THE USE OF *XENOPUS* OOCYTES FOR THE STUDY OF ION CHANNELS

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I. INTRODUCTION

Early embryological studies on amphibian oocytes, among them those of the South African clawed frogs *Xenopus laevis*, concentrated on morphological and cytological processes that occur during the growth, maturation, and fertilization of the oocyte and the development of the embryo.[1-3] In the 1960s and 1970s, extensive use of the oocytes for the elucidation of the mechanisms of regulation of cell division was initiated (for reviews see References 4 and 5). In parallel, these cells have been successfully used in the uncovering of gene expression mechanisms (for reviews see References 6 to 8).

In the studies of meiotic reinitiation, oocyte maturation, and fertilization, it has been found that the membrane potential, intracellular concentrations of ions, and various membrane properties of the oocyte change in the course of growth and development. These findings have stimulated the investigation of the membrane properties of the oocyte, including its ion channels. Soon the oocytes became a preparation in which ion channels could be studied almost without connection to any possible biological role that these channels could have in the functioning and development of the oocyte. In addition, membrane responses to neurotransmitters were demonstrated in *Xenopus* oocytes in 1977,[9] and in the following years these cells proved themselves a useful model for the study of molecular mechanisms of second messenger-mediated neurotransmitter responses.

The studies on genetics and molecular biology contributed to the understanding of some of the fundamental principles of cell development and eukaryotic gene expression. A finding of particular importance (as far as the ion channels are considered) was the demonstration by Gurdon and colleagues of the ability of the oocytes to synthesize exogenous proteins when injected with foreign messenger RNA.[10] In the beginning of this decade, the expression of functional neurotransmitter receptors and ion channels in the oocytes was demonstrated.[11,12] At present, the oocyte is used as a model system for the study of neurotransmitter- and voltage-operated ion channels by many groups. A particularly attractive approach is the use of the oocytes as an assay in the investigation of structure-function relationships of membrane proteins. Here, the properties of the channels encoded by a "mutant" message (e.g., mRNA transcribed from a cloned cDNA that has been modified by site-directed mutagenesis[13]) are compared to those encoded by the "wild-type" message. No doubt this

kind of research will contribute invaluably to the understanding of the molecular basis of ion channel function, and one may expect a quantitative and qualitative "explosion" of research in the field.

Two features make amphibian oocytes particularly attractive for application of a multidisciplinary approach (exploiting methods of cytology, biochemistry, molecular biology, and electrophysiology): (1) oocytes are large (up to 1.3 mm in diameter in *Xenopus*). This allows microdissection of single oocytes, penetration with several microelectrodes to perform voltage-clamp and other electrophysiological manipulations (see Section IV), and injection of various substances into these cells and thus control of the content of intracellular regulatory substances, etc. Biochemical processes initiated by transmitter or hormonal stimuli can be studied by pooling just a few (sometimes in single) cells. (2) Oocytes readily synthesize exogenous proteins when injected with foreign RNA.

In view of the growing importance of the oocytes as a model system for the study of molecular mechanisms underlying the action of ion channels, it seems timely to summarize what is known today about the endogenous ion channels and neurotransmitter responses in the oocytes and what has already been done in the use of these cells as an "assay system" for exogenous membrane proteins. To provide such a summary is the goal of this review.

II. MORPHOLOGY AND DEVELOPMENT OF THE AMPHIBIAN OOCYTE

The oocyte is a germ cell from which the whole organism is later derived. For historical reasons, this cell receives different names, according to its stage of development. During development, it acquires a high degree of specialization. The characteristics of the ion channels and neurotransmitter responses of the amphibian oocytes also change. Therefore, in order to facilitate the understanding of the following sections, a condensed summary of the stages and of some of the processes that take place during the development is provided. Also, a short description of the morphology of the oocyte is given. More detailed information on these issues can be found in References 1 to 4 and 14 to 19.

A. Morphology

In the ovary of a frog, the female germ cells enter meiosis at the larval stage. At the time of first prophase, the meiosis halts ("first meiotic arrest"), and the cells start growing. At this stage, the germ cells are called "oocytes" or "immature oocytes".[4] Such oocytes have very large nuclei (germinal vesicles [GV]), containing lampbrush chromosomes, that are actively engaged in RNA synthesis. In *Xenopus*, the oocyte growth is described in terms of six stages.[16] Most studies concerning ion channels are performed on fully grown immature oocytes or on eggs (see following).

The fully grown oocyte (stage 6) is surrounded by the following layers (beginning at the innermost):[16,17]

1. The vitelline membrane, a noncellular fibrous layer.
2. A layer of follicle cells. These cells form numerous membranal contacts with the oocyte by means of macrovilli (processes of the follicle cell) and microvilli (processes of the oocyte[20]).These contacts are of the "gap junction" type,[21,22] i.e., they allow free passage of molecules up to 1000 daltons and of course provide a direct electrical connection between the oocyte and the follicular cells. Gap junctions are under hormonal control: their permeability (and possibly their number) increases following stimulation of the frog with human chorionic gonadotropin (luteinizing hormone), which serves as a signal for reinitiation of meiosis.[21-22]
3. The "theca", a connective tissue layer, in which smooth muscle cells, nerve fibers, oogonia, and capillaries are embedded.[17]
4. A layer of epithelial cells, a continuation of the ovary wall.[17]

The oocyte surrounded by all the layers listed previously is called a "follicle", a "follicle-enclosed oocyte", or a "follicular oocyte". All outer layers (excluding the vitelline membrane) can be removed by collagenase or other proteolytic enzymes, or mechanically after treatment with a Ca-free solution (Section III.A). This is termed defolliculation, and the oocytes surrounded by the vitelline membrane alone are called "denuded" oocytes. The vitelline membrane can also be removed mechanically following incubation of the oocyte in a high osmolarity solution.[26] We propose calling such oocytes "devitellinized".

Fully grown (state 6) *Xenopus* oocytes are spheres 1.2 to 1.3 mm in diameter, with a very characteristic appearance. Each oocyte is clearly divided in halves: the dark brown ("animal") hemisphere and the yellowish ("vegetal") hemisphere. The darker color of the animal hemisphere results from the higher concentration of melanin-containing pigment granules. The granules are concentrated in the cortical granule layer, lying just below the granule-free cortical (submembrane) layer of the cytoplasm.[27]

The morphological polarization of the oocytes has many additional manifestations. Thus, there are more microvilli in the animal hemisphere.[28,29] In mature unactivated eggs, the specialized membrane junctions between the endoplasmic reticulum and plasma membrane, which may be important in the regulation of intracellular Ca^{2+}, are also concentrated in the animal hemisphere.[30] Cytoskeletal contractile structures also appear to be stronger in the animal hemisphere.[112] In contrast, most of the RNA of the oocyte is found in the vegetal subcortical region.[31] In the eggs, lateral mobility of membrane lipids is five times higher in the vegetal than in the animal hemisphere.[32] The penetration of sperm into the egg seems to occur only in the animal hemisphere.[33] Manifestations of **functional** polarity (some of which will be discussed in the following sections) are worth mentioning: a higher density of Ca^{2+}-dependent Cl^- channels, a larger response to intracellular Ca^{2+} injection and to application of acetylcholine (ACh)[34-37] in the animal hemisphere, a faster spread of the cortical granule breakdown wave[38] and of the fertilization potential[36,37] in the animal hemisphere upon fertilization, etc. The animal-vegetal polarity of the oocyte predetermines the way in which the postfertilization cleavage proceeds, leading to the polarity of the embryo. The molecular basis of the morphological and functional polarization is not clear. It has been hypothesized that the current flowing through the oocyte along the animal-vegetal axis may be the vector that determines polarity (e.g., by creating the physical force separating the cellular components; see Section VI.A). It is, however, unknown what determines the higher density of the Cl^- channels (which are apparently responsible for the animal-vegetal current) in the animal hemisphere.

B. Maturation

Luteinizing hormone, released from the pituitary, acts on follicular cells, causing synthesis and release of the hormone progesterone. The latter reinitiates meisosis; this "maturation" (reviewed in Reference 4) is a complicated process accompanied by numerous biochemical events such as changes in cAMP levels, alkalinization of cytoplasm, massive protein phosphorylation, *de novo* synthesis of proteins, changes in ion fluxes through the membrane, etc.[4,5,39,40,113] It includes major morphological changes: breakdown of the nucleus (GV breakdown [GVBD]; in vitro, it occurs some 4 to 8 hr after the application of progesterone) and extrusion of the first polar body;[4] formation of endoplasmic reticulum shells around the cortical granules and of new endoplasmic reticulum-plasma membrane gap junctions;[41,42] retraction of microvilli;[16] ovulation (see following); etc. The molecular mechanism of triggering and regulation of maturation have been extensively studied. The biochemical information obtained in these studies is important for the understanding of some of the aspects of regulation of ion channels by neurotransmitters. These data are considered in more detail in Section XI.B.

In vivo, maturation proceeds inside the body cavity. Maturing oocytes are first extruded

from the ovary ("ovulation") into the body cavity and then into the oviduct, in which they are covered by jelly, and eventually into the water, ready for fertilization (insemination). At this point, meiosis halts again (second meiotic arrest) at metaphase II, and reinitiates following fertilization. The oocytes at this stage are no longer called oocytes, but eggs.

C. Fertilization

Fertilization triggers the reinitiation of meiosis, extrusion of the second polar body, and, eventually, cleavage of the egg. Most of the biochemical and morphological events taking place after fertilization are not discussed; some are briefly outlined. Amphibian eggs are fertilized in water; therefore, most of the studies in vitro on eggs are done in low-osmolarity solutions (e.g., 10% Ringer's solution). During the first minutes after insemination, a complex series of morphological changes takes place:

1. In Anurans (frogs and toads), the egg undergoes a fast depolarization of the membrane potential (usually to positive potentials). This is called the "fertilization potential" and is believed to provide a fast block to polyspermy.[43,44] Urodeles (salamanders) are naturally polyspermic; their eggs do not produce fertilization potentials.[44,45] The basis of the fertilization potential is discussed in Section VIII.
2. The engulfment of the fertilizing sperm.
3. Starting at the point of sperm entry, a wave of breakdown of cortical granules and fusion of their membranes with the membrane of the oocyte occurs (a process called sometimes "exocytosis" or "cytolysis"). In *Rana* eggs, this is accompanied by a twofold rise in the capacitance of the egg, suggesting an increase in the total membrane area; the capacitance returns to the unfertilized level in about 30 min.[46] Cortical granule exocytosis is believed to participate in the formation of the fertilization membrane that provides a mechanism to block polyspermy.[19]
4. Two or more waves of surface contraction.[27,37]
5. Formation of the grey crescent (an area opposite to the site of sperm entry that attains a grey coloration).[19]
6. Formation of the first cleavage furrow; cleavage.

All these events can also be induced by artificial stimuli (the egg can be "activated" by): pricking, application of the divalent cation ionophore A23187, intracellular iontophoretic injection of Ca^{2+}, and the injection of the Ca-mobilizing agent inositol 1,4,5-trisphosphate (IP_3). Intracellular Ca^{2+} concentration increases upon fertilization or activation in *Xenopus* eggs from 0.4 to 1.2 μM.[47] These and other findings gave rise to the Ca theory of egg activation, i.e., that an increase in free intracellular Ca^{2+} concentration triggers the early events of fertilization (References 47 and 48 and references cited therein). Ca^{2+} is probably released from the intracellular stores, and it has been proposed that the mechanism of Ca mobilization involves, as with several hormonal stimuli in this and other cells (see Section XI.A), activation of phospholipase C and the production of IP_3.[48] A similar mechanism was proposed for fertilization of sea urchin eggs.[49,52]

III. INTRACELLULAR CONCENTRATIONS AND ACTIVITIES OF IONS

Correct interpretation of electrophysiological data necessitates knowledge of ionic composition of the interior of the cell. In amphibian oocytes, the latter has been extensively studied. Yet, there is controversy concerning the exact concentrations and activities and the degree of sequestration of the various ions in amphibian oocytes. There are differences between follicle-enclosed oocytes and those defolliculated ("denuded") by various methods; these differences are not always understood or unequivocally explained.

Table 1 presents a summary of the measured or estimated values of intracellular concentrations of K^+, Na^+, and Cl^- found in the various studies in *Rana*, *Bufo*, and *Xenopus*. It can be seen that estimates of sodium concentration and activity vary almost sevenfold; estimates of potassium concentration and activity have been less controversial, with threefold variations among different reports; the values reported for Cl^- activity are more uniform (only a twofold difference). One reason for these controversies might be that physiological parameters vary vastly among individual frogs. In the following, we shall (1) describe possible other sources of discrepancy and (2) comment on the most realistic estimates of ion activities.

A. The Effect of Defolliculation

Two main methods of defolliculation are used: manual and enzymatic. In the former, the oocytes are incubated for up to 30 min in Ca-free and EDTA- (or EGTA)-containing medium, and then the outer layers are manually removed with fine forceps.[53] The latter method involves treatment with a proteolytic enzyme (usually collagenase; more rarely a nonspecific protease such as pronase or dispase) for various periods of time (with collagenase, the concentration is 1 to 6 mg/mℓ and treatment time is one to several hours). In both cases, the denuded oocyte is still surrounded by the noncellular fibrous vitelline membrane.

Manual defolliculation has been unanimously reported to elevate the intra-oocyte Na^+ concentration, $(Na)_{in}$, when the latter was measured soon after defolliculation.[54-56] Frank and Horowitz[56] have divided the population of manually denuded oocytes into three groups: undamaged, damaged, and seriously damaged (Table 1); they claimed that the more serious the damage caused by defolliculation, the higher the $(Na)_{in}$. The notion that the defolliculation procedure causes damage to oocytes is supported also by the accompanying changes in resting potential (see Section V.B). This damage may result from rupture of the membranal connections between the oocyte and the follicular cells, thereby producing numerous little holes in the membrane of the oocyte. These holes would be expected to serve as "nonspecific ion channels", allowing passive influx of Na^+ and Ca^{2+} and passive efflux of K^+. Thus, defolliculation may be expected to cause a decrease in the intra-oocyte concentration of K^+, $(K)_{in}$. Such a decrease, correlated with the increase in $(Na)_{in}$, was indeed reported by Frank and Horowitz.[56] However, the group of Morrill reported an **increase** in $(K)_{in}$.[54,55] This discrepancy might arise from the fact that Frank and Horowitz did not use the EDTA treatment before the mechanical defolliculation while Morrill's group did; yet, the precise mechanism of the increase in $(K)_{in}$ in the experiments of the latter group is not known.

