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Thesis approval

The abstract and thesis of Alexander Braun for the Master of Science in Physics were presented July 12, 1995 and accepted by the thesis committee and the department.

Committee Approvals:

[Redacted Signature]

Jonathan J. Abramson, Chairperson

[Redacted Signature]

Pavel K. Smejtek

[Redacted Signature]

Carl C. Wamser,
Representative of the Office of Graduate Studies

Department Approval:

[Redacted Signature]

Erik Bodégom, Chair, Department of Physics

Accepted for Portland State University by the Library

by [Redacted Signature] on 12 September 1995

ABSTRACT

An abstract of the thesis of Alexander Braun for the Master of Science in Physics presented July 12, 1995, and accepted by the thesis committee and the department.

Title: The Interaction between a Thiol Specific Probe (OPA) and the Single Channel Characteristics of the Reconstituted Ca^{++} Release Protein from Skeletal Muscle Sarcoplasmic Reticulum.

One advantage of higher life-forms over less developed organisms is their ability to respond to signals from their environment with motion. This requires highly specialized contractile cells and a whole locomotion apparatus. In vertebrates, the cells responsible for movement are the skeletal muscle cells. They receive signals from the autonomic nervous system in the form of an action potential, and they contract in an appropriate manner. Calcium is a vital intracellular passenger whose role in muscular function is to initiate contraction. It is released via specific channel proteins from an internal Ca^{++} store, the sarcoplasmic reticulum, and triggers muscular contraction, the actual interplay of actin and myosin filaments.

The step that is still not fully understood is the coupling process between arrival of an action potential and the subsequent contraction, called excitation-contraction coupling. Several theories have been proposed to explain this process. Some years ago, our laboratory introduced the hypothesis that an oxidation-reduction reaction of critical sulfhydryls associated with the Ca^{++} channel protein are involved in the regulation of channel gating.

In an effort to understand more about the Ca^{++} channel gating mechanism at the molecular level, this thesis focuses on the interaction between *o*-phthalaldehyde, a reagent which specifically forms an isoindole derivative with the amino acids cysteine and lysine, and the Ca^{++} release channel complex.

In this thesis, the planar lipid bilayer technique was used to study the Ca^{++} release channel protein from skeletal muscle sarcoplasmic reticulum at the single channel level. Utilizing this experimental technique, the direct interaction between OPA and the channel was investigated. In this study, it was shown that the interaction of *o*-phthalaldehyde with the channel increases the channel's open probability as well as its mean open time. Furthermore, the covalent nature of *o*-phthalaldehyde binding to the calcium release channel complex is shown and its inhibiting effects on chloride channels are demonstrated.

**The Interaction between a Thiol Specific Probe (OPA) and the Single Channel
Characteristics of the Reconstituted Ca^{++} Release Protein from Skeletal Muscle
Sarcoplasmic Reticulum**

by

Alexander Braun

A thesis submitted in partial fulfillment of the requirements for the degree of

**Master of Science
in
Physics**

**Portland State University
1995**

This thesis is dedicated to my parents, Carla and Gerhard.

You had an ear for my wishes and made my stay in the Portland, Oregon possible.

I thank you both.

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I will never forget my time in Portland, my work and my studies.

It's been a great time.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
CRC	calcium release channel
BLM	bilayer lipid membrane
DHP	dihydropyridine
ECC	excitation contraction coupling
EGTA	[ethylenebis(oxyethylenenitrilo)]tetraacetic acid
DMSO	dimethylsulfoxide
OPA	<i>o</i> -phthalaldehyde
PE	phosphatidylethanolamine
PS	phosphatidylserine
RRed	ruthenium red
RYR	ryanodine receptor
SR	sarcoplasmic reticulum

CHAPTER I: PHYSIOLOGICAL BACKGROUND

1. Mammalian Muscles

1.1. Muscles in General, Types of Muscle

There are three different types of muscle tissue, smooth, skeletal, and heart or cardiac muscle. Skeletal muscle is primarily concerned with effecting adjustments to the organism's environment, while smooth muscle is responsible for movements in response to internal changes.

Figure I: Types of Muscle A) Smooth fibers; B) Portion of two skeletal muscle fibers, each with many nuclei and crossed by alternating light and dark bands, or striations; C) cardiac muscle, the dark black lines, called interlaced discs, now known to be the places where one cell ends and the next begins

(Adapted from W.T. Keeton "Biological Science")

Smooth muscle forms a muscle layer in the walls of the digestive tract, bladder, various ducts, and other internal organs. It is also present in the walls of arteries and veins.

The individual smooth muscle cells (or fibers as they are commonly called) are thin and usually pointed at their ends. Each has a single nucleus and is not striated. Smooth muscle interlaces to form sheets of muscle tissue rather than bundles. Smooth muscle is innervated by the autonomic nervous system.

Skeletal muscle is the best understood muscle. It is responsible for the movements of limbs, trunk, face, eyeballs and jaws. It is by far the most abundant tissue in the vertebrate body. Each skeletal muscle fiber has a cylindrical shape. It contains many nuclei and is crossed by alternating light and dark bands called striations. The fibers are usually bound together by connective tissue into bundles rather than sheets. These bundles, in turn, are bound together by connective tissue and form the composite structure called muscles. Skeletal muscle is innervated by the somatic nervous system. Fibers contract when stimulated by nerve impulses and relax in the absence of electrical stimulation. This thesis will deal only with skeletal muscle contraction.

Cardiac muscle, or heart muscle, shows some characteristics similar to both skeletal and smooth muscle fibers. Its fibers, like those of skeletal muscle, are striated and contain more than one nucleus. Like smooth muscle, it is innervated by the autonomic nervous system, and its activity is more like that of smooth muscle. Electron microscope studies have revealed that cardiac muscle is composed of separate fibers or cells, and that adjacent surfaces of these fibers are interdigitated.

Striated muscle contracts faster than smooth muscle, but does not remain contracted as long.

1.2. Internal Active Structure of Striated Skeletal Muscle

Skeletal muscle is composed of bundles of multinucleate cells (muscle fibers) which are longitudinally aligned in the direction of contraction. Each fiber is composed of many contractile units, called myofibrils, or the myosin filament, which stretch the length of the fiber. Closer examination reveals that each myofibril is further subdivided into repeating alternating light and dark band units of contractile machinery called sarcomeres. The sarcomere is the functional unit of contraction. It is about 2 μm long in resting

muscle. Contraction of the sarcomere shortens the length of the myofibril, and the length of a entire muscle. In contracted muscle, the sarcomere shortens to 70 percent of its uncontracted, resting length.

Electron microscopy and biochemical analysis have shown that each sarcomere contains two types of filament: thick filaments composed of *myosin*, and thin filaments, containing *actin*. Near the center of the sarcomere, actin filaments overlap with myosin filaments.

The thick filament of the dark band is bipolar, with its myosin heads lying at the distal tips of the filament and its tails at the center. The light band however, is a bundle of thin filaments. All the filaments in a light band are of the same length. Biochemical studies have shown that thin filaments are an array of actin molecules plus two additional proteins that are involved in regulating interactions with myosin.

Figure II: The component parts of a skeletal muscle. A) A whole muscle B) A small part of the muscle magnified to show the muscle cells, or fibers. C) Part of the fiber much magnified. D) Myofibril removed from a fiber. E) A sarcomere of the myofibril much magnified to show the pattern of striations. F) The myosin and actin filaments that give rise to the pattern of light and dark bands. The A-band corresponds to the length of the thick myosin filaments; the H-zone is the region where only the thick filaments occur, while the darker ends of the A-bands are regions where thick and thin filaments overlap. The I-bands correspond to regions where only thin actin filaments occur. (Adapted from H.E. Huxley, 1958)

2. Muscle Contraction

2.1. General Features

Individual muscle fibers resemble individual nerve cells in firing only if an impinging stimulus is of threshold intensity, duration, and rate. Vertebrate muscle fibers also seem to exhibit this all-or-nothing property. However, an individual muscle can give graded responses depending upon the strength of the stimulation reaching it. In the laboratory this can be demonstrated by removing a leg from a frog and attaching it to a device that will measure the extent of contraction of the muscle when it is stimulated. If a stimulus barely above the threshold intensity is administered, the muscle gives a very weak twitch. A slightly stronger stimulus gives a slightly stronger twitch. One can keep increasing the strength of the stimulus and getting a stronger contraction from the muscle until we reach a point where further increases in the stimulus do not increase the strength of the response.

