch on its surface that is capable of interacting with and binding to a similar patch on either the target enzyme or the anti-

psychotic drugs that block the activation of calmodulin-dependent enzymes (24) (Figure 3).

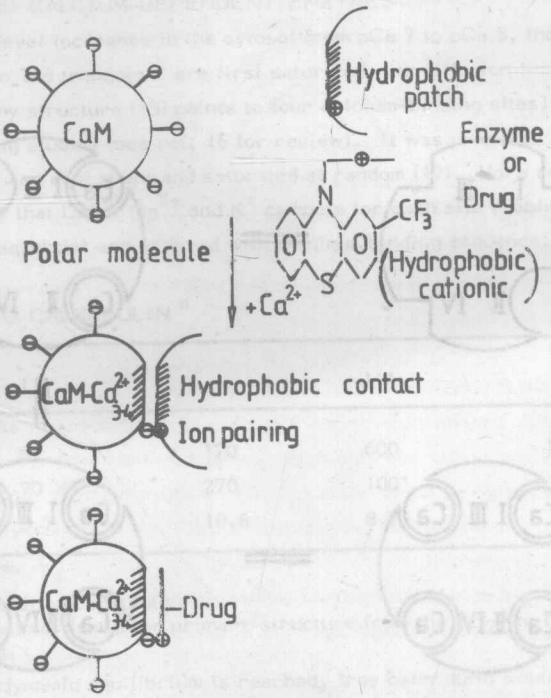

Fig. 3. Calmodulin binding to target enzymes and to tricyclic antipsychotic drugs. CaM, calmodulin. The putative scheme stresses the importance of both hydrophobic and electrostatic bonds.

The calmodulin species that binds to the target enzyme may well be different from the species that activates it. For instance, the tightly bound calmodulin of phosphorylase kinase, i.e. its δ subunit, remains attached to the enzyme in the absence of Ca^{2+} (10) whereas calmodulin dissociates from myosin light chain kinase or cyclic