The effects of enzymatic defolliculation on ionic concentrations is less well documented. In one case, an increase in intracellular K activity (measured by cation-selective electrodes) was reported in collagenase-treated oocytes (Reference 57; Table 1). It seems that the membrane was damaged in this case, too. The changes in the resting potential and in Na/K pump activity (see following) agreed with this notion. Furthermore, the response to ACh disappears in many collagenase-treated cells.[9,69] Ca^{2+} influx is increased for at least 3 hr following the pronase treatment, while the steady-state resting Cl^- current (see Section VI.A) is reduced by 80 to 90%.[34] The rate of Ca^{2+} efflux is reduced 30 to 70-fold, suggesting that Ca extrusion mechanisms have been seriously damaged.[70]

To date, there are no data on the effect of defolliculation on the level of Ca^{2+}. The intracellular Cl^- activity appears to be unchanged in collagenase-treated oocytes.[57]

In conclusion, both manual and enzymatic defolliculation of the oocytes appear to damage the membrane. As a result, there is an increase in $(Na)_{in}$. $(K)_{in}$ also changes, but there is controversy as to the direction of this change. The data on effects of defolliculation on Cl^- and Ca^{2+} activities are either scarce or absent.

B. The Changes in Intracellular Concentrations of Ions during the Growth of the Oocyte

In general, in the few studies in which such changes were followed, a continuous increase

Table 1
INTRACELLULAR MONOVALENT ION CONCENTRATIONS (m*M*) AND ACTIVITIES IN AMPHIBIAN OOCYTES AND EGGS

Species	Method of denuding	Size	Other features	Na^+				K^+				Cl^-				Ref.
				Follicles		Denuded		Follicles		Denuded		Follicles		Denuded		
				Conc	Activ	Conc	Activ	Conc	Activ	Conc	Activ	Conc	Activ	Conc	Activ	
				Measured												
Bufo, Rana		Small		10—30				100—150								58
Bufo		Large	Ovulated	121				17								59
Rana	Manual	Large				80	29			120	88					60
Rana		Large		62												61
Rana		Intermediate		26	9.3			113	82							62
Rana	Manual	Large		74	44	90		93		117	80					54
Rana	Manual	Large		78		102		83		133						55
Rana		Large		73	6			93	120							63
		Small		35	16			105	70							
Rana	Manual	Large	Undamaged	29		30		50		46						56
		Large	Damaged			41				32						
		Large	Seriously damaged			62				10						

Xenopus	Collagenase	Large	18	120	150	62	62	57
	Manual	Large	22.5					
		Matured			117		62	
Xenopus	Collagenase	Large	6		92		33	64
Rana		Eggs			121		44	46
			Calculated from E_{rev} of Various Currents					
Xenopus	Collagenase	Large	1—6					65
Xenopus	Collagenase	Large	2—4					66
Xenopus		Large				33		67
Xenopus		Large		88				68

in $(Na)_{in}$ and a decrease in $(K)_{in}$ were observed[58,63] (Table 1). Changes in $(Na)_{in}$ are accompanied by an increase in passive Na^+ influx and in passive Na^+ permeability.[58] The percentage of water decreases as the oocyte grows, from 90% (w/w) in small oocytes to 50% in the largest oocytes.[63] The data on the decrease in $(K)_{in}$ are quantitatively variable; Cannon et al.[58] reported a threefold decrease in large as compared to small *Rana* oocytes, while Palmer et al.[63] observed only a 10% change. The intracellular activity coefficients γ_{Na} and γ_K apparently change in the opposite direction during oocyte growth:[63] γ_{Na} decreases (suggesting extensive Na^+ sequestration; see following) while γ_K increases (Table 1). No data on possible changes in concentrations of other ions during oocyte growth are available. Since most of the electrophysiological studies were performed with the fully grown (stages 5 and 6 in *Xenopus*, "large" *Rana*) oocytes, in the following sections we shall refer to the data concerning these oocytes.

C. Activities of Ions and Their Sequestration and Compartmentalization in the Oocyte

Most investigators agree that a significant proportion (more than 50%) of intra-oocyte Na^+ is sequestered while most of K^+ is free.[56,59-61,63] This conclusion is supported by the fact that the activity coefficient for Na^+ is usually much lower than in the extracellular solution (the latter is 0.75; see Reference 62); the value of 0.08 reported by Palmer et al.[63] is the lowest known activity coefficient for intracellular Na. In most studies in which the intracellular activities were measured with ion-selective electrodes, values of $a(Na)_{in}$ between 6 and 22 m*M* were reported[57,62-64] (see Table 1). The lower values measured in denuded oocytes agree well with the estimate of $1 \leq a(Na)_{in} \leq 6$ m*M* as calculated from the reversal potential of the Na^+ current in *Xenopus* oocytes.[65,66] In the work of Morrill et al.,[54] a rather high value (44 m*M*) for $a(Na)_{in}$ was reported, and it was suggested that only 6% of intracellular Na^+ is sequestered. Palmer et al.[63] pointed out that this result could be in error due to the use of Na^+-selective electrodes with long uncoated tips that might be partially exposed to the extracellular solution. If this is correct, there still remains an unexplained threefold variation among the values of $a(Na)_{in}$ measured with Na^+-selective electrodes (see Table 1).

The estimates of K^+ activity vary by a lower factor than those of $a(Na)_{in}$ (Table 1). The actual value lies probably somewhere between 80 and 120 m*M*.

In studies of K^+, Na^+, and Ca^{2+} influx and efflux kinetics in follicles at least two components can be detected, suggesting the presence of at least two fractions.[54,56,70] Defolliculation results in the disappearance of the faster phase of Ca^{2+} uptake, in a strong reduction of the faster phase of Ca efflux,[70] and in the disappearance of the faster phases of Na^+ and K^+ uptake.[54] These findings have been interpreted as suggesting that the fast fraction reflected the pool of ions of the follicle cells while the slowly exchanging fractions represented the pool of the oocyte.[54,70] However, these conclusions have been challenged in a later report by Frank and Horowitz,[56] who examined the exchange of K^+ in whole oocytes and, separately, in cytoplasm and nucleus. The results of this study have revealed that in both denuded ooxytes and in follicles K^+ exchange has two kinetic components. The authors suggested that the loss of the faster fraction of K^+ exchange reported by Morrill et al.[54] could be due to the treatment of the oocytes with the Ca-free solution before defolliculation. They have also shown that there are two fractions of K^+ in the oocyte. The rapidly exchanging fraction is common to the cytoplasm and the nucleus and behaves like an ordinary aqueous solution, with the only barrier between this fraction and the extracellular medium being the passive transport properties of the membrane. The second fraction is confined to the cytoplasm (possibly an organelle) and has a higher Na^+ and a lower K^+ concentration than the first fraction (i.e., it actively sequesters Na^+ and extrudes K^+). The existence of this second fraction is the reason for the low value of Na^+ activity coefficient and for the unusually high (higher than unity; Reference 63) value of the apparent K^+ activity coefficient.

A similar intracellular distribution of Na^+ and K^+ have been found in the oocytes of a salamander, *Desmognathus*.[71] In this preparation, the intracellular water is compartmentalized: two thirds are in a slowly exchanging fraction, perhaps absorbed (together with the sequestered ions) in hydrated crystalline structures. These structures are probably the yolk platelets.[72]

The intracellular Ca^{2+} activity ($a[Ca]_{in}$) has not escaped contradiction. An early estimate by Moreau et al.[73] was 0.7 μM (measured with Ca-selective electrodes). More recent measurements gave lower values: 0.14 μM in *Xenopus* oocytes[74] and 0.4 μM in *Xenopus* eggs.[47] The lower estimates are also close to the value of 0.32 μM measured in 2- to 64-cell *Xenopus* embryos.[75] Thus, the actual $a(Ca)_{in}$ in the oocytes is probably between 0.1 and 0.4 μM. The intra-oocyte **concentration** of Ca^{2+} has not been measured, and no studies on Ca^{2+} sequestration in these cells have been performed. The mechanisms of Ca^{2+} extrusion have not been carefully investigated either. The results of a study by Charbonneau et al.[76] indicate that Na-Ca exchange takes place in the membrane of the oocyte. Studies of Ca^{2+} metabolism appear necessary now that the role of Ca^{2+} release from the intracellular stores in neurotransmitter responses and fertilization (see following) has been established.

IV. APPLICATION OF ELECTROPHYSIOLOGICAL METHODS FOR THE STUDY OF OOCYTES

Most modern electrophysiological methods are applicable in oocytes. In the simplest arrangement, the membrane is penetrated with a single microelectrode and the membrane potential is measured. The oocytes can be easily penetrated with two microelectrodes (Figure 1A). This arrangement allows the use of one of the two classical methods: current clamp or voltage clamp. Most electrophysiological studies on oocytes and eggs were performed using the two-electrode voltage clamp. The large size of the oocytes also permits recording of extracellular currents flowing through the cell membrane at various locations using the vibrating probe (see Section VI.A). Recently, the patch clamp method[77] (Figure 1C) has been successfully applied in devitellinized oocytes (Section II.A) and in eggs for the study of single channels. Finally, Leonard et al.[25] recently introduced a new method, called "the big patch" (Figure 1B), suitable for high temporal resolution of macroscopic currents.

V. PROPERTIES OF THE MEMBRANE OF THE OOCYTE AT REST

As in the oocytes of other species (reviewed by Hagiwara and Jaffe[78]), highly variable values of membrane resting potential (RP) in amphibian oocytes were reported (Table 2).

There is a significant variation among oocytes of different frogs (e.g., see Reference 67) and among frogs from different batches and sources.[57] However, this does not mean that a healthy oocyte may have a RP anywhere between -20 and -90 mV (the range of values in Table 2). As will be shown in the following, several factors may introduce errors or discrepancies in the estimate of RP, and elimination of these errors significantly narrows the range in which the actual RP can lie. The membrane of the oocyte inherits an active Na-K pump.[58,59,87] The changes in pump activity following various treatments, and its contribution to RP, have also been a matter of confusion and controversy and will be discussed in the following.

A. Errors Introduced by Electrode Penetration

Oocytes of various animals undergo hyperpolarization after electrode penetration (for review see Reference 78). The amphibian oocytes and eggs are no exception.[57,62,67,79,88] It has been suggested that this process reflects "healing" of the membrane that was damaged by the electrode.[78] Thorough studies performed later in amphibian oocytes and eggs con-

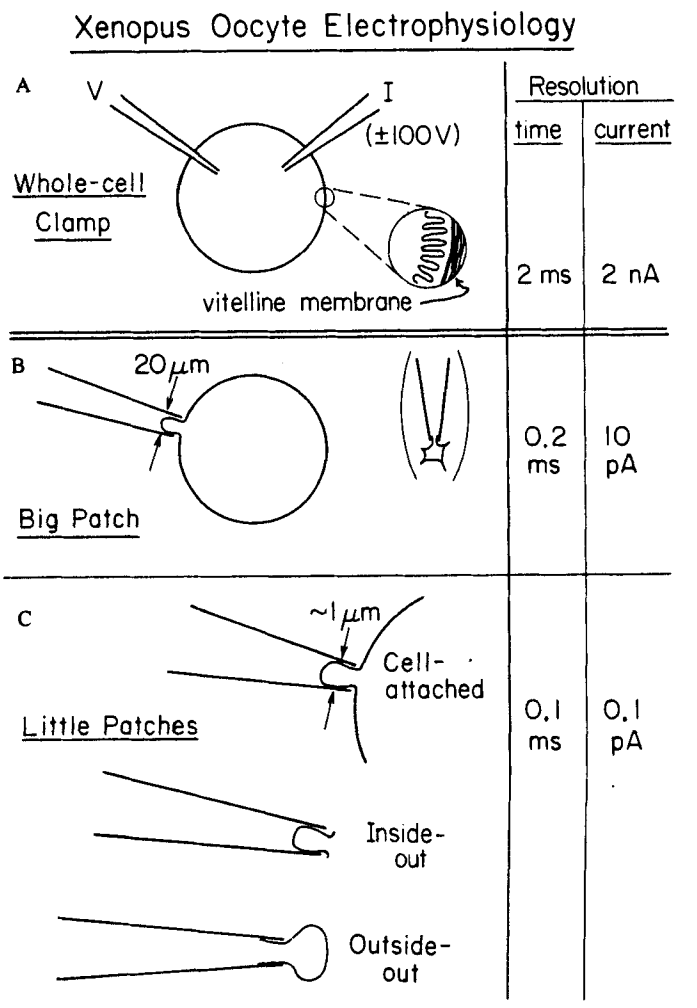

FIGURE 1. Methods used to study the electrophysiology of the oocyte. (A) Two electrode voltage clamps with two intracellular electrodes. Can be performed in follicles and denuded or devitellinized oocytes. Capacity transient lasts for about 2 msec, minimal detectable current 2 nA. (B) The "big patch" method. Performed with a large (20 to 40 μm outer diameter of the tip) fire-polished micropipette in devitellinized oocytes. Capacity transient lasts for about 0.2 msec, minimal resolvable current 10 pA. (C) Little patch: the regular patch techniques. Performed in devitellinized oocytes. Time resolution 0.1 msec or less, minimal resolvable current 0.1 pA. (Figure courtesy of Dr. H. A. Lester).

firmed this hypothesis. Thus, Dascal et al.[67] have shown that, when the oocyte was penetrated sequentially by two or three electrodes (the membrane potential was measured by the first one or by the first and the second one), each penetration caused an instantaneous depolarization and a drop in the input membrane resistance (R_m). This was followed by a slow (time course of minutes) partial recovery of both RP and R_m. The steady-state values of RP and R_m reached after the penetration with the additional electrode were always lower than before the penetration. This suggested that even the values of RP and R_{in} measured with the first electrode underestimated the actual R_m and that the actual RP was more negative than that measured by the microelectrode. This conclusion is supported by the observations

Table 2
VALUES (IN mV) OF RP IN FROG OOCYTES AND EGGS[a]

Oocytes

Species	Follicles	Denuded	How denuded	Ref.
Rana		−20—−70	Manually	60
Xenopus		−52	Collagenase	73
Rana	−20—−30	−60—−80	Manually	79
Xenopus		−63	Collagenase	80
Rana		−48—−77	Manually	55
Rana		−43		63
Xenopus		−49	Collagenase	81
Xenopus	−42; −52[b]	−52—−56	Manually	57
Xenopus		−37—−50	Collagenase	57
		−90	Dispase[c]	65
Xenopus		−60	Dispase[d]	66
Xenopus		−66	Dispase[e]	82
Xenopus		−60	Dispase[f]	82
Rana		−59	Manually	83
Xenopus		−60—−90	Collagenase	64
Xenopus	−48	−47	Collagenase	67

Eggs[g]

Rana		−30—−50		84
Rana (metaphase I)		−25		85
Rana (metaphase II)		−30		85
Rana		−24		45
Rana		−30		46
Xenopus (overshoot)[h]		−33		86
Xenopus (steady)[h]		−24		86
Xenopus (2 electrodes)[h]		−19		86

[a] Either ranges or mean values (where available) are given.
[b] Mean values for two different batches of frogs.
[c] Immediately after the enzymatic treatment.
[d] At 2 hr after the treatment with enzyme.
[e] First day after treatment.
[f] Second day after treatment.
[g] In low-osmolarity solutions such as 10% Ringer; the ovulated eggs do not need to be defolliculated since they are already free of the surrounding cellular layers.
[h] See the text for details.

of Webb and Nuccitelli[86] made in *Xenopus* eggs in which the potential measured by the first electrode immediately upon penetration (the "overshoot", see Table 2) was rather negative but then "dropped" to a less negative value. Penetration by the second electrode caused a further drop and an incomplete recovery of RP[86] and an increase in total membrane conductance from 0.14 to 0.7 μS.[46] Dascal et al.[67] have further shown that withdrawal of the electrode was followed by an immediate depolarization followed by a relatively fast return of RP and R_m to values observed **before this electrode was inserted**. The recovery of RP and R_m was faster in denuded oocytes than in follicles, possibly due to the extra effort needed to penetrate the latter thus causing greater damage when the electrode was eventually pushed in. A linear correlation was found between RP and R_m measured in a single oocyte which underwent depolarizations and recoveries due to penetrations and withdrawals of electrodes.