How does this fit with the all-or-nothing theory? One possible explanation is that the threshold values of different muscle fibers, of which a whole muscle is composed, are not the same. Alternatively, different muscle fibers may be innervated by different nerve fibers, and these nerve fibers may not all fire at the same time. Thus, although single fibers show an all-or-nothing response to electrical stimuli, an increase in the strength of the stimulus above the threshold level may elicit a greater response from the whole muscle by stimulating more muscle fibers.

It must be stressed that the description given above applies only to vertebrate skeletal muscle. The striated-muscle fibers of invertebrates seldom exhibit an all-or-nothing property; the strength of their contraction is proportional to the stimulating frequency.

2.2. The Sliding-Filament Model

It has been known for many years that during a muscle contraction heat is generated. If a contracting muscle can perform work and if it releases heat in the process, an energy supply must be involved. This energy, in the form of ATP (adenosine triphosphate) comes from oxidation of food such as glycogen, glucose, and fatty acids. In fact, a high percentage of the oxidative phosphorylation in a mammal's body occurs in the muscle.

In the 1950s a model was proposed to explain how muscle contraction occurs. The central point of this model is that ATP-dependent interactions between thick filaments (myosin) and thin filaments (actin) generate a force which causes thin filaments to slide past thick filaments. The force is generated by myosin heads, which make cross-bridge attachments with actin. Conformational changes in a cross-bridge cause the myosin heads to walk along the actin filament. The overlap region of the actin and myosin molecules is the only part of the sarcomere where myosin heads can bind to actin filaments. The sliding-filament model predicted that the force of contraction should be proportional to the overlap between the two filament systems.

To understand how a muscle contracts, one has to look at the interactions between one myosin head and one thin actin filament. During the steps of contraction, the myosin head binds ATP, detaches from the thin filament, and undergoes a conformation change to its new position. After hydrolysis of ATP, the head rebinds to a new position on the thin filament and executes the conformational changes that are associated with the power stroke. At the completion of the cycle a myosin head has moved two subunits closer to the (+) end of the filament. Because the myosin heads are physically connected to the rigid rodlike backbone of the thick myosin backbone, myosin remains near actin and probably dissociates very briefly from the filament during contraction. Thus the heads, once they dissociate from a thin filament, are available to rebind to the thin actin filament. It is

assumed that each oscillation of a cross bridge would require the energy of one ATP molecule.

Figure III: Model of the function of cross bridges between myosin and actin filaments. A) The cross bridges are thought to be part of the thick myosin filaments **B)** Movement of the distal portion of the cross bridges pulls the actin filaments in a ratchetlike mechanism.

(Adapted from W.T. Keeton "Biological Science")

Muscle contraction is the collective pull of hundreds of myosin heads of a single thick filament on one actin filament, amplified by the hundreds of thick filaments in a sarcomere and thousands of sarcomeres in a muscle fiber. Because the thick myosin filament is bipolar, the action of the myosin heads at opposite ends of the thick filament draws the thin actin filament toward the center of the thick myosin filament and therefore

toward the center of the sarcomere. This movement shortens the sarcomere until the ends of the thick filaments reach the end of the thin actin chains or the (-) ends of the thin filaments jam together at the center of the sarcomere.

2.3. The Stimulus For Contraction, Sarcoplasmic Reticulum

Like the membrane of a resting neuron, that of a resting muscle fiber is polarized, the outer surface being more positively charged than the inner one. Stimulatory transmitter substances released by a nerve axon at the neuromuscular junction cause a brief reduction of this polarization. If the reduction reaches the threshold level, an impulse, or action potential, is triggered which then propagates over the surface of the fiber. It has been known for a long time that the action potential somehow activates the contraction process, but only in the last few years some of the steps involved have been described.

Unlike sodium ions in nerve cells, the depolarizing phase of an action potential in muscle cells depends on an inflow of calcium ions. The repolarizing phase is marked in both cases by an outward flow of potassium ions. It was reasonable to propose that the calcium ions that flow into the cell during the cardiac action potential as the wave of depolarization moves across the cell surface causes the myosin cross bridges to become active. When this idea was first put forward, however, there were at least two major objections to it. First, contraction of a vertebrate muscle fiber requires the essentially simultaneous shortening of all its many myofibrils, but the myofibrils in the center of a fiber are so far from the surface membrane that calcium ions from outside could not possibly diffuse fast enough to reach them in the short interval between stimulation and contraction. Second, there was evidence that not enough Ca^{++} entered the cell to account for the contraction of all involved myofibrils.

It was the rediscovery in 1955 of an extensive network of tubules in muscle that opened the way to a solution of this problem. Tubules were soon found to comprise two separate but functionally related systems: the *sarcoplasmic reticulum* (SR), an internal membrane system which does not open to the exterior, and the transverse or *T-system*, which is a continuation of the surface membrane and does open to the outside. (see Figure

IV.) Furthermore, the T-tubule membrane contains a high percentage of calcium channels which are known as dihydropyridine (DHP)-sensitive calcium channels (Sabbadini and Dahms, 1989). The function of the DHP receptor is to respond to the action potential and to deliver a signal to the SR to release its stores of Ca^{++} and induce muscle contraction.

Not all muscles are activated the same way, but all require an increase of the free Ca^{++} concentration. The sarcoplasmic reticulum is the muscle cell's highly specialized version of the endoplasmic reticulum. Its membranous canals form a cufflike internal Ca^{++} store network around each of the sarcomeres of each myofibril (Peachy 1965) (Van de Graff and Fox, 1986). The structure of SR can be divided into two morphologic regions. At the end of a sarcomere the reticulum forms a series of sacs called *terminal cisternae* or *junctional SR* or *heavy SR*. Between the junctional SR stretches the *longitudinal SR* (Van Winkle, 1986) or *light SR*, which surrounds the bulk of the sarcomere, and contains predominantly Ca^{++} pump proteins (Steward and MacLennan 1974). Between the terminal cisternae at the distal end of one sarcomere and at the proximal end of the next sarcomere is the transverse tubule or the T-system. Though the terminal cisternae and the T-tubules are in direct contact, there is no interconnection between their cavities and hence no mixing of their contents. The repeating structure composed of junctional SR-T-tubule-junctional SR is called a triad (Peachy, 1965).

The tubules of the T-system can be shown to be deep invaginations of the cell membrane. When an action potential is propagated across the cell surface, it also penetrates into the interior of the fiber via the T-tubule. The action potential moves much faster than diffusing ions, fast enough to reach all the myofibrils at nearly the same instant. As a consequence myofibrils near the surface and those in the center of the fiber contract at practically the same time.

Figure IV: Relationship between the Sarcoplasmic Reticulum and the T-system. Portion of a muscle fiber. The longitudinal section reveals the intimate association between the terminal cisternae of the sarcoplasmic reticulum and the T-tubules. The cross section at the level of a Z-line shows that the tubules of the T-system are invaginations of the plasma membrane.

(Adapted from W.T. Keeton "Biological Science")

Figure V: The Role of Calcium in Stimulation of Contraction. **A)** A sarcomere in the resting (relaxed) condition. Calcium ions are stored in high concentration in the terminal cisternae of the sarcoplasmic reticulum. **B)** The polarization of the membranes of the T-tubules is momentarily reversed during an action potential impulse, and this reversal of polarization induces the release of the calcium ions, which spread over the sarcomere and stimulate contraction.