On the basis of these observations, Dascal et al. suggested that pricking by the electrode

produces a hole in the membrane, which acts as a shunt to the membrane resistance. Extrapolation of the plot of RP vs. R_{in} to $R_m = 0$ (an infinitely large hole in the membrane) gave an estimate of RP = −12 mV (the membrane potential that the oocyte would have immediately after such a hole was produced and before any changes in intracellular ion concentrations could occur). This is very close to the value of −13 mV as calculated for a theoretical cell equally permeable for all monovalent ions using the Goldman's equation and the most realistic estimates of the intracellular concentrations of ions. It follows that the hole produced by the electrode serves as a large nonspecific ion channel. Ca^{2+} will probably penetrate through such a hole, too; indeed, in many instances pricking causes Cl^- current fluctuations in the oocytes and activation in the eggs; both events are characteristic consequences of elevated $a(Ca)_{in}$. We notice parenthetically that in *Fucus* (an alga) embryo, the influx of Ca^{2+} caused by pricking brings about the opening of Ca-dependent K^+ channels and hyperpolarization;[89] this does not appear to happen in amphibian oocytes.

In summary, penetration by the electrode damages the oocyte membrane and leads to understimation of the values of RP and R_m. Thus, the most reliable estimates of the actual RP and R_m are those obtained with a single electrode when the membrane is allowed to seal around the site of pricking for at least 15 min.[57,67,86] When this requirement is fulfilled, the RPs of *Rana* and *Xenopus* follicle-enclosed oocytes are found in the range between −40 and −60 mV; the eggs usually have RPs between −25 and −30 mV (Table 2). More variations are observed between oocytes from different donors than from a single frog;[67] an additional source of variability may be the use of frogs obtained from different suppliers.[57] Further discrepancies are introduced by defolliculation; the effects of defolliculation on RP are discussed in the following.

B. RP in Denuded Oocytes and the Role of the Electrogenic Na-K Pump

Manual defolliculation (preceded by incubation of the oocytes in Ca-free, EDTA-containing medium) shifts RP to much more negative values than in follicles; values range from −65 to −90 mV.[55,79] Wallace and Steinhardt[79] have monitored RP following mechanical defolliculation. In freshly defolliculated cells, there was an initial hyperpolarization from a value of about −30 mV (immediately after penetration with the electrode) to −70 or −80 mV, followed by a slower decay to less negative values. In oocytes stored at room temperature for 24 hr, RP was close to −50 mV. Similarly, in oocytes denuded by collagenase or dispase, immediately after the defolliculation RP was very negative[64,65] (down to −90 mV) but "repolarized" to less negative values 2 to 48 hr later[66,82] (−60 to −66 mV). In many studies in which the dependence of RP on the time elapsed after collagenase defolliculation was not specifically studied, values of RP close to those seen in the follicles (−45 to −60 mV) were reported.[57,67,73] In these cases, RP was measured at least 1 hr after defolliculation.

Ziegler and Morrill[55] and Wallace and Steinhardt[79] have found that, in manually denuded oocytes with very negative RPs, the Na-K pump inhibitors ouabain, strophanthidin, or K^+-free solution reduced RP to −25 to −40 mV, i.e., to values less negative than even in follicular oocytes. Elevation of extracellular K concentration caused hyperpolarization. There was little or no effect of low $(K)_{out}$ or Na-K pump inhibitors on RP in follicle-enclosed oocytes. The authors did not look for a possible difference in the effects of pump inhibitors on freshly denuded oocytes, on which the study was performed, vs. oocytes incubated for several hours after the dissection. These results were interpreted to suggest that in follicle-enclosed oocytes the electrogenic Na-K pump is almost or fully inactive[55,79] and that removal of follicular cells leads to activation of the pump, possibly by "uncovering" some specific pump sites.[79] This hypothesis, although attractive, does not explain some of the facts observed by the same authors, or later by others, that: (1) there is a decrease in RP during long-term incubation of denuded oocytes without a loss of viability (e.g., the ability to undergo maturation); (2) the values of RP in denuded vs. folicle-enclosed oocytes in the presence of

pump inhibitors are less negative; (3) in both follicle-enclosed and denuded oocytes, none of the ions is in equilibrium at rest (see Section V.B), thereby necessitating some active mechanism for maintaining the intra-oocyte ionic concentrations. Morover, it was shown later[67] that ouabain did depolarize follicles, although less than collagenase-denuded oocytes; in the presence of ouabain, RP in collagenase-treated oocytes was more positive than in follicles (−33 vs. −42 mV).

Dascal et al.[67] concluded that the defolliculation-related phenomena described above can be explained if one takes into account the damage done by these procedures to the membrane and the accompanying changes in intracellular concentrations. The increase in $(Na)_{in}$ that accompanies defolliculation (see Section V.A) would certainly activate the Na-K pump, and no "uncovering" of pump sites would be necessary. The same permeability increase that underlies the increased Na^+ influx in denuded oocytes may explain their lower RP in ouabain. Indeed, the ratio of Na^+ to K^+ permeabilities (P_{Na}/P_K) in collagenase-treated oocytes is twice as high as in follicles. The slow return of RP to less negative values during the long-term incubation of the denuded oocytes is the result of membrane healing, reduction of $(Na)_{in}$ (due to the preceding enhanced extrusion by the pump), and the subsequent reduction in pump activity.

To summarize, it appears that defolliculation of oocytes by various methods results in changes in RP that can be accounted for by the damage caused to the membrane. The latter seems to heal over a period of several hours, and it would probably be advisable not to use freshly defolliculated oocytes for experiments intended for the study of ion channels. The "healed", healthy denuded oocytes have resting potentials between −45 and −65 mV.

C. Current-Voltage Characteristics, Resistance, and Capacitance of the Oocyte

The resting voltage-current characteristic of the oocytes is linear or close to linear between −100 and −20 mV;[57,67] at more positive potentials, an outward rectification is observed that can be partially eliminated by intracellular injection of the Ca-chelator, EGTA.[35] Thus, the outward current activated at voltages more positive than −20 mV is Ca dependent and is probably carried mostly by Cl^-.[35,64,90] At potentials more negative than −100 mV, an inward rectification was observed.[57,90] The features of the resting current-voltage relationship were studied only superficially, and the appearance of some of the recently described voltage-dependent currents might have been overlooked (Sections VII.A and IX). However, to a first approximation, the relationship can be considered linear in the region close to the resting potential (−40 to −60 mV). The membrane of *Xenopus* eggs displays a strong rectification at voltages more positive than +5 mV and more negative than −30 mV;[86] the conductances underlying these rectifications have not been determined.

When speaking of R_m, one should be aware of the possible errors introduced by the procedure utilized to measure it: the leak due to pricking and the possible nonlinearity of the I-V curve. In general, any value of R_m measured with a microelectrode is probably an underestimate. Also, the values of R_m in denuded oocytes would be expected to be higher in oocytes that were allowed to heal after the defolliculation procedure. All things considered, it appears that one should take into consideration only the measurements of R_m made (1) in oocytes with RP between −40 and −60 mV, (2) using a single electrode, and (3) by injection of small currents. No such measurements have been done in follicle-enclosed oocytes; in fully grown denuded oocytes, R_m is between 1 and 3 MΩ.[66,81] The total capacitance of a fully grown *Xenopus* oocyte is about 230 nF.[91] The specific capacitance of the membrane of the oocyte, calculated under the assumption that the oocyte is a perfect sphere, lies between 4 and 7 $\mu F/cm^2$, which is several times higher than in most other kinds of cells. These findings confirm the data of morphological studies and show that the surface of the oocyte is significantly (at least by a factor of 4) increased due to the presence of microvilli and cristae.[81,91] It is unlikely that microscopic potential differences exist between

the bulk of the cell and the basis and the tops of microvilli on a time scale of 1 msec or greater.[92] On a very fast time scale, such as is needed for the recording of voltage-sensitive Na^+ current (Section XVI.A), inadequate voltage control has been reported.[26] It is not clear whether this resulted from the spatial nonuniformity of the surface of the cell or from the inadequate procedure of subtraction of the capacity transient, which is very large and long lasting.

Both resistance and capacitance change during the growth of the oocyte. The specific capacitance increases from 2.3 $\mu F/cm^2$ in stage 1 *Xenopus* oocytes to 6 to 7 $\mu F/cm^2$ in stage 4 and 5 oocytes and slightly decreases again to about 4 $\mu F/cm^2$ in fully grown (stage 6) oocytes.[91] All these changes, including the decrease in specific capacitance between stages 5 and 6, parallel the pattern of development of microvilli.[16] The input resistance of the cell decreases as it grows, as expected from the increase in surface area.[66]

A very high resistance (13 MΩ on average) was reported for *Xenopus* eggs. The calculated specific resistance in this case was 0.7 $M\Omega/cm^2$, one of the highest values ever reported in any cell type.[86] The total capacitance of an egg is about 50 nF; the specific capacitance decreases in the eggs to 0.8 to 1.4 $\mu F/cm^2$, suggesting that the surface is now smooth.[86,93] These resistance and capacitance changes fit with observations that microvilli disappear during maturation.[94] The higher specific resistance in the eggs may reflect not only the general decrease in the total surface area, but also an actual loss of some of the ionic conductances: thus, a decrease in membrane selectivity to K^+ over Na^+ and a decrease in K^+ fluxes during maturation were reported (Section V.E), which may mean that some of the "resting" K^+ channels were eliminated in the course of maturation.

D. Oocyte Membrane Selectivity and the Contribution of Different Ions to RP

In his early work on toad (*Bufo*) oocytes, Maeno[88] showed that their membrane behaved as a K^+ electrode and that changes in $(Na)_{out}$ did not affect RP. Interestingly, RP displayed a strong dependence on $(Ca)_{out}$, more than could be expected from the surface charge effects; however, no further study of this phenomenon followed. The situation in frog (*Rana* and *Xenopus*) oocytes with respect to the monovalent cations turned out to be more complicated. At $(K)_{out} > 10$ m*M*, the slope of the plot of RP vs. $\log([K]_{out})$ has been reported to be either 38 mV per decade change in$(K)_{out}$,[55,59] 46 mV/decade,[47] or 53 mV/decade;[60] all these values are lower than the 58 mV/decade predicted for a perfect K^+ electrode. The permeability ratio P_{Na}/P_K, obtained from the measurement of fluxes of monovalent cations, was 0.1.[70] These findings suggested that permeabilities other than K^+ may partially determine RP. Clearly, if one is interested in the relative roles of the monovalent cations in a cell with such a high P_{Na}/P_K, one should not study the effect of $(K)_{out}$ on RP by replacing Na^+ with K^+, as it has been done in the works cited above.

In the work of Dascal et al.,[67] the relative roles of Na^+ and K^+ were studied with either $Tris^+$ or Mg^{2+} substituted for one or the other of the monovalent cations. In the presence of ouabain (to eliminate the effects of additional Na-K pump activation at higher K^+ concentrations), the dependence of RP on $(K)_{out}$ was correctly described by Goldman's equation. As predicted by the latter for the case when P_{Na}/P_K is not negligible, the relationship RP vs. $\log(K)_{out}$ was nonlinear; its slope increased with increasing $(K)_{out}$, being about 20 mV/decade at $(K)_{out} = 20$ m*M*. The dependence of RP on $(Na)_{out}$ also conformed to Goldman's equation at $(Na)_{out} > 40$ m*M*, but deviated from the predictions of constant field theory at lower $(Na)_{out}$. This finding suggests that at these concentrations changes in $(Na)_{out}$ may have an effect on conductances or fluxes of ions other than Na^+ (e.g., through Na-Ca exchange mechanism), or that the effect of Na^+ on its own conductance is complex. The former assumption is strengthened by the fact that a complete elimination of extracellular Na^+ caused potential fluctuations probably carried by Cl^-.[67] The value of P_{Na}/P_K calculated from the experiments described above was 0.12 in follicles and 0.24 in collagenase-denuded

oocytes. Thus, the membrane of denuded oocytes appears to be relatively more permeable to Na^+ than that of follicle-enclosed oocytes, supporting the "damage" hypothesis. Another possibility is that K channels are in the follicle cells, as suggested by loss of some responses (see Section XIII.F). The calculated P_{Cl}/P_K was 0.4 both in follicles and denuded oocytes; however, no direct measurements of the effect of Cl^- on RP were performed in this study and this value should therefore be taken only as a tenative estimate. In fact, the dependence of RP on Cl^- has not been systematically studied, and it is not clear whether changes in $(Cl)_{out}$ affect RP at all. Thus, Tupper and Maloff[60] observed only transient changes in RP when Cl^- was replaced by propionate. This suggests a purely passive distribution of Cl^- resembling, e.g., skeletal muscle.[95] In *Xenopus* eggs, changing of $(Cl)_{out}$ between 1 and 100 m*M* did not affect the RP.[96] On the other hand, Kusano et al.[57] observed variable results (both depolarizations and hyperpolarizations) when Cl^- was replaced by SO_4^{2-}.