(Adapted from W.T. Keeton "Biological Science")

The intimate association discovered between the T-tubules and the junctional SR suggested that an action potential moving along the membrane of a T-tubule directly alters the properties of the cisternal membrane. The cisternae contains very large amounts of calcium, which as seen in Figure V, cause the myofibrils to contract. The action potential induces a sharp, very marked increase in the permeability of the terminal cisternal membrane to calcium ions, resulting in an increase in the myoplasmic Ca^{++} concentration. It is this suddenly released intracellular calcium that leads to muscle contraction. Once in the myoplasm, the Ca^{++} ions bind to specific high-affinity Ca receptors, the regulatory proteins calmodulin and troponin. These proteins have several Ca^{++} binding sites and respond very sensitively to free Ca^{++} ion concentration changes between 0.1 and 10 μM . They in turn activate enzymes or release them from inhibition. Whole cascades of nucleotide metabolism and protein phosphorylation can also be called into play. In muscle the most obvious result is the shortening following activation of actomyosin ATPase, the

enzymatic unit of the contractile filament. The transient opening of the Ca^{++} release channel (increase of permeability) is complete prior to the onset of muscle contraction and lasts 3 to 5 ms in duration (Martonosi, 1984).

Relaxation occurs when the Ca^{++} , Mg^{++} ATPase calcium pump in the longitudinal SR actively causes the calcium ions to move back into the lumen of the SR (cisternae). Hereby, the myoplasmic Ca^{++} concentration decreases to submicromolar levels. The reabsorption of Ca^{++} by the SR results in the debinding of Ca^{++} from the troponin binding sites, and as a result the actin and myosin filaments dissociate from one another.

2.4. Excitation Contraction Coupling

The term excitation contraction coupling (ECC) stands for the critical link between *excitation* by the motorneuron and the final *contraction* of the muscle fiber. The event takes place in the 120 Å gap between T-tubules and SR. Although this link has been investigated for a long time the actual mechanism still remains unsolved and controversial. The question of how the T-tubule communicates with the SR and causes the release of Ca^{++} stored in the lumen of the SR has not yet been answered.

Two different approaches have been taken in an attempt to understand the mechanism underlying ECC. Physiologists have tried to understand how the entire system works as a whole, hoping to identify the responsible structural elements later on (Eisenberg, 1987). Biochemists on the other hand have tried to identify the components involved and correlate their observed properties in an isolated assay with their biological function. Using this approach many important proteins have been sequenced in the last few years. The patch clamp technique has been a very powerful technique for understanding the functional roles of ion transport proteins. Unfortunately, the CRC is an internal membrane channel and can not be examined in this manner. However, using subcellular fractionating techniques, vesicles of SR or T-tubules have been isolated. It has been found that these vesicles maintain much of their structural and functional characteristics (Campbell et al., 1980). Depending on the isolating technique, SR and T

tubules may remain in a triad formation. Electron microscopy on vesicles provide insight into the structure of the triad (Franzini-Armstrong, 1970). Even with another technique, the bilayer method (see Chapter II), one cannot determine what protein(s) constitute the CRC because whole vesicles composed of a number of proteins are used. Purified proteins can also be added to the bilayer. However, correlation and comparison of results of different approaches will surely help to identify the CRC and characterize its behavior.

Clarification of the CRC's identity is ultimately necessary for a complete understanding of EC coupling. However, in its absence, many models have still been proposed to describe the communication between T-tubules and SR. These models fall into three categories: namely electrical, mechanical, and chemical.

2.4.1. Mechanical Coupling

In 1986 Franzini-Armstrong suggested a mechanical EC coupling between the T-tubules and the SR membrane. This work is based on electron micrographs which reveal a physical connection referred to as junctional feet protein (JFP) or simply "feet" proteins linking the T-tubules with the SR. Considerable effort has been directed toward investigation of these "feet" proteins (Franzini-Armstrong, 1986).

According to Chandler (1976) the coupling mechanism works on a rigid rod and plug interaction. Although simple, this model has recently gained credibility. The linking protein between the T-tubule and the SR could be the JFP which when electrically depolarized undergoes a conformational change opening the CRC. In this model the foot part of the JFP acts like a plunger, whereas the DHP receptor in the T-tubule membrane constitutes the voltage sensor (Block et al., 1988).

Junctional T-tubules show diamond-shaped clusters of particles that correspond in position to the subunits of the foot protein. The finding of a protein that was identified as the high affinity ryanodine receptor which forms a four-leaf clover structure similar to the feet structures supports the idea that the JFP and the ryanodine receptor and the Ca^{++} release channel are synonymous.

2.4.2. Electrical Coupling

Neurons transmit electrical signals to muscle via propagating action potentials. Therefore, it is reasonable to propose a direct electrical coupling between T-tubule and SR. The electrical connection between these two membranes could be the JFP. However, no evidence of electrical depolarization of the extensive SR membrane was found in high impedance measurements of muscle cells (Eisenberg, 1987).

An exclusively electrical coupling hypothesis claims that depolarization of the T-tubule causes a potential change at the SR membrane which then opens the CRC. This also seems unlikely because the SR membrane is leaky to most ions and no ion gradient other than Ca^{++} exists across the SR membrane. It seems therefore unreasonable to believe that an electrical potential difference could be maintained across the SR membrane (Martonosi, 1984). Furthermore, the CRC in the SR when fused into an artificial lipid bilayer shows no strong voltage dependency (Smith et al., 1986).

2.4.3. Chemical Coupling

Chemical coupling between the T-tubule and the SR is based in part on the fact that there is a small latency between the arrival of the action potential in the T-tubules and the actual beginning of Ca^{++} release from the SR. Among several chemicals that control the Ca^{++} channel under physiological and non physiological conditions, Endo in 1970 proposed the *Ca^{++} -induced Ca^{++} release* theory. In this very popular model, Ca^{++} is the linking factor between polarization of the T-tubule and Ca^{++} release from the SR. This seemed quite reasonable and simple since Ca^{++} is clearly present and well regulated. Free Ca^{++} has been shown to induce the release of calcium from isolated SR vesicles and skinned fibers (Ford and Podolsky, 1970). In addition, the presence of Ca^{++} channels in the T-tubules supports this idea. Recent evidence demonstrates the weakness of this hypothesis. Contraction in whole muscle fibers was shown to take place in the absence of extracellular calcium and, furthermore, in the presence of Cd^{++} which blocks Ca^{++} flux through T-tubules (Lüttgau, 1987). Another fact that speaks against the Ca^{++} linkage is

that the kinetics of the DHP-sensitive Ca^{++} channels in the T-tubules does not timely fit with the activation of contraction. DHP receptors gate slower and longer than needed (Agnew, 1988). Thus, calcium appears not to be the critical link in ECC.

2.4.4. Transmitter Coupling

The intracellular transmitter inositol -1,4,5-triphosphate (IP_3) may serve as the missing bridge between depolarization of the T-tubule and release of Ca^{++} from the SR. IP_3 has been shown to release Ca^{++} from endoplasmic reticulum as well as it induces Ca^{++} release from purified SR fractions from skeletal muscle (Volpe et al., 1985). The application of this chemical resulted in contraction, and electrical stimulation of muscle causes the production of IP_3 (Vergara et al., 1985). The breakdown of phosphatidylinositol -4,5-diphosphate (PIP_2) in the T-tubular membrane upon depolarization has been proposed. The breakdown of PIP_2 results in the production of IP_3 which could travel across the junctional gap and stimulate Ca^{++} release. However, the kinetics of this model disagree with experimental observations. It is doubtful if the IP_3 can be made and transported to the SR in a time scale consistent with ECC.