Removal of extracellular Ca^{2+} (in the presence of a Ca^{2+} chelator) causes a strong depolarization and a large increase in membrane conductance, apparently due to an increase in P_{Na}.[60,80] The mechanism of this effect is not clear. One possible explanation is that the removal of Ca^{2+} enables Na^+ flux through the voltage-dependent Ca channel (e.g., see References 97 to 99); this hypothesis is supported by the fact that high extracellular Mg^{2+} prevents the depolarization and conductance increase caused by the absence of Ca^{2+}.[67]

One conclusion from these data is that in normal physiological conditions Na^+ contributes significantly to RP. The contribution of Cl^- is not clear. However, the main point is that in an amphibian oocyte at rest none of the ions are at equilibrium. Indeed, the RP is around -50 to -60 mV; the equilibrium potentials of the various monovalent ions are (approximately): K^+, -100 mV;[64,68] Cl^-, -25 mV;[47,64,67,100] Na^+, $+80$ mV.[8,82] It follows that Donnan equilibrium conditions are not fulfilled in this system. Some kind of active machinery is necessary to preserve the intracellular milieu. Gradients of monovalent cations are maintained by the electrogenic Na-K pump. The mechanism that keeps $(Cl)_{in}$ constant is not known. The means of ensuring the Ca^{2+} homeostasis have not been elucidated (see Section III.C).

E. Changes in Membrane Properties during Maturation

The complex process of oocyte maturation is beyond the scope of this review. The changes that occur in ion fluxes and ion channels during maturation will be considered only briefly. These changes present an impressive example of regulation of the membrane properties of a cell in the course of development, but, unfortunately, little is known about the actual events occurring at the level of ion channels. Various processes that take place during maturation, like increased protein synthesis or morphological changes in the membrane, may play a role in the appearance of new channels or the disappearance of old ones, but to date no correlations of this kind have been established.

Depolarization of the membrane of the oocyte is invariably observed about halfway between the triggering of maturation (by the application of progesterone or another maturation-inducing agent) and GVBD.[55,79,81,101] This is most probably the result of a change in membrane selectivity in favor of Na^+:[88] indeed, the permeability ratio P_{Na}/P_K increases from 0.1 before progesterone application to 0.43 at GVBD,[70] and the slope of the curve RP vs. $\log(K)_{out}$ changes from 38 to 7 mV/decade.[55] It is not clear whether this process is due to a **decrease in K^+ conductance** and, correspondingly, in passive K^+ flux (no changes in Na^+ exchange were observed by Morrill and Ziegler[102]) or to an **increase in Na^+ conductance** and Na^+ influx (an initial **increase** in K^+ efflux, followed by a sharp decrease at GVBD, was reported by O'Connor et al.[70]). There are conflicting reports on whether the input resistance increases,[39] permanently decreases,[101] or transiently increases[81] during maturation. The disappearance of channels is favored by the fact that total oocyte surface area is decreased in the course of maturation due to retraction of microvilli[94,81] (see also Section V.C and Table 2).

Following GVBD, a second depolarization was reported in maturing oocytes; this has been attributed to the disappearance of Cl^- conductance.[103] This finding has not yet been confirmed in other studies.

The electrogenic Na-K pump activity and high-affinity ouabain binding have been reported to disappear in the course of maturation.[83] These findings contradict an earlier report that the ouabain-sensitive Na^+ efflux is unchanged in the course of maturation;[59] an increase in total Na^+ efflux following progesterone treatment has been reported.[70] The reason for these discrepancies is not clear.

There is a controversy concerning the changes in intracellular free Ca^{2+} in the course of maturation. An increase in Ca^{2+} efflux starts ≤40 min postprogesterone, followed by an increase in Ca^{2+} uptake about 1 hr later and a permanent and significant decrease in the efflux.[70,104] It was proposed that this process reflects an increase in $a(Ca)_{in}$. Indeed, Wasserman et al.[105] observed a transient (up to 5 min long) increase in Ca-dependent aequorin light signal in albino *Xenopus* oocytes immediately after the application of progesterone. However, only 50% of the tested oocytes responded in this way; the absence of response in the other 50% was attributed to spontaneous maturation that started before the application of progesterone (and thus the transient Ca^{2+} signal would have been missed). A different result was obtained by Moreau et al.,[73] who used Ca-sensitive electrodes: they observed an increase in $a(Ca)_{in}$ from 0.7 to 7 μM that started only 30 min postprogesterone; the high level of Ca^{2+} was then maintained till GVBD. Finally, in a recent thorough study with Ca-sensitive microelectrodes, Robinson[74] did not observe any changes in $a(Ca)_{in}$ throughout the period between progesterone application and GVBD. He suggested[361] that progesterone may activate Ca^{2+} efflux by activating a Ca-extrusion pump or another extrusion mechanism, with no actual increase in $a(Ca)_{in}$. This view is supported by the notion that, at least during the first minutes after progesterone application, no changes in RP[81] or holding current in voltage-clamped oocytes[106] are observed, whereas any increase in a $(Ca)_{in}$ would be expected to evoke a Cl^- current (see Sections VI and X). However, membrane potential fluctuations resembling the Cl^- currents induced by various $a(Ca)_{in}$-elevating agents (ACh, Ca^{2+}, or IP_3 injection; see Sections VI, X, and XII) were observed 30 to 60 min postprogesterone.[81] Thus, at present it appears that progesterone does not produce an immediate increase in $a(Ca)_{in}$; the proposal of a later increase cannot yet be discarded. The inability of a Ca-sensitive electrode to detect changes in $a(Ca)_{in}$ may be explained if one assumes that this change is confined only to the submembrane layer of cytoplasm, which is about 5 μm thick;[27] the minimal distance between the tip of the Ca-sensitive electrode and the membrane is about 50 μm.[74]

VI. ENDOGENOUS Ca^{2+} CHANNEL AND Ca-DEPENDENT CURRENTS AND CHANNELS IN THE MEMBRANE OF THE OOCYTE

A. Ca-Dependent Cl^- Current Flowing through the Oocyte at Rest

The pattern of currents leaving and entering the oocyte at various points has been studied using an extracellular electrode that vibrates between two points.[107,108] The vibration converts any steady potential difference into a periodic signal, with a peak-to-peak value equal to the steady potential difference. This peak-to-peak value is then measured with a lock-in amplifier tuned to the vibration frequency. The method allows the measurement of the current density between the two points and thus maps the local currents flowing through the oocyte at a series of locations.

Using this method, Robinson[34] has shown that a constant net current flows through the oocyte, entering the animal hemisphere and leaving the vegetal hemisphere. (By convention, the direction of the current is defined as though the current is carried by positive ions. If the carrier is negative, an inward current means outflow of the carrying ion.) The density

of the current was maximal at the poles and lowest at the equator. It disappeared soon after the induction of maturation by various agents such as progesterone or inorganic Ca-channel blockers. The time course of the inhibition of the current by the Ca-channel blockers La^{3+} and Mn^{2+} was much faster than by progesterone, although the induction of maturation by the Ca-channel inhibitors was slower. These observations were interpreted to suggest that the current flowing through the oocyte at rest was Ca dependent. The current was increased by 50 to 100% by removal of external Na^+, contrary to what would be expected if the current were carried by Na^+. It was increased by 50% by the removal of external Cl^-, increased by 20% following the removal of extracellular Mg^{2+}, and decreased by 25% following the removal of extracellular K^+.

The author's conclusion was that the main carrier of the observed current is Cl^- and that the opening of the Cl^- channel is controlled by Ca^{2+}. A similar current was found earlier in developing embryo of an alga, *Pelvetia*,[108] and it was also known that a Cl^- channel existed in *Xenopus* oocytes.[9] Later findings, such as the Cl^- current evoked by intra-oocyte injection of Ca^{2+} (see References 35 and 109) or by membrane depolarization that causes Ca^{2+} influx through voltage-dependent channels,[64,90] supported this conclusion.

Robinson further suggested that all the current through the oocyte is carried by Cl^-, i.e., there is a passive efflux of Cl^- all around the oocyte, but the density of this flux is much higher (at least 2.5 times) in the animal pole. According to this view, then, the current is inward at any point of the surface of the oocyte, but its higher density in the animal pole leads to the generation of the total macroscopic current through the oocyte in the observed direction. The higher density of Ca-dependent Cl^- channels in the animal hemisphere was confirmed later in the experiments with Ca^{2+} injection: the latter elicited a much larger Cl^- current when injected in the animal than in the vegetal hemisphere.[35] It is also in line with the observation that iontophoretically applied ACh evokes larger Cl^- currents in the animal hemisphere;[47] this response is mediated by Ca^{2+} mobilization[109,110] (see Section XII.D). A similar distribution of Cl^- currents has also been found in the mature eggs[36,37] (see Section VIII.B). Robinson proposed, as also suggested earlier for *Pelvetia*,[108] that the intracellular electrical field produced by the observed current provides a physical force that segregates the cellular components along the animal-vegetal axis, thus maintaining of polarity of the oocyte.

Several points remain unclear. First, if all the current is the result of Cl^- efflux, then how is the electroneutrality of the oocyte interior preserved? The loss of negative charges, even if active, cannot last long because it will very soon create an electrostatic force opposing Cl^- movement and stop any current flow. Yet, the current flows through the resting oocyte all the time. One possible solution to this problem is to assume that the current observed in the vegetal hemisphere is indeed outward and that it is carried by K^+. Indeed, in the eggs, there seems to be a uniform distribution of K^+ channels that are opened at the time of activation potential;[37] thus, the density of Cl^- current may be higher in the animal hemisphere, while K^+ current may prevail in the vegetal hemisphere. The removal of external K^+ will not significantly affect the driving force for the outward current, particularly if the K^+ channel is at least slightly permeable to Na^+. However, as correctly predicted by Robinson,[34] it will cause a strong depolarization due to the inhibition of the Na-K pump (the oocytes in his experiments were denuded manually and apparently used soon after defolliculation) and a strong reduction in the driving force for Cl^- and thus of the total current through the oocyte. The role for K^+ as the outward current carrier was also proposed by Nuccitelli and Jaffe[108] in *Pelvetia*.

Another problem to be solved is the role of Na^+. The increase in the current across the oocyte in Na^+-deficient solution has been explained by Robinson as the result of removal of the inhibition of Ca^{2+} entry by Na^+. A similar phenomenon in *Pelvetia* was assumed to result from the elimination of Na-Ca antiport and thus an increase in $a(Ca)_{in}$.[108]

B. Voltage-Activated, Ca-Dependent Cl^- Current

Miledi[90] and Barish[64] described a novel type of voltage-dependent current in the membrane of the oocyte: when, under voltage clamp conditions, the oocyte is depolarized to a potential more positive than −20 mV, a slow transient outward current emerges that has a time-to-peak of about 200 msec, a decay time constant of about 1 sec, and an overall duration of 1.5 to 2 sec. The maximal amplitude of this current in a normal physiological solution usually varies between 30 and 100 nA. The current is observed both in denuded oocytes and in follicles. The amplitude and the direction of this current depend on $(Cl)_{out}$, but are affected very little by changes in K^+ or Na^+ concentrations. The relaxations ("tails"), seen when the membrane potential is stepped to different voltages at the time of the peak, reverse their direction (in a normal physiological solution) between −25 and −30 mV, which is close to the equilibrium potential for Cl^- in the oocytes. Moreover, this reversal potential does not depend on $(K)_{out}$, but shows a dependence on $(Cl)_{out}$ as predicted by the Nernst relation.[64] Thus, Cl^- appears to be the main carrier of this current. The channel is rather selective for Cl^- over cations; selectivity for other small anions has not been tested. At physiological Cl^- concentrations, it is the **influx** of Cl^- that produces the observed **outward** current when the membrane is depolarized to voltages positive to −20 mV. Opening of Cl^- channels at membrane potentials that are close to the resting values will result in the appearance of an **inward** current due to **efflux** of Cl^-.

The depolarization-evoked Cl^- current is suppressed by: (1) substitution of external Ca^{2+} by Mg^{2+} (see Reference 64), (2) addition of Ca-channel blockers Mn^{2+}, Co^{2+}, La^{3+}, and Ni^{2+} (see References 64 and 90), (3) intracellular injection of the Ca-chelator EGTA.[35] It is enhanced by increasing the extracellular Ca^{2+} concentration.[64,90] Ba^{2+} and Sr^{2+} cannot effectively substitute for Ca^{2+} in evoking it.[64] The depolarization-evoked Cl^- current exhibits a bell-shaped voltage dependence, peaking at about +10 mV and disappearing at voltages positive to 50 mV; on the other hand, Cl^- current evoked by intracellular Ca^{2+} injection still increases linearly in this range.[35] These observations led Miledi[90] and Barish[64] to the conclusion that the opening of the Cl^- channels underlying this current is controlled by the entry of extracellular Ca^{2+} through a voltage-dependent Ca^{2+} channel. Therefore, it was called the Ca-dependent Cl^- current ($I_{Cl(Ca)}$). However, the underlying voltage-dependent Ca^{2+} current was not detected. The presence of a voltage-operated Ca^{2+} channel in *Xenopus* oocytes has been demonstrated only recently, with high extracellular Ba^{2+} as the charge carrier[111] (see Section VI.C). The rather small amplitude of $I_{Cl(Ca)}$ probably results from the limited entry of Ca^{2+} rather than from a limited number of Ca-activatable Cl^- channels since the injection of Ca^{2+} evokes Cl^- currents that are manyfold larger than the maximal $I_{Cl(Ca)}$ in the same oocytes.[35]

At physiological external Ca^{2+} concentrations, the decline of $I_{Cl(Ca)}$ can be fitted by a single exponential component.[64] Cl^- currents evoked by intracellular Ca^{2+} injection may last for tens of seconds, whereas $I_{Cl(Ca)}$ usually does not outlast 3 sec.[35] These facts suggest that the time course of decay of $I_{Cl(Ca)}$ is defined by the decay of the underlying Ca^{2+} current; although the time course of the latter is not known, Ba^{2+} current through the Ca channel inactivates in a voltage-dependent manner, with a time constant of about 0.5 sec.[111]

When $I_{Cl(Ca)}$ is elicited by two sequential depolarizing pulses, the current elicited by the second pulse is smaller than the first one unless a certain time has passed between the two. This recovery of $I_{Cl(Ca)}$ from decay has a two-component time-course: a 70% recovery is achieved in 5 to 6 sec, but 30 sec are needed for a 95% recovery.[64] On the other hand, Ba^{2+} current through the Ca^{2+} channel displays no long-lasting refractoriness and can be repeatedly evoked every 10 to 15 sec with no decrement.[374] Thus, the refractoriness of $I_{Cl(Ca)}$ may be a Ca-dependent feature of either Ca^{2+} or Cl^- channels, although other possibilities cannot be excluded.