2.4.5. Sulfhydryl Oxidation

Calcium release is sensitive to oxidation of internal sulfhydryl (SH) groups. This suggests another physiologically relevant Ca^{++} release mechanism. Abramson and Salama (1989) proposed that these SH groups regulate the gating of the CRC by oxidation-reduction reactions. Oxidation of two SH to a S-S opens the channel, while the reduction of the disulfide forms closed channels. Oxidation by compounds that specifically react with SH groups stimulates Ca^{++} release from SR vesicles and chemically skinned muscle fibers. Reducing agents reverse the effect and favor calcium uptake (Zaidi et al., 1989). Binding of heavy metals to endogenous SH groups causes calcium release specifically from terminal cisternae SR vesicles. Silver ions have been studied in most detail. Calcium release by Ag^+ is most effective under physiological conditions, i.e., pH 7.0 and 1 mM free

Mg^{++} (Salama and Abramson, 1984). More recently it has been shown that irrespective of the Ca^{++} releasing agent added to stimulate Ca^{++} release a high molecular weight disulfide - linked multiple protein complex is formed. The closure of the Ca^{++} release channel results in the dissociation and reduction of this complex. It appears as if underlying ECC is the oxidation of critical thiols associated with a number of key SR proteins.

3. Ion Channels

3.1. General Features

Ionic channels are excitable macromolecular protein pores in the membranes of nerve, muscle, and other tissue. Their known functions include establishing a resting membrane potential, shaping electrical signals, gating the flow of messenger Ca^{++} ion, controlling cell volume, and regulating the net flow of ions across cell membranes.

Each channel may be considered as an excitable molecule as it is specifically responsive to some stimulus: a membrane potential change, a neurotransmitter or other chemical stimulus, a mechanical deformation, and so on. The channel's response, called *gating*, is apparently a simple opening or closing of the pore. The open pore has the important property of selective permeability, allowing only some restricted class of small ions to flow passively down their electrochemical activity gradients at a rate that is very high ($> 10^6$ ions per second) when seen from a molecular viewpoint.

Naming of ionic channels has not been systematic. It seems rational to name a new channel after the most important permeant ion. This fails however, if the ions involved are not adequately known, or when no ion is the major ion, or when several different kinetic components are all clearly carried by one type of ion.

3.2. Calcium Channels

Upon sensing membrane potential changes, Ca^{++} channels found in the muscle cell's surface membrane simultaneously generate an electrical signal (the net inward depolarizing

current) and create an intracellular chemical messenger. The inward, depolarizing current results in an accumulation of Ca^{++} in the cytoplasm, which can act as a chemical trigger for secretion of hormones and neurotransmitters, contraction of muscle and a variety of Ca^{++} -sensitive events. This dual ability is unique among the family of ion channels and allows Ca^{++} channels to play a variety of roles in excitation-secretion and excitation-contraction coupling.

Ca^{++} channels have been classified into three different types (McCleskey, 1986). There are L-type, T-type and N-type Ca^{++} channels. They differ in characteristics like steady-state inactivation, activation threshold, selectivity and pharmacology. Channels of the L-type are found in heart and in T-tubules of skeletal muscle fibers. These channels have long lasting, non-inactivating currents, are activated by high voltage and have a relatively high Ba^{++} conductance of 25 pS (the conductance of barium ions is higher than that of calcium). In contrast, T-type channels have transient currents which inactivate completely in about 50 ms. They are activated by low voltages and have a relatively small Ba^{++} conductance of 8 pS (here $I_{\text{Ca}} > I_{\text{Ba}}$). Generally, T-type channels are found in neurons. Recently, evidence to support the existence of a third type of Ca^{++} channel has been presented. This channel is the N-type channel and appears to be an intermediate of the L- and T-type channels. It is thought to be responsible for neurotransmitter release from presynaptic terminals. The N-type channel inactivates rapidly, is activated by high voltage and has an intermediate Ba^{++} conductance (13 pS). Although kinetics provide some good distinguishing criteria for comparing Ca^{++} channels, discrepancies still exist. The literature suggests that many cells have several kinds of Ca^{++} channels.

Many Ca^{++} channels are both voltage sensitive and chemical sensitive. Calcium channels commonly allow Ca, Ba, Sr, and Cs ions to pass through them. Mg, Mn, Cd, Co, Ni, and La ions, however, all block the current flowing through an open Ca^{++} channel. Some organic blockers have been identified as well. Many of them are DHPs (nifedipine and nitrendipine) and phenylalkamines (verapamil and D600). Bay K8644 as a synthetic DHP, is an L-type Ca^{++} channel agonist.

In skeletal muscle, voltage sensing dihydropyridine (DHP) receptors in the plasma membrane contact ryanodine receptors (RYRs) located in the membrane of the sarcoplasmic reticulum. In response to a change in voltage, the dihydropyridine receptor undergoes a conformational change. This produces a change via an unknown mechanism in the associated RYRs, so that Ca^{++} ions can exit the SR into the cytosol. The ryanodine receptors have received their name for their sensitivity to the plant alkaloid ryanodine which is naturally occurring in certain South American trees.

The highest concentration of Ca^{++} release channels exists in the junctional SR (terminal cisternae) which are positioned closest to the T-tubule. This CRC is composed of four identical 565 kDa subunits, which form a large conductance channel that is strongly cation-selective and permeable to both monovalent and divalent cations (Smith et al., 1988). Since this is not an outer membrane channel the CRC cannot be studied by patch clamping. To investigate this membrane internal channel one can fuse it into a artificial lipid bilayer membrane. This channel has been shown to be activated by Ca^{++} and ATP and to be inhibited by ruthenium red and Mg^{++} . Compared with other Ca^{++} channels, the CRC has a high conductance. Commonly reported values for conductance are, $G_{\text{Ca}} = 100$ pS, $G_{\text{Cs}} = 380$ pS, and $G_{\text{Na}} = 400$ pS (Smith et al., 1986; Smith et al., 1988; Fill, 1990).

3.3. Chloride channels

Chloride is by far the most abundant physiological anion. In many animal cells it is distributed almost at equilibrium so that the equilibrium potential U_{Cl} is near the resting potential. Even if U_{Cl} is not at the resting potential in some excitable cells, it is at least many tens of millivolts negative from zero. Thus, like K^{+} channels, chloride channels would be expected to oppose normal excitability and to help repolarize a depolarized cell - a stabilizing influence. Chloride ions also play an important role in intracellular pH regulation and in cell volume regulation.

Most Cl^- channels lack electrical excitability. However, one could divide Cl^- channels into three categories: steeply voltage dependent channels, weakly voltage dependent “background” channels, and transmitter operated synaptic channels.

Background Cl^- channels are found in vertebrate muscle fiber cells. A search for voltage or time dependence of these channels reveals only slow and minor changes that are emphasized in very alkaline and very acid media (Hutter and Warner, 1972; Warner, 1972). Therefore, in a typical voltage clamp experiment, Cl^- currents are usually lumped into the background leakage current. Nevertheless, pharmacological experiments suggest that Cl^- ion channels can be blocked by external Zn^{++} and, in mammals, by a variety of aromatic monocarboxylic acids, particularly anthracene-9-carboxylic acid (Palade and Barchi, 1977). Chloride channels are generally permeable to most small anions. Inorganic anions, including Br^- , I^- , NO_3^- , and SCN^- , and perhaps small organic acids are permeant in these vertebrate muscles (Palade and Barchi, 1977; Edwards, 1982). Their fluxes are also inhibited by Zn^{++} or anthracene-9-carboxylic acid.

3.4. The Gating Mechanism

The gating of biological channels is not simple. All the well studied voltage-sensitive and transmitter-sensitive channels show delays, inactivations, or desensitizations in their macroscopic time course.

A multiplicity of closed states is observed as multiple kinetic time constants in gating currents, in fluctuation measurements, and in histograms of closed times obtained from single channel recordings. In addition, some channels have two or more open states, which can differ in their single channel conductance.

In Na^+ , K^+ , Ca^{++} , and endplate channels the open-shut transition does not merely close a pathway of atomic dimensions, but it also changes the binding energies and access

for a wide variety of drugs and toxins to sites on the channel macromolecule (state dependent binding). Hence gating can not be regarded as a event involving only a few atoms but undoubtedly involves major tertiary and quaternary conformational changes of the channel protein.

The study of gating has so far lacked an essential ingredient, a detailed correlation of structure and function. Once we learn more about the chemistry and physical structure of channel proteins, we will be able put more physical reality into the present-day abstract ideas about gating and gating states.