The amplitude of the voltage-dependent Ca^{2+} current in a normal physiological solution

probably does not exceed 1 nA (see the next section). A simple calculation shows that a 1-nA Ca current corresponds to a flux of 5 fmol/sec/oocyte; assuming a 0.5-$\mu\ell$ water volume of an oocyte,[63] this gives a change in $a(Ca)_{in}$ of 0.01 μM, which is less than a 3% increase with respect to the resting level. Since Ba^{2+} current is inactivated in a voltage-dependent manner in <1 sec,[111] it is reasonable to assume that the Ca^{2+} current would not last long either; thus, the change in free intracellular Ca^{2+} is even smaller than 3% per depolarizing step. Yet, this Ca^{2+} influx is enough to activate the Cl^- channels. This suggests that it is the free Ca^{2+} concentration in the close vicinity of the membrane (which may be expected to be much higher than that calculated assuming a uniform distribution of Ca^{2+} over the cell) that determines the amplitude of $I_{Cl(Ca)}$. In other words, the molecular apparatus underlying the opening of Cl^- channels by Ca^{2+} is situated in or near the plasmatic membrane. An additional possibility is the existence of a mechanism of Ca^{2+} signal amplification through Ca-dependent Ca^{2+} release.[64] This hypothesis is strengthened by several observations: (1) at high extracellular Ca^{2+} concentrations, the decay of $I_{Cl(Ca)}$ can no longer be described by a single exponential;[64] (2) a second "hump" of Ca-dependent Cl^- current appears in oocytes injected with brain or heart mRNA;[111] (3) a long-lasting second phase of Cl^- current appears following an injection of rather large amounts of Ca^{2+} into oocytes.[109,114]

The slow rise and the strong temperature dependence of $I_{Cl(Ca)}$ suggest a complex mechanism of activation,[64] about which nothing is known at present.

C. Voltage-Dependent Ca Channel

As mentioned above, the results of the studies on $I_{Cl(Ca)}$ implied that it is probably caused by a Ca^{2+} influx through a voltage-dependent Ca^{2+} channel; however, the small Ca^{2+} current escaped detection because it was obscured by $I_{Cl(Ca)}$. To overcome this problem, Dascal et al.[111] used Ba^{2+} at a high concentration (usually 40 mM) as the current carrier (e.g., see Reference 115); Ba^{2+} does not replace Ca^{2+} in eliciting $I_{Cl(Ca)}$.[64] In addition, all extracellular Cl^- was replaced with methane sulfonate; this procedure proved useful in abolishing a large noninactivating outward current that appeared upon depolarization in high-Ba^{2+} solution.[374] Under these conditions, a depolarization from a holding potential between -100 and -60 mV to a test potential more positive than -30 mV elicited a transient inward current with a decay time constant of about 0.5 sec. The rise time was less than 2 msec and could not be clearly resolved because of the capacity transient at the beginning of the depolarizing pulse. The current was suppressed by Cd^{2+} and Co^{2+} and also appeared in a solution in which all Na^+ was substituted by *N*-methyl-D-glucamine. The current-voltage characteristic was bell shaped, with a peak at about $+10$ mV. The current was almost fully inactivated at holding potentials more positive than -40 mV. These results suggested that the observed current was a Ba^{2+} current through a voltage-dependent Ca^{2+} channel. Moreover, the similarity of the voltage dependence of activation and inactivation of this current to those of $I_{Cl(Ca)}$ suggested that the Ca^{2+} influx through this channel in a normal physiological solution underlies $I_{Cl(Ca)}$.

Inactivation of the classical slow Ca^{2+} current in heart and neurones is, at least in part, the result of increases $a(Ca)_{in}$ (Ca-dependent inactivation; e.g., see References 116 to 118). When Ba^{2+} is used as a charge carrier, this current is significantly prolonged. More recently, in various cell types, "fast" Ca^{2+} currents were discovered; their inactivation appears to be mostly voltage dependent; their time course does not depend on the kind of divalent cation used as the charge carrier (e.g., see References 119 to 123). The Ca^{2+} channel of the oocyte appears to belong to this second class. It is probable that the increase in the submembrane free Ca^{2+} concentration caused by the current outlasts the current itself.

Assuming a similar permeability for Ca^{2+} and Ba^{2+}, with 40 mM Ba^{2+} outside and about 0.4 μM Ca (Section III.C) inside the cell, a reversal potential of about 150 mV (positive

inside) for the Ba^{2+} current will be expected if Ba^{2+} and Ca^{2+} are the only permeable ions. However, from an extrapolation of the current-voltage curves for the Ba^{2+} current, we have obtained an estimate of the reversal potential between +50 and +70 mV.[374] A similar extrapolated reversal potential was reported for $I_{Cl(Ca)}$.[90] These discrepancies presumably arise from the nonlinear current-voltage relation expected from the very large ratio of external to internal $[Ca^{2+}]$.[115] An additional factor may be a nonuniform distribution of Ca^{2+} inside the oocyte, with a higher Ca^{2+} concentration just below the membrane.[90] It would be also reasonable to assume that monovovalent cations also can permeate the channel,[99,124,125] thus shifting the reversal potential of Ba^{2+} current to less positive values.

The actual amplitude of the voltage-dependent Ca^{2+} current in a normal physiological solution is not known. Under the assumption that the permeabilities of Ba^{2+} and Ca^{2+} are similar, and keeping in mind that in a 40-m*M* external Ba^{2+} solution the Ba^{2+} currents have amplitudes of 5 to 20 nA, one would expect a Ca^{2+} current of less than 1 nA with 2 m*M* extracellular Ca^{2+}. In a cell that for a voltage step of 60 to 100 mV produces leak currents of several tens of nanoamperes, such a current would be indistinguishable, even if one does not take into account the masking effect of $I_{Cl(Ca)}$.

To summarize, the voltage-dependent Ca^{2+} channel in the oocytes has now been only very perliminarily characterized. A number of questions concerning its properties remain open, and further study is obviously necessary.

D. Hyperpolarization-Evoked Cl^- Current

Peres and Bernardini[126] have studied the voltage-activated channels in ovulated oocytes. The membrane of immature oocytes usually responds passively to hyperpolarizations (down to −120 mV). In oocytes taken from female *Xenopus* that were stimulated to ovulate by the injection of human chorionic gonadotropin and tested in a solution containing 60 m*M* NaCl, 1 m*M* KCl, and 4 m*M* $CaCl_2$, the RP was usually around 0 mV; these oocytes underwent GVBD and displayed no active currents in response to hyperpolarization. However, some oocytes had RPs of about −20 mV and had no white spot on the animal hemisphere (the sign of a completed GVBD), and histological examination showed an intact GV. The authors do not report the size of these oocytes, and it is not clear whether the reason for incomplete maturation might be the stage of development (e.g., Dumont's stage 5). The authors assumed that these were maturing (but pre-GVBD) oocytes. Hyperpolarization from a holding potential of −20 mV to more negative values evoked a transient current that resembled in its time course the Ca-dependent Cl^- current, $I_{Cl(Ca)}$ (see Section VI.B). The "tails" of this current reversed at about −15 mV, and the reversal potential changed with the change in $(Cl)_{out}$ as predicted for a Cl^- electrode. It was concluded, therefore, that this transient current is carried by Cl^-. No further characterization of this current followed. The authors claimed that this current does not appear in post-GVBD oocytes; however, low-osmolarity solutions that are routinely used in experiments with ovulated oocytes and eggs (see Section VIII.A) have not been tested. Thus, it is not clear whether this conductance may explain the inward rectification observed in the current-voltage characteristic of the eggs at membrane potentials more negative than −20 to −30 mV.[85] Parker et al.[127] later reported that a similar or identical current can be elicited occasionally by hyperpolarizations to voltages more negative than −50 mV in immature oocytes. In some instances, it could be observed only immediately after the penetration of the oocyte with the microelectrodes, but disappeared a few minutes later; the authors assumed, therefore, that it could depend on the temporary increased $a(Ca)_{in}$ (due to the electrode-induced leakage). A similar hyperpolarization-evoked Cl^- current always appeared in oocytes injected with rat brain mRNA during and shortly after the application of serotonin, glutamate, or ACh (see also Sections XV.D and E). The authors assumed that this is the same current as observed in oocytes not injected with RNA and further characterized it. The tail currents

had a reversal potential of about −20 mV. The current was enhanced in low-Cl^- solution. They concluded, therefore, that this current was carried by Cl^-. It developed with an appreciable delay after the beginning of the hyperpolarizing pulse; its amplitude displayed a strong temperature dependence. An interesting observation concerned the recovery of the current from inactivation upon repetitive stimulation: although the current itself lasted for less than 3 sec, it took more than 10 sec for a full recovery from inactivation (subsequent stimuli applied at shorter intervals elicited smaller currents). In this respect, this current resembled the depolarization-evoked $I_{Cl(Ca)}$ in "normal" oocytes (see Section VI.B). Another aspect of similarity was the dependence on $(Ca)_{out}$: like $I_{Cl(Ca)}$, the hyperpolarization-evoked Cl^- current was abolished in Ca-free solution or by the addition of inorganic Ca^{2+} channel blockers (Mn^{2+}, Co^{2+}, and La^{3+}), as well as by intracellular injection of EGTA. Thus, it appears that it is a Ca-dependent Cl^- current; a rather surprising conclusion is that this current apparently results from a hyperpolarization-evoked **influx** of Ca^{2+}, possibly through a voltage-dependent Ca^{2+} channel, and if so, it is a channel of a kind not yet found in any other system. The authors emphasized that the long latency, the stong temperature dependence, and the fact that the current is much more pronounced during the application of transmitters which may initiate production of intracellular second messengers all indicate that the activation and regulation of this current are multistep processes. Whether the Ca-activated Cl^- channel is the same as that activated by other Ca-elevating stimuli is discussed in Section X. There are no indications whatsoever as to the biological role (if any) of this current in the oocytes.

E. Ca-Activated Channels Reconstituted in Lipid Bilayers

Young et al.[128] described nonspecific Ca-activated channels in a planar bilayer into which purified fractions of membranes of *Xenopus* oocytes or mature eggs were incorporated. These channels have an average conductance of 380 pS (in 1*M* NaCl, and 1 m*M* $CaCl_2$) and an average open lifetime of 1.5 sec at 40 mV. They are activated by low Ca^{2+} concentrations (half-maximal activation at 0.5 μ*M* Ca^{2+}) at the *trans* (presumably intracellular) side of the membrane). The channels are relatively more permeable to anions than to cations, with permselectivity ratios for Cl^-:K^+:Na^+:Li^+:Ca^{2+} of 1.00:0.71:0.42:0.40:0.31, respectively. In some aspects, these channels resemble the high-conductance anion channels found in several tissues,[129-135] although the anion selectivity in all these preparations is higher: P_K/P_{Cl} or P_{Na}/P_{Cl} is always <0.2.[130-132]

The current-voltage relationship of the bilayers containing the nonselective channels is linear between about −30 and +30 mV, but beyond this range a decrease in the conductance of the bilayer occurs (negative slope of I-V curve) which results from a decrease in the open probability of the channel. The current-voltage curve of a **single open channel** is linear over a wider range of membrane voltages (from −60 to +60 mV; the authors have not tested lower or higher potentials). Another interesting property of these channels (unfortunately not described in detail) is their tendency to form aggregates behaving as single channels.

Young et al. suggested that the channels described above are the entities responsible for the Ca-activated Cl^- currents observed in intact oocytes at rest and following depolarization of the membrane. However, the Cl^- channels underlying both $I_{Cl(Ca)}$ and the muscarinic response appear to be highly selective to Cl^- compared with cations.[64,67,100] Also, the Cl^- conductances activated by either Ca^{2+} injection or muscarinic stimulation do not display a region of negative slope beyond the −30- to +30-mV range; the I-V curve is linear between −80 and +60 mV, and a mild rectification (decrease in conductance) is observed at voltages negative to −80 mV.[35,57] It appears, therefore, that the Ca-activated nonselective channels, at least as they are expressed in the planar bilayer, differ significantly from Cl^- channels controlled by Ca^{2+} in intact oocytes. Because of their high single channel conductance and

low selectivity, they also differ from the Ca-activated nonspecific cation channels described by Colquhoun et al.[136] and by Byerly and Hagiwara.[137] One cannot disregard the possibility that the different lipid environment of the bilayer vs. the real membrane is responsible for the observed differences. In this case, however, the correspondence of any of the observed properties of the channel in the bilayer and in the real membrane would be questionable. It seems more reasonable to assume that the channels described by Young et al. coexist in oocyte membrane with the small Ca-dependent Cl channels (see Section X). Indeed, cell-attached patch recordings in immature oocytes reveal (albeit rarely) single channels with very large unitary conductance and long-lasting openings, resembling the channels seen in the bilayers;[362] the ionic selectivity of these channels was not studied yet.

The biological role of the high-conductance nonspecific channel is also unclear. Young et al.[128] have suggested that it may underlie the fertilization potential (Section VIII). However, using the patch clamp technique, Jaffe et al.[36] did not observe any channels of the type described above. To the contrary, Cl^- channels involved in fertilization potential seem to have very small unitary conductance (see Section VIII.C). However, until the identity of the Cl^- channel underlying the fertilization potential is resolved at the single-channel level, an involvement of the high-conductance nonspecific Ca-dependent channel cannot be discarded.

VII. ENDOGENOUS VOLTAGE-DEPENDENT CURRENTS AND CHANNELS

A. The Slow K Current

When $I_{Cl(Ca)}$ is inhibited by an inorganic Ca-channel blocker (e.g., Mn^{2+} or Co^{2+}), a slowly developing depolarization-activated outward current is observed in denuded *Xenopus* ooxytes.[64,90] Miledi[90] suggested that this current is the delayed rectifier found in many nerve and muscle cells. However, the voltage sensitivity of this current (appears at membrane potentials ≥ -50 mV[64]) and some other properties described in the following argue against this possibility.

The "tails" of the slow Ca-insensitive current reverse their direction at about -90 mV,[64,363] which is close to the equilibrium potential of K^+. Lotan and Dascal have performed a series of experiments intended to characterize this current better.[363] These experiments show that the reversal potential (measured by the tails) is affected by the changes in $(K)_{out}$ in a way expected for a K^+ electrode. Thus, this current is carried mostly by K ions; however, it was only partially inhibited by external or by intracellularly injected tetraethylammonium (TEA). The amplitude of this current usually does not exceed 30 to 40 nA at $+10$ to $+30$ mV; it shows no signs of inactivation for many seconds and even tens of seconds. A similar slow K^+ current has been described recently in the oocytes of *Rana esculenta;*[138] it was activated at voltages positive to -40 mV, partially reduced by 50 m*M* TEA, did not inactivate, and was carried mostly by K^+. Unfortunately, the experiments were performed while $I_{Cl(Ca)}$ was not blocked. Thus, a decision on the identity of this current and that in *Xenopus* oocytes should await further study. As to the biological role, Peres et al.[138] have suggested that this channel serves to keep the RP more negative than -40 mV: at more positive voltages, the noninactivating K^+ current will start flowing, hyperpolarizing the membrane.

B. Slow Voltage-Dependent Na^+ Channel

Studies by Baud, Kado, and colleagues have shown in the membrane of immature *Xenopus* oocytes a rather unusual property not yet found in other cell types: a prolonged depolarization causes the appearance of voltage-activatable Na^+ channels.[65,82,139] These channels are not detectable unless the membrane potential is made more positive than $+20$ mV for several seconds or even minutes. Under current clamp conditions, such a stimulus triggers a long-lasting (up to several minutes) depolarization: the membrane potential reaches $+70$ to $+90$

mV, then declines slowly to +55 to +60 mV, and finally repolarizes more rapidly to the resting value. Under voltage clamp conditions, the prolonged depolarization to ≥ +20 mV results in a slow development of an inward current that reverses its direction at about +80 mV. Upon repolarization of the membrane to a normal RP (−60 mV), the tail current decays rather abruptly, indicating a fast closure of the channels. However, if the cell is depolarized again a few seconds later, the inward current is reestablished, this time with a very fast (tenths of a second) rise time. These findings have been interpreted as indicating that a long-lasting strong depolarization "induces" the appearance of voltage-dependent Na^+ channels or facilitates the expression of normally "quiescent" channels; once induced, the channels can be activated (opened) by stepping the voltage to potentials ≥ −20 mV. The "induced" channels slowly disappear (i.e., cannot be opened by a short test depolarizing pulse) if the membrane potential is held in the repolarized state for several minutes; the half-time of the disappearance of the channels is about 4 min at 16°C. Baud and Kado[82] proposed, therefore, that the Na^+ channels exist in at least three states: closed and noninduced; closed and induced; and open. The time courses of induction and disappearance of the channels are much slower than those of opening and closure (see following). An additional state (intermediate between noninduced and closed) is suggested by the fact that, although keeping the membrane at +10 mV does not cause the appearance of the Na current, it does facilitate the induction obtained later with a more positive pulse.

The time course of induction is faster at more positive membrane potentials: the rate of induction changes e-fold for a 20-mV change in membrane potential. The induction process is temperature sensitive: the Q_{10} is 4.9, which is very high compared with 2 to 3 found usually for ion channel gating.[82] This may indicate that a complex biochemical process is involved in channel induction. However, the nature of this process is unclear. Synthesis *de novo* of the channel protein does not appear to be involved because cycloheximide does not change the rate of induction.[65] Phosphodiesterase inhibitors IBMX and theophilline, as well as elimination of external Ca^{2+}, do not have any significant effect on the induction process.[81] It should be noted, however, that the changes in external Ca^{2+} concentration tested by the authors were rather small (between 0 and 2 m*M*); any possible effects of changing intracellular Ca^{2+} concentration were not tested at all. Similarly, the doses of the phosphodiesterase inhibitors used (0.1 m*M*) are too low to produce significant changes in the cAMP content of the oocytes.[140] Thus, an involvement of one of these two second messengers (or possibly of some others) cannot be disregarded at present.

The disappearance of the channels has an even stronger temperature dependence than the induction (Q_{10} = 7.5). The rate of disappearance does not depend on the membrane potential in the range −30 to −90 mV, implying that the channel can convert into its noninduced state only from the closed (but not from the opened) state.[82]

The reversal potential of the relaxation "tails" of the depolarization-evoked current (after the channels have been "induced") changes by 52 mV per tenfold change in $(Na)_{out}$ instead of 58 mV as predicted from the Nernst equation for a perfect Na^+ electrode. Thus, the channel is rather selective for Na^+ but probably possesses a certain amount of permeability for another ion(s). An alternative is a concomitant activation of a separate, nonspecific cation channel (see Reference 82; see Section IX).

As already mentioned, once induced, the Na^+ channel opens upon depolarization with a rise time of tenths of a second (more accurate estimations have not been made). If depolarization is maintained, the current does not inactivate. Tetrodotoxin inhibits the Na^+ current but only at an extremely high concentration (1 m*M*);[65] on the other hand, it is blocked in a dose-dependent fashion by Li^+.[65,141] In all or some of these aspects, the underlying channel differs significantly from the tetrodotoxin-sensitive voltage-dependent Na channel of nerve and muscle cells[142] and from the fast tetrodotoxin-insensitive Na^+ channel in tunicate eggs.[78] Thus, it seems doubtful (to say the least) that this channel is the precursor of the voltage-

dependent Na^+ channels observed in the adult tissues. It appears, though, that slow Na^+ channels are common in amphibian oocytes. Thus, a slow voltage-activatable Na^+ current appears in axolotl (*Ambystoma*) oocytes during the progesterone-induced maturation; however, a Na channel could not be "induced" in the membrane of immature oocytes of this species.[143] Na^+- and Cl^--dependent action potentials were observed in maturing *Rana* oocytes;[144] the underlying Na^+ channels were voltage sensitive and disappeared after fertilization.[46] The properties of these Na^+ channels resemble those of the "induced" channels in *Xenopus* oocytes in some aspects (a relatively slow activation, absence of inactivation); however, the existing data do not provide the basis to decide whether the two channels are identical. To date, no depolarization-induced slow Na^+ channels were detected in oocytes of other vertebrates, even when special efforts were made to find such channels.[145]

The biological role of the depolarization-induced Na^+ channel in *Xenopus* oocytes is not clear. Meiosis-arrested immature oocytes do not appear to encounter, in normal physiological conditions, depolarizations that are strong enough to induce the channels; however, it has been suggested that they might be activated by another, unknown physiological (e.g., hormonal) stimulus.[66] In this case, these channels might serve to load the cells with Na^+: an increase in intracellular Na^+ concentration is observed in *Rana* and *Bufo* oocytes during cell growth,[58] and an abrupt increase in the amplitude of the depolarization-induced Na^+ current has been observed in *Xenopus* oocytes at the late stages (Dumont's 5 and 6) of growth.[66] A similar Na-loading role has been proposed for the spontaneous Na^+ action potentials observed during the maturation of *Rana* oocytes;[146] however, no regenerative activity of any kind was detected in maturing *Xenopus* oocytes,[139] although the slow Na^+ channels can still be induced in maturing oocytes.[82] It has been proposed that the failure to see such spontaneous activity in maturing *Xenopus* oocytes may arise from the fact that the right stage of oocyte development (post-GVBD but before extrusion from the body) has not been tested. The use of the right solution (while in the body cavity, the oocytes still sense a Ringer-like solution) is also important: the spontaneous Na^+ spikes are not observed in *Rana* oocytes kept in 10% Ringer, but do appear in normal Ringer.[46] It has been reported that, like the spontaneous Na^+ currents in *Rana* oocytes, the voltage-induced Na^+ channels in *Xenopus* oocytes disappear after fertilization and cannot be detected in ovulated eggs or in embryonic cells at early stages of development.[66] However, Peres and Mancinelli[96] have shown recently that these channels can still be induced in *Xenopus* eggs; moreover, they seem to participate in the decay phase of the activation (fertilization) potential. Since during the fertilization potential the membrane voltage stays positive for minutes, the induction of a repolarizing Na current may play a role in preventing excessive membrane depolarization and in providing a balance of inward and outward currents (see Section VIII.B). Thus, the depolarization-induced Na^+ channels in immature oocytes may be the precursors of the channels found in the eggs.

VIII. FERTILIZATION-RELATED ELECTRICAL EVENTS

A. Fertilization (Activation) Potential: Ca Dependence

As mentioned previously (Section II.C), insemination or activating stimuli like pricking, ionophore A23187, Ca^{2+}, or IP_3 injection evoke in the mature egg membrane depolarization called fertilization (for sperm) or activation (for other activating stimuli) potential. Although there are some minor differences between the fertilization and activation potentials (see following), in most aspects they are very similar.[84,86]

It has been shown, using the vibrating probe and patch clamp methods,[36,37] that a wave of fertilization current spreads over the oocyte starting at the point of sperm entry or of the artificial activation (pricking). This wave **precedes** the wave of increase in free Ca^{2+} concentration measured with Ca-sensitive electrodes immersed about 100 μm deep into the

cytoplasm; the timing of the latter roughly corresponds to that of cortical granule exocytosis.[47] It is possible that a free Ca^{2+} rise in the cortical layer precedes that in the subcortical layer (in which the tip of the Ca-sensitive electrode is placed). The latter may be the result of Ca-dependent Ca^{2+} release, possibly according to the phospholipase C-IP_3 scheme[48] (Section XI.A), where the rise in cortical Ca^{2+} concentration is the trigger; the increase in subcortical Ca^{2+} concentration is probably the direct stimulus for the breakdown of the granules.[47] This hypothesis is supported by the findings concerning two contraction layers (cortical and subcortical) and two corresponding contraction waves[27] that may reflect Ca-initiated actin-myosin interaction.[37] Additional indirect support comes from the existence of two peaks of inward current in the response of the egg[37,47] and of the immature oocyte[110] to IP_3 injection; it was proposed that Ca^{2+} is released from two different stores (see Reference 336; cf. Reference 109). Although plausible, this explanation still remains hypothetical, and no direct proof of a cortical Ca^{2+} concentration rise that **precedes** fertilization potential is available as yet. It has been proposed that the source of released Ca^{2+} is in cortical endoplasmic reticulum.[30,41]

B. Ionic Conductances that Participate in Fertilization Potential

RP of a mature ("ready for fertilization") amphibian egg is about -30 mV in a low-osmolarity solution, such as 10% Ringer,[46,85] 5 to 27% De Boer solution,[37,147] or Fl solution.[86] In fact, it may be somewhat more negative, taking into account the leak caused by electrode penetration (see Section V.A). Under these conditions, the calculated equilibrium potential for Cl^- is about $+20$ mV, and for K^+ about -160mV;[46] therefore, opening of Cl^- channels will be expected to produce depolarization. Interestingly, the equilibrium potential of Na^+ may be **negative** (assuming an intracellular Na^+ concentration of about 20 m*M*, see Reference 146). Therefore, Na^+ current at the peak of the fertilization potential will be outward, causing repolarization.

Application of sperm causes, after a 30- to 60-sec delay, a positive-going shift in membrane potential that reaches maximum in about 0.5 min (data from Webb and Nuccitelli;[86] *Xenopus*). Maximal positive level that the membrane reaches during the fertilization potential depends on the species of the frog and on experimental conditions; recently, it was reported that the size and shape of the fertilization potential may also depend on the species of the fertilizing sperm.[148] Values of $+3$ mV in *Xenopus* eggs[86] and $+5$ to $+13$ mV in *Rana*[46,85] have been reported. The amplitude of activation potential induced by the ionophore A23187 is the same[46] or somewhat less positive (maximal level 0 mV in *Xenopus* eggs; see Reference 86), and the rise time is slower.[85,86] On the other hand, potentials as positive as 40 mV were observed in prick-activated *Xenopus* eggs.[96] It is not clear whether these differences can be entirely accounted for by the differences in experimental conditions (such as somewhat different solutions, more or less sharp electrodes, etc.) or whether other factors like types of frogs, their ages, etc. also may play a role. In *Xenopus* eggs, a difference was noticed in the shape of the rising phase of the two types of potentials: A23187-induced activation potential rises smoothly, while the sperm-evoked fertilization potential starts with several positive-going fluctuations before it reaches a steady state, during which the membrane potential remains stable for minutes.[86] The duration of the fertilization potential is about 15 min. It is accompanied by a 20-(*Rana*[43,46]) to 200-(*Xenopus*[86]) fold conductance increase; the conductance reaches a maximum of 13 to 40 μS.[46,86]

It has been known since Maeno's study in 1959[88] that the current underlying fertilization (activation) potential in amphibian eggs is carried mostly by Cl^- (*Bufo*). Neither Ca^{2+} [86] nor Na^+ [37,84,86,88] appear to carry the inward current. During fertilization potential, the permeability of the egg membrane for halides increases; order of permeability is $F < Cl < Br < I$.[43,86,147] This correlates with the hydrated size of these anions, suggesting that the anion interacts with the selectivity filter of the channel while hydrated.[86]

The amplitude (maximal positive value) of the fertilization potential changes by 38 to 48 mV/decade change in the extracellular Cl^- concentration,[84,86] instead of 58 mV/decade as expected from the Nernst equation for a pure Cl^- current. The calculated Cl^- equilibrium potential in *Xenopus* eggs is more positive than the peak fertilization potential.[46,86] These facts imply that, in addition to Cl^-, another ionic conductance is activated during fertilization potential. Indeed, a hyperpolarization usually follows and sometimes precedes the dominant depolarizing change in membrane potential (Talevi et al.:[149] *Discoglossus;* Iwao:[148] *Xenopus*). Using the vibrating probe method, Kline and Nuccitelli[37] (*Xenopus*) found that an outward current precedes and follows the inward current carried by Cl^-. A similar result was obtained by Jaffe et al.[36] (*Rana*) with the patch clamp technique. (It should be emphasized that the last two methods provide measurements of currents flowing through restricted areas of the membrane, while the "classical" measurements, made with intracellular electrodes, give information about the currents flowing through the whole cell.) Jaffe and colleagues have shown that the "other" conductance activated by fertilization is a K^+ one.[36,46] Potassium ions flow through a separate channel because the hyperpolarizing component of the fertilization potential can be selectively inhibited by 10 m*M* TEA applied extracellularly; under TEA, the reversal potential of the fertilization current approaches the Cl^- equilibrium potential.

Another outward current has been observed by Peres and Mancinelli[96] in the eggs before, during, and several minutes after the activation potential. This current displays most of the features of the voltage-induced Na^+ current described earlier in the immature oocytes: it appears only when the membrane is polarized to voltages $\geq +20$ mV; it develops slowly; its amplitude and reversal potential are dependent on extracellular Na^+ concentration. The Na^+ current appears to be enhanced during activation and fades more slowly than Cl^- current. Peak amplitude of the activation potential is sensitive to external Na^+ (a change of about 10 mV/decade), confirming the idea that there is a Na^+ component in the current underlying the activation potential, which may participate in the decay of the latter.

Thus, at least three separate ionic conductances are activated in the egg membrane by insemination or activation: a K^+ conductance, a Na^+ conductance, and an anionic conductance. Since in normal physiological conditions the only anion expected to participate in this conductance is Cl^-, we shall refer to the latter as the Cl^- conductance or the Cl^- channel. Since fertilization potential is believed to be Ca dependent, this channel should probably be termed "a Ca-dependent Cl^- channel". Its exact relation to the Ca-dependent Cl^- channels activated in the immature oocytes by voltage, Ca-injection, and other means is not clear; the various possibilities will be discussed in Section X. The Cl^- conductance activated by fertilization displays an outward rectification at positive voltages.[46,96] In *Rana*, it was not affected by either SITS or DIDS,[46] while in *Xenopus*, the same concentration (1 m*M*) of both agents was reported to block the fertilization potential and the Cl^- current almost completely.[37,86] Peres and Mancinelli[96] reported a lack of effect of SITS and another Cl^- channel blocker, 9-anthracenecarboxylic acid (9-AC), on activation potential in *Xenopus* eggs; unfortunately, the authors did not mention the concentrations of the substances.