Figure VI: Possible Mechanisms for Channel Gating. A,B,C) A gate could rotate or slide; D,E) The pore might pinch shut or twist; F,G) A free or tethered particle might block the pore; H) The pore might swing out of the membrane; I,J) or assemble from subunits; K) The passage of ions might be stopped by an unfavorable charge in the channel.

(Adapted from Bertil Hille, "Ionic Channels of Excitable Membrane")

4. Biological Membranes

4.1. General

All biological membranes, regardless of their source, contain proteins and lipids. The protein to lipid ratio varies greatly: the inner mitochondrial membrane is 76 percent protein; the myelin membrane is only 18 percent.

The lipid composition varies among different membranes. All membranes contain a substantial proportion of phospholipids, predominantly phosphoglycerides, which have a glycerol backbone. All membrane phospholipids are amphipathic, having both hydrophilic and hydrophobic portions. The basic structural unit of virtually all biomembranes is the *phospholipid bilayer*: a sheetlike structure composed of two layers of phospholipid molecules whose polar head groups face the surrounding water and whose fatty acyl chains form a continuous hydrophobic interior about 3 nm thick. Each phospholipid layer in this lamellar structure is called a leaflet.

4.2. Membrane as Capacitor

The lipid bilayer of biological membranes separates internal and external conducting solutions by an extremely thin insulating layer. Such narrow gaps between two conductors form a significant electrical capacitance.

To create a potential difference between objects requires just a separation of charge. The capacitance C is a measure of how much charge Q needs to be transferred from one conductor to another to set up a given potential V , and is defined by

$$C = \frac{Q}{V} \quad (\text{I-4-1})$$

In an ideal capacitor the passage of current simply removes charge from one conductor and stores it on another in a fully reversible manner and without evolving heat.

If the voltage across a membrane of capacitance C varies at a rate dV/dt then a capacitive current I_C will be generated.

$$\frac{dV}{dt} = \frac{I_C}{C} \quad (\text{I-4-2})$$

The capacity to store charges arises from their attraction across the gap and from polarization of the insulating medium. Furthermore, the capacitance depends on the dielectric constant and on the geometry of the conductors. In a simple capacitor formed by two parallel plates of area A and separated by an insulator of dielectric constant ϵ and thickness a , the capacitance is given by:

$$C = \frac{\epsilon\epsilon_0 A}{a} \quad (\text{I-4-3})$$

where ϵ_0 , called the polarizability of free space, is $8.85 \times 10^{-12} \text{ AsV}^{-1}\text{m}^{-1}$. A cell membrane can be described as a parallel-plate capacitor with a specific capacitance of approximately $1.0 \mu\text{F}/\text{cm}^2$, just slightly higher than that of a pure lipid bilayer, which measures $0.8 \mu\text{F}/\text{cm}^2$ (Almers, 1978). According to equation (I-4-3), this means that the thickness a of the insulating bilayer is only 2.3 nm (23 Å), assuming that the dielectric constant ϵ of hydrocarbon chains is 2.1. Hence the high electrical capacitance of a biological membrane is a direct consequence of its molecular dimensions.

The high capacitance gives a lower limit to how many ions (charges) must move (eq. I-4-1) and how rapidly they must move (eq. I-4-2) to generate a given electrical signal. In general, capacitance slows down the voltage response to any current by a characteristic time τ that depends on the product RC of the capacitance and any effective resistance R . From Ohm's law the current in the resistor is $I=V/R$, which discharges (hence the "-" sign) the capacitor at a rate of

$$\frac{dV}{dt} = \frac{I_C}{C} = -\frac{V}{RC} \quad (\text{I-4-4})$$

The solution of this first-order differential equation has an exponentially decaying time course

$$V = V_0 \exp(-t / RC) = V_0 \exp(t / \tau) \quad (\text{I-4-5})$$

where U_0 is the starting voltage, and t is time in seconds.

For biological membranes the product, $R_M C_M$, of membrane resistance and capacitance is often called the membrane time constant, τ_M . It can be determined, using eq. (I-4-5), from measurements of the time course of membrane potential changes as small steps of current are applied across the membrane. In different resting cell membranes, τ_M ranges from 10 μ s to 1 s, corresponding to resting R_M values of 10 to $10^6 \Omega\text{cm}^2$. This broad range of specific resistances shows that the number of ionic channels open at rest differs vastly from cell to cell.

CHAPTER II: METHODS AND MATERIALS

1. Vesicle Preparation

1.1. Isolation of SR Vesicles

SR vesicles were prepared according to the method of MacLennan (1970) from rabbit skeletal muscle. All steps were carried out either on ice or in the cold room (8°C).

White muscle was taken from the back and the hind legs from New Zealand white rabbit. Fat and connective tissue were trimmed off. The muscle tissue was diluted into three volumes of buffer A (120 mM NaCl, 10 mM imadazole, pH 7.4) and homogenized in a Waring blender alternating low and high speed for two to three minutes, before it was centrifuged for 10 min at 1,600 g (3100 rpm in a GSA rotor, Sorval centrifuge). The supernatant obtained was strained through four layers of cheesecloth and afterwards adjusted to pH 7.4 with dry imidazole. The pellet was discarded. The supernatant was again centrifuged at 10,000 g (8,000 rpm in the same GSA rotor) for 14 min. The brown pellet, which mainly contained mitochondria, was discarded, and the resulting supernatant was again strained through cheesecloth. The solution was then centrifuged at 44,000 g (19,000 rpm) in a Beckman Ti19 rotor for 70 min using a L2-65B ultracentrifuge. The pellet was scrapped off, resuspended in buffer A at an approximate concentration of 10mg/ml and homogenized with a glass homogenizer. Then the mixture was subjected to centrifugation at 7,500 g (11,000 rpm in a Ti60 rotor in a Beckman ultracentrifuge) for 10 min. The mainly myosin pellet was discarded and the supernatant was then centrifuged at 78,000 g (35,000 rpm in the Ti60 as above) for 30 min. The resulting pellet contained SR vesicles. It was suspended into a buffer (100 mM KCl, 20 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), adjusted to pH 7.0 with KOH) to obtain the final protein concentration of between 15 to 25 mg/ml.

For bilayer experiments SR vesicles were incubated with 300 mM sucrose at a protein concentration of 1.5 mg/ml. These vesicles when added to the bilayer setup creates an osmotic gradient which swells the vesicles promoting fusion.

The resulting SR suspension was distributed into microtubes and stored in liquid nitrogen for later use.

All buffers had been prepared with distilled, deionized water.

1.2. Protein Concentration Determination

The concentration of SR protein was measured by absorption spectroscopy as described by Kalckar (1947).

Primarily due to the presence of tyrosine and tryptophan residues proteins strongly absorb UV light at 280 nm. Kalckar's method uses three wavelengths as a simple tool to determine the protein concentration, while also accounting for possible contamination with nucleic acids which absorb most strongly at 260 nm. Kalckar and Thorley-Lawson (1977) established the following two equations:

$$\begin{aligned}\text{protein}[\text{mg/ml}] &= 1.45 A_{280} - 0.74 A_{260} \\ \text{protein}[\text{mg/ml}] &= 0.183 A_{230} - 0.075 A_{260}\end{aligned}\tag{II-1-1}$$

2. The Bilayer Technique

Reconstitution of vesicles into an artificial lipid bilayer enables extended study of ion channels in vesicles. Vesicles fuse with the bilayer in a reproducible manner (Mitchell, 1988). The cytosolic face of the SR points in the direction from which the vesicles were added (cis side). The luminal side faces the other side of the bilayer (trans side).

Once in the bilayer, chemical treatments are easy because the researcher has now access to both sides of the membrane and consequently to both sides of the proteins in question. In addition, this technique enables the researcher to monitor ion currents at different holding potentials.

Lipid bilayer membranes were made from a mixture of phosphatidylethanolamine (PE) and phosphatidylserine (PS) at a 5:3 ratio by weight and a final concentration of 50

mg/ml in decane. The bilayer separating two aqueous solutions is made by painting the lipid-decane mixture across a $\sim 150\ \mu\text{m}$ hole drilled in a polystyrene cup. The bilayer lipid membrane (BLM) behaves like a parallel-plate capacitor in an unbranched circuit. Thus the size and thickness of the bilayer can be monitored electrically following eq. (I-4-3) with $A=(150\ \mu\text{m}/2)^2\pi$. The distance between the two phospholipid heads, the separation of the two capacitive plates in the BLM is about $60\ \text{\AA}$. The electrodes and the solutions on both sides have intrinsic resistance which can be described as a resistor and a capacitor in series, an RC unit. Therefore, when a sawtooth waveform is applied across the lipid membrane it produces its derivative, a square wave. Membranes corresponding to 16-32 pF measured from peak to peak are thin and thus acceptable.

A perfect lipid membrane is thin and forms a tight seal (conductance less than 10 pS, i.e. 1 pA/100 mV) across the hole and does not allow the penetration of gradient-driven leakage currents. The formation of a bilayer was a critical step in the reconstitution experiment. To achieve easy membrane formation and stable membranes cups were stored overnight in 1% HCl and the aperture was pre-painted with lipids before each experiment. For membranes to thin spontaneously, the use of excess amounts of lipid was avoided. Experience has shown that membranes with leakage currents less than 3 pA remain stable over the period of an experiment (typically 20-30 min). An overly thick membrane prevents fusion of SR vesicles into the bilayer membrane and if fusion does occur subsequently alters or inhibits channel gating. The capacitance of the bilayer was constantly checked, in order to insure that the bilayer thickness remained the same throughout one experiment.

The *cis chamber* is defined as the side to which SR vesicles are added; the opposite side is referred to as the *trans chamber*. All experiments were performed at a temperature of about 15°C . In the experiments carried out, the trans chamber (0.7 ml) contained 100 mM CsCl, 10 mM Hepes (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) brought to pH 7.25. The cis chamber (0.7 ml) contained 500 mM CsCl, 10 mM Hepes at pH 7.25. The final SR protein concentration in the cis chamber at the beginning of each experiment was between 5 and $10\ \mu\text{g/ml}$.

The two main factors that influence fusion of SR vesicles to the lipid bilayer are the osmotic gradient across the SR and the lipid that the bilayer is made out of. PE is a dipolar phospholipid. PS is negatively charged and thought to promote fusion in the presence of micromolar concentrations of Ca^{++} . A bridge between the PS, Ca^{++} and negatively charged proteins or phospholipids on the SR vesicle has been proposed. Charge and charge-carrying ions seem to be crucial to fusion since Mg^{++} also facilitates fusion (Chernomordik et al., 1987). However, the proposal that Mg^{++} facilitates fusion is somewhat controversial. Mg^{++} only at concentrations above physiological levels promotes fusion. Under more physiologically relevant conditions, Mg^{++} does not promote fusion, in fact, it inhibits the process.

Fusion of SR vesicles was accomplished by adding 200 μM CaCl_2 to the cis chamber with constant slow stirring. By monitoring the current across the bilayer lipid membrane, single fusion events can be observed. Once fusion was detected the conditions were altered to make continued fusion unfavorable. Thus, in order to stop fusion by lowering the free Ca^{++} concentration, 500 μM EGTA ([ethylenebis(oxyethylenenitrilo)] tetraacetic acid) was added to the cis chamber. It was then perfused with four volumes of an identical buffer (see above) with no added Ca^{++} or EGTA, and excess SR was removed.

Single channel gating was measured with a CsCl gradient (cis 500 mM CsCl, trans 100 mM CsCl). Cs^+ was chosen as a conducting ion because Coronado et al. (1980) had shown it to inhibit K^+ channels which are found in SR preparations. The high Cs conductance of the SR CRC also improved the signal to noise ratio. The effect of Cl^- channels was minimized by monitoring the Ca^{++} channel activity near the reversal potential of Cl^- at +25 mV. The disadvantage to this method was that it was not possible to monitor channel activity at negative voltages.

A Warner Instrument Corp. Bilayer Clamp BC-525A headstage and amplifier was used to detect and amplify the pA-currents of the Ca^{++} channel. Connections from the headstage to the baths were not made directly. The leads from the headstage were short

silver chloride (Ag/AgCl) wires connected to the solutions via agar/CsCl salt bridges. The unmanipulated data were digitized with an Instrutech Corp. VR-10 Digital Data Recorder and stored on videocassette tape (Zenith VHS HQ). The stored data was processed by the VR-10 passed through an adjustable Krohn-Hite model 3202 filter, and viewed on a Kikusui 20MHz Digital Storage Oscilloscope (model 5020A). The following analysis of the channel activity was done using pCLAMP Version 5.5 software (Axon Instruments, Burlingame, CA) on an IBM-XT computer.

Figure VII: Schematic of Experimental Setup.

While collecting data the assembly was shielded from outside interference by a Faraday cage. A vibration-isolation table was used to protect the system from physical vibrations. A direct current stirring motor with Teflon stir bars was placed directly under the bilayer chamber to achieve fast mixing of solutions without mechanical disturbance.

3. Chemicals

3.1. *o* -Phthalaldehyde

In the 1960s α -amino acids were detected and tested with ninhydrin. In these tests bright intense colors were produced which served as basis for numerous analytical procedures, both qualitative and quantitative. Because the sensitivity of these ninhydrin reactions soon reached their limits, Roth in 1971 developed a new technique with

o-phthalaldehyde (OPA). This procedure is based on the fluorescing properties of amino acid-OPA compounds. Best results were obtained when OPA (as a solution in ethanol) was mixed with a solution of an amino acid in the presence of a strong reducing agent like potassium borohydride or 2-mercaptoethanol buffered at pH 9.0. For the fluorescence of OPA derivatives, the critical wavelengths occurred at $\lambda_{\text{ex}} = 340 \text{ nm}$ and $\lambda_{\text{fl}} = 455 \text{ nm}$. However, the amino acids proline and cysteine do not react, and the basic amino acids lysine (even at the optimum pH 6.0 to 7.0) and ornithine give weak signals. A sharp decrease in fluorescence was observed if the buffered amino acid was first mixed with OPA and mercaptoethanol was added last. This indicates that in the absence of a reducing agent, OPA is capable of reacting otherwise with amino acids forming nonfluorescent products. All reactions mentioned were done at room temperature and reached their maximal fluorescing intensity 5 to 25 min after mixing the reagents. All these features made the reaction well suited to the automatic assay of amino acids fractionated by ion exchange chromatography.

In 1985 Puri et al. demonstrated the usefulness of OPA in obtaining information about cysteine and lysine residues in protein. In this case OPA with its two CHO functions acts as a homobifunctional crosslinking reagent and binds to sulfhydryl (in cysteine) and ϵ -amino (in lysine) functions that are about 3 Å apart. This reaction converts the OPA's benzenoid ring into a fluorescent isoindole ring system, a ten overlapping π -electron system. This compound proved stable over a period of 24 hours.

Figure VIII: Schematic Representation of the Reaction of *o*-phthalaldehyde.

(adapted from Puri et al., 1985; *Biochemistry*)

OPA with a purity of about 99% was bought as a dry powder from Sigma Chemical Company. The molecular weight is 134.1 g/mol. It was always prepared fresh in DMSO (dimethylsulfoxide).

3.2. Ryanodine

Ryanodine is a natural plant alkaloid that occurs in several South American trees. It is toxic to insects and higher animals because it produces irreversible muscle contraction. It affects cardiac and skeletal muscle in different ways. In skeletal muscle ryanodine causes irreversible contraction, whereas in cardiac muscle it produces a decline in the contractile force (Meissner, 1986). Confusion regarding the effects of ryanodine exist. They are due to the large variation in effects reported under different experimental conditions. Ryanodine blocks EC coupling by binding to the open SR calcium release channel. In general, the plant alkaloid binds with at least two different affinities to skeletal muscle SR. Nanomolar concentrations of ryanodine cause the CRC to slowly fluctuate in a

full conductance state. At micromolar concentrations, it produces subconductance states which fluctuate on a still slower time scale. At even higher (hundreds of micromolar) concentrations, ryanodine can close the CRC altogether.