The fertilization current precedes cortical granule exocytosis.[46,93] Treatment of the oocytes with CO_2 eliminates cortical granule exocytosis but not activation potential.[93] At early stages of the development of the oocyte, the ionophore A23187 induces cortical granules breakdown, but fails to elicit fertilization potential.[38] These findings suggest that the "appearance" of Cl^- channels is not due to the addition of new membrane derived from the cortical granules.

The amplitude of activation current measured with a patch pipette[36] or vibrating probe[37] was much smaller in the vegetal than in the animal hemisphere. In this respect, the Cl^- channels of the activation current resemble the Ca-dependent Cl^- channels in the immature oocytes (see Section VI.A). Patch pipette measurements have shown that the opening of the

outward current channels during the activation also appeared to propagate as a wave, but there was no major difference in the density of the outward currents in animal and vegetal hemispheres. At the plateau of the activation potential, inward and outward currents are equal (the potential does not reach the Cl^- equilibrium potential; yet it remains stable for minutes, see References 36 and 46). However, the net current that was recorded by a patch pipette was usually outward.[36] Jaffe et al.[36] interpreted these findings as suggesting that the procedure of patch clamping may introduce artifacts, e.g., opening of stretch-activated channels (cf. Section IX). On the other hand, in the vibrating probe recordings done by Kline and Nuccitelli, the inward current was dominant. These recordings also demonstrated that the outward current appears in the vegetal hemisphere in less than 1 sec after the rise of the activation potential in the animal hemisphere (evoked by IP_3 injection with a sharp electrode, to minimize the distorting effects of the leak). This is a much faster spread than would be possible for the wave of the inward current, which may mean that the K^+ channels of the activation potential are spread over a large area of the egg.[37] More importantly, these facts may indicate that the outward current, or part of it, may be activated by depolarization. Because the egg is equipotential,[67,87] the depolarization produced by the efflux of Cl^- in the animal hemisphere will activate any voltage-operated channels anywhere in the membrane. It should be emphasized, however, that at least a part of the K^+ conductance is probably activated by Ca^{2+} rather than by voltage since the TEA-sensitive conductance increases during the activation of the egg under voltage clamp, when the membrane potential is kept constant.[46] TEA reduced the membrane conductance that developed during the activation only if the membrane was kept at potentials more negative than -5 mV; the effect of TEA increased with increasing depolarization.[46] Taken together, these facts suggest a dual regulation of K^+ channels involved in the fertilization potential by voltage and by Ca^{2+}.

C. Single-Channel Recordings

Jaffe et al.[36] performed patch clamp studies on *Rana* eggs. In the study, Cl^- channels underlying the fertilization potential were not detected at the single-channel level. Even with a patch pipette 1 μm in diameter (small enough to record single K^+ channels), the Cl^- current component was still seen, but no single-channel activity could be resolved. In this respect, too, Cl^- channels involved in fertilization potential resemble the Ca-activated Cl^- channels in immature *Xenopus* oocytes (see Section X); in both cases, it seems probable that the conductance of the channel is very small and that the concentration of the channels in the membranes is relatively high.

Two types of single outward current channels were recorded at the time of activation. A smaller channel was not characterized, and it is not known what ions carry the current through this channel. The calculated reversal potential of the larger channel was -150 mV, suggesting that it was a K^+ channel (the calculated K^+ equilibrium potential was -161 mV). It had a single channel conductance of 25 pS and an average open time of 25 msec. The probability of opening this channel increased with depolarization. Although preliminary, this is the first characterization of an endogenous single-channel activity in this preparation. It remains to be seen whether such channels are observed in immature oocytes, and what their relationship is to the K^+ channels described in other preparations.

D. Biological Role

Numerous observations suggest that fertilization potential in amphibian eggs serves to prevent polyspermy (fast polyspermy block) until the fertilization membrane (the mechanical polyspermy block) is raised. The existence of an electrical polyspermy block was first demonstrated in sea urchin eggs and later in starfish[151] and in other species. In amphibians of the order Anura, this role is supported by the following findings.

In the eggs of frogs and toads, fertilization potential is ubiquitous. Frog eggs which did not complete maturation do not develop fast polyspermy block and do not respond to sperm by a fertilization potential; rather, they produce multiple brief depolarizations that correlate with multiple sperm penetrations.[85] Prevention of the fertilization potential by various means renders the eggs polyspermic.[43,45,147] Clamping the membrane of a mature egg at positive potentials prevents fertilization.[44,45] As already mentioned, the naturally polyspermic eggs of the Urodeles (salamanders) do not display fertilization potential; fertilization of salamander eggs does not depend on membrane potential.[45] Moreover, voltage clamping of *Xenopus* eggs at positive potentials does not prevent their fertilization with salamander (*Notophtalmus*) sperm, suggesting that the voltage dependency of polyspermy block is defined by the species of the sperm.[44]

E. Conclusion

Although the biological role of fertilization potential as the fast polyspermy block is well established, the electrical events underlying this potential are not sufficiently characterized yet. Thus, on the single-channel level, the Cl^- and Na^+ channels have not been resolved, the K^+ channels were only preliminarily characterized, and the properties of yet another outward current channel have not been characterized. The relative roles of Na^+ and K^+ channels in the determination of the properties of the fertilization potential are not clear. A very interesting question is how the different channels participating in the fertilization potential are distributed over the surface of the egg, and what are the regulatory and molecular mechanisms underlying their distribution (which, at least in the case of Cl^- channels, appears to be very asymmetric). Molecular mechanisms of activation and regulation of the channels mentioned above also await further study.

IX. OTHER IONIC CONDUCTANCES

In this short section, the ionic currents that were only preliminarily characterized are described. It is possible that a thorough characterization of these currents will allow identification of the underlying conductances with those already described.

In immature *Xenopus* oocytes, a very slowly developing nonsaturating outward current is activated at positive membrane voltages; it apparently involves a nonselective cation channel.[82] This current has not been further characterized.

Miledi[90] observed an inward current activated at very negative (≤ -120 mV) voltages; he assumed that it was the inward (or "anomalous") rectifier, i.e., a hyperpolarization-activated K^+ current similar to that found earlier in eggs of various other species.[78] On the other hand, Baud et al.[65] claimed that there was no sign of inward rectification in *Xenopus* oocytes. We did observe a current similar to that reported by Miledi, but its reversal potential was close to 0 in V, which implied that it might be a nonspecific cation current.[374]

In maturing *Rana* oocytes, a voltage-activated Cl^- current underlies the repolarization phase of the spontaneous action potentials. This Cl^- conductance disappeared as the oocytes underwent maturation, and it was not observed in eggs.[144,146] It is not known whether the opening of this chloride channel is mediated by (1) the entry of Ca^{2+} or (2) voltage.

In their recent paper, Methfessel et al.[26] describe a stretch-activated channel that can be detected using the patch clamp technique. The channels are activated by negative or positive pressure, or appear spontaneously, in almost all cell-attached or inside-out patches, but less frequently in outside-out ones. Two classes of such channels can be distinguished, with single-channel conductances of 18 and 25 pS. The conductance does not depend on membrane potential in the range tested (-100 to $+30$ mV). Both seem to be nonspecific cation channels.

X. Ca-DEPENDENT Cl CHANNELS IN THE OOCYTE: A GENERAL DISCUSSION

There are several ways to elicit a Cl^- current in the membrane of an amphibian oocyte or egg:

1. Depolarization that allows the influx of Ca^{2+} through voltage-dependent channels (Section VI.B)
2. Hyperpolarization of maturing oocytes, of oocytes injected with brain mRNA during and following exposure to serotonin and other transmitters; influx of Ca^{2+} appears to be involved in this case, too (Sections VI.D and XV.E)
3. Application of a muscarinic agonist that evokes a two-component Cl^- current due to Ca^{2+} release from intracellular stores (Section XII)
4. Direct intracellular injection of Ca^{2+}, IP_3, or extracellular application of the Ca ionophore A23187 (Section VI.A)
5. Fertilization or activation of an egg (Section VIII.B)

If this is not enough, a "standing" asymmetrical Cl^- current is recorded at rest (Section VI.B), and a Ca-dependent nonspecific channel, preferentially permeable to Cl^-, is reconstituted in bilayers into which the membrane fraction of the oocyte has been incorporated (Section VI.E). A question naturally arises: how many Ca-dependent Cl^- channels are there in the membrane of the oocytes? Obviously, there are fewer such channels than items in the above list; on the other hand, it will probably be an oversimplification to presume that all the aforementioned currents are the result of opening of the same Cl^- channel. For instance, it appears that the channel reconstituted in the planar bilayer differs in its conductance from the Cl^- channel activated during fertilization, and from $I_{Cl(Ca)}$ in its ionic selectivity (Section VI.E).

We now summarize the similarities among the various Ca-dependent Cl^- currents:

1. The Cl^- current flowing through the oocytes at rest and those evoked by injection of Ca^{2+}, by fertilization, or by iontophoresis of ACh are all asymmetrically distributed in the membrane, with a significantly higher density in the animal hemisphere (Sections VI.A and B and VIII.B).
2. As already mentioned, the Cl^- channel activated in the course of fertilization apparently has a very low single-channel conductance (Section VIII.C). Similarly, an inward current (but not single-channel activity) was recorded in immature oocytes with relatively small (about 2 μm diameter) patch pipettes in the cell-attached configuration when Ca^{2+} was injected intracellularly.[367] Thus, in this case, too, the channel appears to have a very low unitary conductance.
3. A slight elevation of $a(Ca)_{in}$, using low concentrations of A12187 and Ca^{2+} in the bathing medium, significantly reduced both (1) fast and long-lasting components of the response to ACh application and to IP_3 injection and (2) the Cl^- current appearing in response to elevation of $(Ca)_{out}$ itself.[360] Thus, apparently, there is a process of Ca-dependent inactivation of the Cl^- channel and it is common to all responses mentioned here. Such desensitization may explain the relatively long-lasting inactivation of the Ca-dependent Cl^- currents elicited either by depolarizing or hyperpolarizing stimuli (Sections VI.B and D): if one assumes that the increase in $a(Ca)_{in}$ caused by the influx of Ca^{2+} through the voltage-dependent Ca^{2+} channel is prolonged, then Ca-dependent inactivation of the Cl^- channel will last at least as long as the elevation in $a(Ca)_{in}$ does. It seems, then, that all Cl^- currents mentioned in this paragraph share a common feature: a Ca-dependent inactivation.

There is a disagreement among different authors as to the ability of SITS and DIDS to inhibit the Cl^- channel involved in fertilization potential. However, species variability or the use of suboptimal concentrations may have caused these discrepancies (Section VIII.B). The effect of SITS and DIDS on Cl^- currents evoked by other means should be tested. We have found that 9-AC blocks both depolarization- and A12187-induced Cl^- currents;[357,360] the effect of this blocker on Cl^- currents evoked by other treatments awaits testing.

A final answer to the question of "how many Ca-dependent Cl^- channels are there in the oocyte membrane" cannot be obtained at present; we need more data about the single-channel characteristics of the different currents and about their pharmacology. On the basis of evidence we have at present, it seems that one channel may be the carrier of most currents mentioned previously, and this channel is not the one found in the bilayer.

The molecular mechanism that underlies the activation of Cl^- by Ca^{2+} is unknown; the list of possibilities includes Ca-dependent phosphorylation (with or without the involvement of calmodulin),[152,153] a direct effect of Ca^{2+} on Cl^- channel, or triggering by Ca ions of a yet unknown chain of intracellular events.

Better characterization of the channel is necessary and not only for the understanding of the processes taking place in the oocytes. Ca-dependent Cl^- currents have been found in secretory cells,[154] photoreceptors,[155] and neurones.[156-158] It would be of interest to see whether these channels are similar to those in the oocytes, and if this is so, the oocytes may be used as a model system for the study of molecular mechanisms of activation and regulation of these channels.

XI. SECOND MESSENGER-MEDIATED NEUROTRANSMITTER RESPONSES AND THE SECOND MESSENGER SYSTEMS OF THE OOCYTE

Since the discovery of muscarinic, β-adrenergic,[9,57] and later purinergic[68] responses in the oocyte, mechanisms of these responses have been extensively studied. It is now clear that at least two distinct second messenger systems, the IP_3-Ca^{2+} and the cAMP systems, are involved in mediation of the membrane events evoked by these transmitters. Both systems have been hypothesized, by different groups, to be involved in yet another process of crucial importance: the reinitiation of meiosis. Due to the studies of the latter process, a large body of knowledge on the adenylate cyclase-cAMP system of the oocyte and on oocyte biochemistry in general has accumulated. This knowledge, taken together with the other advantageous features of the oocytes such as the large size and the ease with which voltage clamp and intracellular injection of regulatory substances are done, contributed to recent progress in understanding the molecular mechanisms involved in mediation of the transmitter responses in these cells. Moreover, since the introduction of methods for expressing foreign transmitter receptors and ion channels in oocyte membranes, it appears that the oocyte may become a preparation of choice as a model system for the study of neurotransmitter-regulated ion channels and of signal transduction in general.

The following sections deal with the endogenous neurotransmitter responses and their mediation by second messengers. In order to facilitate the understanding of this subject and to introduce the terminology, we provide in the following a very short summary of the molecular basis of signal transduction. Then we briefly summarize the (presumptive) involvement of second messengers and the related regulatory proteins in the triggering of meiotic reinitiation in amphibian oocytes.

A. Second Messengers, G-Proteins, and Neurotransmitter Responses and Ion Channels

The best-characterized second messengers regulated by neurotransmitters and hormones are cyclic adenosine monophosphate (cAMP), IP_3, Ca^{2+}, diacylglycerol, and possibly cyclic guanosine monophosphate (cGMP). Several transmitters and hormones (such as norepi-

nephrine through β-adrenergic receptors, adenosine through P1 receptors, dopamine through D1 receptors, VIP, etc.) elevate the cAMP level by activating the enzyme adenylate cyclase that converts ATP to cAMP (see References 159 to 164). Several neurotransmitters and neuromodulators inhibit adenylate cyclase and suppress cAMP accumulation. Among these are ACh acting through muscarinic receptors, norepinephrine acting through α_2-adrenergic receptors, dopamine at D2 receptors, opiate agonists, etc.[165,166] Regulation of adenylate cyclase activity by both stimulatory and inhibitory agents involves guanine nucleotide-binding proteins (G-proteins).[167] In the absence of an external signal (inactive state of the G-protein), the guanine nucleotide moiety bound to the G-protein is GDP. Binding of an agonist (A) to the receptor (R) is followed by the binding of the AR complex to a G-protein (G_s in the case of stimulatory agents, G_i in the case of inhibitory agents). This allows the displacement of GDP by GTP, transforming the G-protein into its active form. G_s may then **activate** the catalytic subunit of adenylate cyclase, C, thus elevating cAMP level. Activated (GTP-bound) G_i **inhibits** C; it is not clear whether this is a direct or an indirect interaction. Activation of G is terminated by the hydrolysis of GTP by the GTPase activity of the G-protein itself, leaving GDP bound to the G-protein.