The ryanodine, used in the following experiments, was purchased from Sigma Chemical Company.

3.3. Phospholipids

Phospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS) dissolved in chloroform were purchased from Avanti Polar-Lipids, Inc. and stored in the freezer at -20°C to decrease oxidation. Lipids were freshly made each day by evaporating chloroform under nitrogen and dissolving the dry lipid in decane yielding a final lipid concentration of 50 mg/ml in a 5:3 ratio by weight of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine and 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine].

CHAPTER III: RESULTS

1. Calcium Channel Behavior

1.1. Control Measurements

The first experiments done were control experiments to test unmodified calcium release channel activity. As shown in Figure VIII, addition of 100 μM Ca^{++} increased the open channel probability P_o , and addition of the Ca^{++} channel inhibitor ruthenium red (20 μM) closed down the single channel.

Figure IX: Increase of Channel Open Probability by Addition of Calcium and Inhibition by Ruthenium Red. A) single channel after fusion into bilayer, $P_{\text{open}} = 0.08$; B) single channel at 100 μM CaCl cis, $P_{\text{open}} = 0.53$; C) single channel with 100 μM CaCl cis and 20 μM ruthenium red cis; the upper (dashed) bars refer to the open state, whereas the lower (solid) bars refer to the closed state; similar experiments were done more than 10 times.

1.2. Calcium Channel Conductivity

Conductance through the channel is dependent on the ions present. The channel is buffered in 100 mM CsCl, 10 mM Hepes, pH 7.2 on the trans side and 500 mM CsCl, 10 mM Hepes, pH 7.2 on the cis side. Under those conditions the single channel conductance is reported as 475pS, whereas its reversal potential is given by:

$$\Psi = (\Psi_1 - \Psi_2) = (RT / zF) \ln(c_1 / c_2) \quad (\text{III-1-1})$$

where ψ is the transmembrane potential, and c_1 and c_2 correspond to the concentrations of the permeant ion on the two sides of the membrane, $R = 8.324 \text{ J K}^{-1} \text{ mol}^{-1}$ is the gas constant, T is the absolute temperature, z is the valency (for Ca = +2 and Cs = +1) and $F = N_a e = 6.022 \times 10^{23} \text{ mol}^{-1} \times 1.602 \times 10^{-19} \text{ As} = 9.647 \times 10^4 \text{ C mol}^{-1}$ is the Faraday constant.

In Figure X one can see that channel fluctuations at higher holding potentials produce larger currents. The reversal potential, or the intercept of the fitted curve with the voltage axis is $-25.3 \pm 4 \text{ mV}$, Figure XI. The selectivity of cations over anions is $P_{\text{Cs}}/P_{\text{Cl}} = 5.6 \pm 1.2$. The conductance calculated via linear regression of the slope yields $500 \pm 50 \text{ pS}$. This is consistent with the reported conductance of 475 pS, mentioned above.

Figure X: Single Channel Current versus Applied Voltage with 100 μM Calcium. A) channel at 30 mV holding potential; B) channel at 25 mV holding potential; C) channel at 20 mV holding potential; D) channel at 15 mV holding potential; each measurement was done 2 times; the upper (dashed) bars refer to the open state, whereas the lower (solid) bars refer to the closed state.

Figure XI: Conductivity for Cs^+ Ions Through CRC. The conductivity was measured at 100 μM free calcium. The entries in the graph do not necessarily reflect the measurements from A) to D) of Figure X, but refer to 2 times repeated experiments; the conductivity is 500 pS.

2. Experiments with OPA

2.1. Control Experiments

As the *o*-phthalaldehyde (OPA) was prepared in dimethylsulfoxide (DMSO), early experiments were done to prove that DMSO itself does not affect the bilayer lipid membrane (BLM) nor the calcium release channel behavior. In the same way, the influence of OPA in DMSO on the BLM alone was shown to be insignificant with respect to the intended experiments. Both traces are not shown in this paper.

2.2. OPA Activates Calcium Channel

In flux measurements OPA was shown to cause calcium release from calcium loaded SR vesicles. This occurred either via the direct interaction between OPA and the CRC or via a process which made the membrane leaky.

The gating of a channel requires the facilitation of conformational changes. This may happen in the quaternary structure of the channel protein and goes along with realization of crosslinking chemical bonds. One physiologically relevant possibility would be the oxidation of two SH functions of neighboring cysteines to an S-S bond. In its usual chemical reaction, OPA binds to two binding sites, to one SH (in a cysteine amino acid) and to one NH₂ (in a lysine amino acid). The idea was to investigate the specific reactions of the two OPA binding sites in regard to the CRC. This was done at different OPA concentrations.

In Figure XI it is demonstrated that OPA stimulates single channel activity. Activation occurs quickly, within less than 1 minute, when stirring. The traces shown were recorded between 1 and 5 min after the addition of OPA.

Figure XII: CRC in Different OPA Concentrations and no Added Calcium. A) Control, channel in a buffer with no added Ca^{++} , $P_{\text{open}} = 0.08$; B) CRC in 200 μM OPA, $P_{\text{open}} = 0.7$; C) CRC in 400 μM OPA, the channel jumps into the open state, $P_{\text{open}} = 0.9$; D) CRC in 600 μM OPA, $P_{\text{open}} = 0.9$; E) CRC in 800 μM OPA, $P_{\text{open}} = 0.85$; F) 20 μM RRed shut the channel down, $P_{\text{open}} = 0.025$; each experiment was done 3-4 times; all additions were made to the cis side only, the upper (dashed) bars refer to the open state, whereas the lower (solid) bars refer to the closed state.

In many cases at high OPA concentrations ($>400\ \mu\text{M}$) the channel locks into the open state, closing only seldomly for short periods of time. This locking into the open state, increased both the probability to find the channel in the open state P_{open} and the mean open time t_{mo} dramatically. Interestingly, Figure XII and Figure XIII indicate that at $800\ \mu\text{M}$, the P_{open} decreases slightly, and more rapid fluctuations reappear. However this trend might be questionable because the standard deviations of each P_{open} and mean open times t_{mc} are of the same order of magnitude as the actual calculated values.

In experiments not shown, OPA in the first step was also added to the trans side of the channel. In this case the calcium release channel did not respond, which indicates that the critical binding site to which, among other chemicals, OPA binds and opens the channel is located on the cis side of the CRC protein.

Figure XIII: Probability of Finding the Channel in an Open and Closed State versus OPA Concentration.

Figure XIV: Mean Open and Mean Closed Time versus OPA Concentration. The high value for mean closed time t_{mc} at zero OPA reflects long times of inactivity due to the lacking of a stimulant.

The increase in dwell time at high OPA concentrations, may result from limitations in the time response of the electronics used. It is likely to be higher than it appears in Figure XIV.

Channel activation by OPA and inhibition by ruthenium red indicate that the conformational change that the channel has undergone must be similar to those when activated by calcium. In Figure XIV, 20 μM RRed was shown to inhibit Ca^{++} channel activity. Similar results were obtained when 10 μM RRed was added to the cis chamber. Ruthenium red inhibition of channel activity was never absolute. Occasional gating events were still present. In some experiments it was difficult to observe RRed inhibition of channel activity because RRed destabilizes the lipid membrane and frequently caused membrane breakage.

Figure XV: RRed shuts down CRC Previously Activated by OPA. A) Control, CRC with 100 μM Ca^{++} , $P_0 = 0.29$; B) Ca^{++} captured by 0.2 mM EGTA, 200 μM OPA, $P_0 = 0.76$; C) 20 μM ruthenium red cis closes the channel down, $P_0 = 0.034$; similar experiments with different OPA concentrations and / or 10 μM RRed were done more than 20 times; the upper (dashed) bars refer to the open state, whereas the lower (solid) bars refer to the closed state.

Motivated by related ryanodine binding assays which indicate a time and OPA concentration dependency of [^3H]ryanodine binding to an open channel, similar bilayer experiments were done. Using the bilayer technique it was expected that OPA activation of channel activity should be followed by a time dependent inactivation of channel activity. Several concentrations (200-1100 μM) of OPA were tested. With only one SR preparation one could observe a consistent channel inactivation after about 4-6 min. One possible explanation for a time dependent inactivation by OPA is that this inactivation OPA binding site is located on the trans side of the bilayer. In other words in order to gain access to the OPA binding site on the trans side of the BLM, OPA must penetrate the lipid bilayer membrane. This was tested by adding low OPA concentrations to the trans side of

Figure XVI: OPA Added to Both Sides of CRC. **A)** CRC stimulated with 200 μM OPA cis, $P_0 = 0.71$; **B)** 100 μM OPA trans does not close the channel, $P_0 = 0.74$; this experiment was done 3 times, the upper (dashed) bars refer to the open state, whereas the lower (solid) bars refer to the closed state.

OPA is believed to form a covalent complex with a thiol and an amine. Exchanging solution for the original buffer with no added Ca^{++} nor OPA and addition of the reducing agent cysteine, which is itself capable of forming a stable isoindole ring with OPA via its SH and NH_2 functions (Puri, et al., 1985), should not influence a channel previously activated by OPA. As shown in Figure XVI no change in P_0 is observed in trace C, after changing solution and addition of 200 μM cysteine.

Figure XVII: Study of Binding Characteristics of OPA to CRC: A) A control channel, $P_{\text{open}} = 0.08$; B) added 200 μM OPA, $P_{\text{open}} = 0.8$; C) OPA washed out, 200 μM Cysteine, $P_{\text{open}} = 0.85$; D) 20 μM RRed shut down the channel ;this experiment was repeated 3 times, the upper (dashed) bars refer to the open state, whereas the lower (solid) bars refer to the closed state.

2.3. Conductivity of OPA-Activated Calcium Channels

By varying the holding potential and measuring the single channel current fluctuations, the single channel conductance can be determined. In the absence of OPA, the conductance equals 500 ± 50 pS, the reversal potential is -22.3 ± 3 mV, and the selectivity $P_{\text{Cs}}/P_{\text{Cl}} = 5.6 \pm 1.2$. In the presence of $200 \mu\text{M}$ OPA, the conductance equals 555 ± 50 pS, as it can be seen in Figure XVII, and the ratio $P_{\text{Cs}}/P_{\text{Cl}} = 4.25 \pm 0.8$. Thus, the selectivity of the CRC for cations over anions decreases slightly, but remained clearly cation selective and almost the same in terms of magnitude and in the acceptable range of error.

The analyses of each voltage/current step was done by fitting digitalized channel current events list to two Gaussian bellshapes. The difference of the two means μ_1 and μ_2 determine the passing current at each holding potential.

Figure XVIII: Conductivity for Cs^+ Ions Through CRC. At $200 \mu\text{M}$ OPA the calcium release channel conducts Cs^+ ions down a 5:1 gradient with 550 pS.

2.4. OPA Inhibits Cl⁻ Channels

Soon after the first few experiments it became obvious that OPA, when added to the cis side, has an effect on Cl⁻ channels as well. OPA added to the trans side, however, does not affect the Cl⁻ channel. The specificity of OPA action of the CRC/ryanodine receptor is unknown. In Figure XIX it is observed that OPA inhibits Cl⁻ conductance in a time dependent manner. The time constant for the decay of Cl⁻ current at 400 μ M OPA is ~ 30 sec. OPA both activates Ca⁺⁺ channels and inhibits SR Cl⁻ channel. As a positive side-effect, the signal to noise ratio becomes better when examining the calcium release channel at +25 mV.

Figure XIX : Decay of Cl⁻ Conductance at 400 μ M OPA. Following addition of 400 μ M OPA cis to the single reconstituted Ca⁺⁺ channel, the voltage was flipped to -25 mV, and Cl⁻ and current was measured versus time, $t = 0$ defines the instant of adding OPA, similar experiments were done 3 times.

As Figure XX reveals, inhibition of Cl^- channels by OPA starts significantly at concentrations between 50-75 μM OPA, slows down, but can be brought further with an additional increase of the OPA concentration until the Cl^- current approaches zero.

Figure XX: Inhibition of Cl^- Conductance in Three Steps. 50 μM OPA does not affect Cl^- channels; 75 μM OPA initiate inhibition, time constant $t = 58$ sec; 200 μM OPA decrease Cl^- conductance drastically, time constant $t = 67$ sec, this experiment was done two times.

3. Experiments with Ryanodine

3.1. Control Experiments

Ryanodine in nanomolar concentrations is known (compare E. Buck doctoral thesis, 1993) to increase calcium release channel open time in a reversible manner. Occasionally subconductance states of approximately 50% of the full conductance value can be seen. Raising the concentration into the micromolar range, ryanodine stabilizes long-lived 1/2 conductance transitions in the Ca^{++} release channel. Ryanodine at concentration greater than 70 μM causes the channel to fluctuate in both 1/2 and 1/4 conducting states.

This behavior of the CRC was confirmed in experiments, not shown, with 1.5 and 5 μM ryanodine added to the cis side. Approximately 3 min after addition of ryanodine, channel fluctuations stabilized, and long-lived subconductance 1/2 states in the millisecond range were observed. These subconductance states could be shut down by 20 μM of RRed.

3.2. OPA does not Reverse Half States

Experiments were carried out to test if OPA is capable to reverse ryanodine's ability to lock the protein in a 1/2 state. Figure XX reveals that 400 μM OPA is not able to bring the channel back to full state fluctuations. Concentrations of 2 μM and 5 μM ryanodine and 200-600 μM OPA have been tested with the same result. This indicates that whether the reaction of ryanodine with the CRC is of a very stable nature or that, due to conformational changes, the SH functions that OPA usually reacts with, are not available any longer.

Figure XXI: OPA is not Capable of Reversing Half States Initiated by Ryanodine. **A)** Control, calcium release channel with 100 μM Ca^{++} ; **B)** CRC locked into long-lived 1/2 state by 5 μM ryanodine, 100 μM Ca^{++} ; **C)** 400 μM OPA does not affect stable 1/2 states; **D)** channel was shut down by 20 μM ruthenium red, this experiments has been done 3 times, the upper (dashed) bars refer to the full open state, the middle bars refer to the half state, whereas the lower (solid) bars refer to the closed state.

Conclusion

The calcium release channel of sarcoplasmic reticulum from white rabbit skeletal muscle was shown to be activated by different concentrations of the thiol specific probe OPA, and to conduct cesium ions down a 5:1 gradient. With the addition of OPA, a large increase in the mean open time of the channel and a large increase in the open probability of the channel was observed.

Channel activation by OPA and inhibition by ruthenium red indicate that OPA was acting directly on the Ca^{++} release channel from SR.

The conductance and the selectivity of the CRC in the absence and in the presence of OPA was only slightly changed. In both cases the channel was cation selective. The conductance of the channel with $100\mu\text{M}$ free Ca^{++} was 500 pS, its selectivity of cations over anions was 5.6. The conductance of the channel with $200\mu\text{M}$ OPA was 550 pS, its selectivity of cations over anions 4.25.

OPA was also shown to inhibit Cl^- conductance at $70\mu\text{M}$ OPA and above. This is interesting in so far as the effective OPA concentration, that inhibits Cl^- channels is approximately the same as the OPA concentration that affects calcium release channel activity. Therefore, it would be difficult to inhibit Cl^- channels without affecting Ca^{++} channel activity in single channel reconstitution experiments. The search for an effective inhibitor of Cl^- channels remains a task for further inquiry.

OPA was shown not to be able to reverse half states invoked by low micromolar concentrations of ryanodine. Even at high concentrations of OPA (up to $600\mu\text{M}$) the half conductance transition induced by ryanodine was not modified.

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