The signal-transducing G-proteins (G_s; G_i; transducing, the G-protein involved in visual transduction; G_0, a G-protein with an unknown function) consist of three subunits: α, β, γ. The α-subunits of the various G-proteins are different, while at least the β-subunit is highly preserved and is interchangeable among the various G-proteins, even from evolutionarily remote sources. The α-subunit is the "active" one, whereas the β-subunit is the inhibitory one; dissociation of α from β is the trigger for the activation of α. Thus, excess of free β-subunits is expected to cause inhibition of adenylate cyclase; activation of G_i by a neurotransmitter like ACh will lead to the release of free β-subunits, reduction of the amount of free α-subunits of G_s, and finally, to a reduction in adenylate cyclase activity in an "indirect" way.[168,169] The activation of G_i by the adenylate cyclase-inhibiting (e.g., muscarinic) agents is blocked by pertussis toxin (PTX), which ADP-ribosylates G_i and apparently prevents the dissociation of β-subunit from the α-subunit. Cholera toxin causes ADP-ribosylation of G_s, leading to irreversible dissociation of α- and β-subunits and, therefore, to permanent activation of adenylate cyclase.[170-172]

In all biological systems studied, cAMP produces its final effect through the activation of a cAMP-dependent protein kinase (A-kinase) by promoting the dissociation of its catalytic subunit from the regulatory one.[160,173] A-kinase phosphorylates the target protein(s); in a few known cases of cAMP-dependent regulation of ion channels, the target protein is the channel itself or a closely associated protein.[174,175]

Another pathway by which certain transmitters may reduce cAMP level is the activation of a cyclic nucleotide phosphodiesterase (PDE). Thus, muscarinic agonists activate PDEs; the effect is secondary to the increase in intracellular Ca concentration[176,177] or in cGMP levels.[178]

The role of cAMP in mediation of neurotransmitter regulation of various ion channels is well established. Outstanding examples include: (1) down-regulation of a molluscan K^+ channel by serotonin, mediated through cAMP-dependent phosphorylation (see References 179 and 180); (2) positive regulation of a K^+ channel (possibly the inward rectifier) in Aplysia R15 neurone, subserved by a similar mechanism (see References 181 and 182 and references cited therein); (3) enhancement by norepinephrine of the voltage-dependent Ca^{2+} current in the heart, mediated by cAMP, and the inhibition of this effect by ACh, apparently due to suppression of adenylate cyclase activation[183,185] and activation of cGMP-dependent PDE.[178]

Many of the agents (including muscarinic) that inhibit adenylate cyclase elevate cGMP levels. This effect is not due to a direct activation of guanylate cyclase; rather, this enzyme seems to be activated by the products of other metabolic reactions initiated by the agonists,

e.g., Ca^{2+}, products of metabolism of arachidonic acid,[186-188] or by protein kinase C.[189] The role of cGMP in the mediation of neurotransmitter effects on ion transport is controversial;[190] recent reports suggest involvement of cGMP in regulation of Ca^{2+} channels.

Many hormones and transmitters regulate (mostly elevate) the intracellular level of another important second messenger, Ca^{2+}. The list of these agents includes many of those already mentioned as adenylate cyclase inhibitors, such as ACh, serotonin, dopamine, and many new names as well.[192,193] This increase in $a(Ca)_{in}$ may be a result of either entry of Ca^{2+} from the external solution through some receptor-operated channels or a mobilization of Ca^{2+} from intracellular stores. The molecular mechanism of the latter pathway has recently begun to be understood.[193-195] In brief, the binding of an agonist to the receptor promotes the activation of an enzyme called phospholipase C by the AR complex. A G-protein is involved in this process, too.[196-201] Activated phospholipase C catalyzes the breakdown of membranal phosphatidylinositol phosphates, preferentially of the phosphatidylinositol 4,5-bisphosphate, with the formation of diacylglycerol and IP_3. The latter diffuses into the cytosol and releases the sequestered Ca^{2+} from intracellular stores, mostly from the endoplasmic reticulum. Diacylglycerol diffuses within the plane of the plasma membrane; in the presence of Ca^{2+} and certain membrane phospholipids, diacylglycerol activates a specific protein kinase (C-kinase). C-kinase may also be activated artificially by β-phorbol esters, known as potent tumor promoters,[194] even in the absence of Ca^{2+}. Although the role of C-kinase is not yet sufficiently explored, some evidence points to the possible involvement of this enzyme in termination of the agonist-induced increase in $a(Ca)_{in}$ (i.e., negative feedback; see References 203 to 205); regulation of Na/H exchange.[206,207] It may also play a role in the regulation of some ion channels: in various neuronal preparations, C-kinase or its activators inhibit the transient K current[208] and the Ca-dependent K current[208,209] and either potentiate[208,210] or inhibit[211,212] the voltage-dependent Ca^{2+} current. Activation of C-kinase may also down-regulate the cell-to-cell channels.[289]

Recently, in addition to the regulation of channels by G-proteins through the formation of the second messengers, a direct coupling of a G-protein to an ion channel has been proposed; the muscarinic stimulation of a K^+ channel in the heart turned out to depend on activation of a PTX-sensitive G-protein,[213,215] possibly through the βγ-subunits.[216]

B. Ca, cAMP, G-Proteins, and Maturation of the Oocyte

A very short (several minutes) exposure to progesterone is sufficient to initiate maturation of frog oocytes.[4] This striking phenomenon led to the concept of maturation triggering, i.e., eliciting by progesterone of a fast biochemical change that would give rise to the long and complicated chain of reactions that constitute maturation. It has been demonstrated by various means that, in contrast with other cases of steroid action, the receptor for progesterone is not cytoplasmatic but membranal (reviewed in References 4, 5, and 113). It seemed plausible that the mechanism of the maturation induction by progestrone might be similar to the action of other hormones and transmitters that activate intracellular processes via interaction with a membrane-bound receptor, possibly through the production of a second messenger.

Two second messengers have been proposed as candidates for the role of maturation triggers: Ca^{2+} and cAMP. The involvement of an elevated level of intracellular free Ca^{2+} was suggested by:

1. The ability of various "membrane-stabilizing" agents to cause maturation. Most of these agents were believed to cause perturbation or release of membrane-bound Ca^{2+}.[217] It has been reported that the initiation of maturation by progesterone is inhibited if intracellular Ca^{2+} stores are depleted.[218]
2. Increase in Ca^{2+} efflux from the oocytes shortly after the exposure to progesterone, and the (apparent) increase in free intracellular Ca^{2+} at various times during maturation (see Section V.E).

3. The apparent ability of calmodulin to induce maturation[219,220] and of intracellularly injected calmodulin inhibitors to suppress it.[220,221]

The Ca-hypothesis of maturation triggering has been seriously challenged in the last few years. As discussed in Section V.E, the data on progesterone-induced $a(Ca)_{in}$ increase are controversial; although it is not clear yet whether there might be an increase in free Ca^{2+} localized to the submembrane layer of the cytoplasm, there is almost no doubt that the bulk $a(Ca)_{in}$ remains unchanged.[74] In the studies in which the effects of the maturation-inducing "membrane-stabilizing" agents were reported, their specificity with respect to their ability to displace membrane-bound Ca^{2+} has never been really tested, and various other nonspecific effects (known and unknown) have not been taken into account. The effects of calmodulin and calmodulin inhibitors turned out to be erratic or irreproducible.[220,222,223] Calmodulin and A23187 have been found to decrease cAMP levels in the oocytes, suggesting an involvement of the latter pathway.[223] Indeed, a Ca, calmodulin-dependent cyclic nucleotide PDE is found in the oocytes.[224,225] Thus, although the role of Ca^{2+} in the triggering of maturation cannot be rejected at present, the case appears to be weak; if Ca^{2+} is involved in the triggering of maturation, it certainly is not the sole inducer.

The involvement of a cAMP system appears to be better substantiated.[4,5,113] In the early works, the investigators attempted to mimic the progesterone effect by cAMP and its analogs but obtained negative results.[226] In 1976, O'Connor and Smith[140] showed that the PDE inhibitor theophylline suppressed maturation, although no significant progesterone-induced changes in cAMP levels have been found by these and other investigators.[227] However, in many later works, a small (10 to 40%) decrease in basal cAMP level during the maturation has been demonstrated.[228-232] Progesterone also reduced cAMP levels that were previously elevated by cholera toxin or forskolin.[231,233] The involvement of cAMP-dependent protein kinase in the regulation of maturation is strongly supported by the classical experiments of Maller and Krebs,[234] who demonstrated activation of maturation by the regulatory subunit of A-kinase and by A-kinase inhibitor and inhibition of maturation by the catalytic subunit of A-kinase. Later, the presence of a progesterone-inhibited adenylate cyclase in the oocyte membrane was demonstrated.[230,235,236] It has been proposed that a high basal level of cAMP in the oocytes serves to maintain a constant high level of phosphorylation of a hypothetical protein (Mp-P), and its dephosphorylation (due to a progesterone-induced drop in cAMP level) is the signal for maturation.[237]

The membrane of the oocytes contains substrates for ADP-ribosylation by cholera toxin (presumably G_s) and by PTX (presumably G_i).[238-240] A G-protein appears to be involved in the inhibition of adenylate cyclase by progesterone;[236] surprisingly, it is not the PTX substrate (G_i), but probably another as yet unknown G-protein.[238,239] Indeed, ACh inhibits adenylate cyclase in the oocytes, and this effect is suppressed by PTX, as it would be expected for a G_i-mediated effect;[238] however, the maturation-initiating action of progesterone is **enhanced** by PTX, again indicating that its effect is not mediated through the PTX substrate.[238,240] The involvement of a G-protein in the initiation of maturation is further supported by the finding of Birchmeier et al.[241] that microinjection into *Xenopus* oocytes of human ras proteins (GTP-binding proteins known as potent oncogens) induces maturation **with no change in cAMP levels**. Moreover, the mutant $ras^{val\ 12}$ (in which glycine in position 12 of the wild type is replaced by valine), which is a much more potent oncogen than the wild-type protein and has reduced GTPase activity,[242-244] is also a more potent maturation inducer. These findings raise the possibility of involvement of a ras-like G-protein in mediation of a progesterone effect by a pathway alternative to the cAMP-dependent one.[241]

XII. THE ENDOGENOUS MUSCARINIC RESPONSE

A. Pattern and General Characteristics of the Response

In 1977, Kusano et al.[9] reported the discovery of a cholinergic response in *Xenopus* oocytes: ACh evoked a depolarization sometimes preceded or followed by a hyperpolarization. The response was usually accompanied by large depolarizing fluctuations of membrane potential. It was blocked by muscarinic but not by nicotinic receptor antagonists. The response to ACh was apparently confined to the oocyte membrane itself because it was still observed in oocytes defolliculated by collagenase, although it was noticed that in some cases defolliculation reduced or abolished the muscarinic response. Using the voltage clamp technique, the authors demonstrated that the depolarizing (inward) current was accompanied by an increase in membrane conductance and that it was not eliminated upon removal of external Ca^{2+}, Na^{+}, or Cl^{-}. In the latter case, the amplitude of the response was even increased. The authors proposed, therefore, that the inward current elicited by ACh is carried mostly by Cl^{-}. Following iontophoretic extracellular application of ACh, the depolarizing response appeared after a substantial (seconds) delay, interpreted by the authors as a sign of either high cooperativity in binding or of involvement of a second messenger in mediation of the response.

Further characterization and study of the mechanism of the muscarinic response in the oocytes was done by the same authors and by a Tel Aviv University group (N. Dascal, E. M. Landau, and Y. Lass, joined later by B. Gillo, Y. Oron, and others). Dascal and Landau[69,100] demonstrated that, with bath application of ACh, as much as four components of muscarinic response in follicle-enclosed oocytes can be detected when the membrane is voltage clamped at a potential close to the resting value, i.e., −50 mV:

1. A transient inward current that has a delay of 1 to 2 sec following iontophoretic application of ACh, a rise time of 1 to 4 sec, a plateau of 1 to 4 sec, and a decay that might last for about 30 sec. This component was termed D1.
2. Next, a slowly developing inward current appears. Its beginning is usually masked by the fast component; however, in oocytes of many frogs, especially during the winter months, the fast component is absent, and it can be seen that the delay of this current (termed D2) is long lasting and highly variable (2 to 3 sec and up to 2 min!) (cf. References 9 and 57). Typically, in the constant presence of ACh, this current decays slowly, but in many cases this decay may be the result of the (slower) development of the concomitant outward current (see below). In some instances, the slow inward current lasted for 30 min with no appreciable inactivation. It usually outlasted ACh application for a significantly longer time than the "dead time" of the perfusion system.
3. The slow response component is usually accompanied by large inward current fluctuations (the F-component) that have irregular delays and amplitudes. In some oocytes, such fluctuations were present spontaneously, and exposure to ACh increased their amplitude and frequency.[9,69]
4. Finally, as already mentioned, an outward current can sometimes be seen, especially soon after the beginning of ACh washout (as if the slow inward current ceased faster than the outward one[67]). The outward current component is more sensitive to collagenase treatment than the inward currents. While with some batches of collagenase the outward current response "survives" defolliculation,[67] with most batches it appeared to be more vulnerable.[249] In some cases, ACh responsiveness disappeared completely after defolliculation but reappeared a day or two later;[245] in these cases, however, the outward current did not reappear.[249] Thus, while there is no doubt that the receptors and the intracellular machinery that underlie the inward current responses to ACh reside in the oocyte, a similar conclusion cannot be drawn unequivocally with respect to the outward current (cf. the discussion in Section XIII.F).

In addition to the differences in shape and time course, the separation of the muscarinic response into four distinct components is justified by the following findings:

1. Each component may appear independently and separately or in different combinations with the others in oocytes of different frogs.[69]
2. The various components can be inhibited or separated by different treatments. Thus, the fluctuations do not appear in low-osmolarity solutions; the outward current (and the fluctuations) is inhibited by the intracellular injection of TEA.[67] The slow and the fast inward currents display different sensitivity to changes in extracellular and intracellular Ca^{2+} concentration[67,109] (see Section XII.D) and are differently affected by progesterone at short exposure times.[106]

Kusano et al.[57] found that iontophoretic application of ACh evokes larger inward currents in the animal than in the vegetal hemisphere, although the response can be elicited in both. This may be the consequence of the nonuniform distribution of the Ca-dependent Cl^- channels (Sections VI.A and X), but a possibility of a similar distribution of the muscarinic receptors cannot be excluded. The distribution of the outward current response was not studied.

Dascal and Landau[69] found that during the winter (from about October to March) oocytes of most frogs did not exhibit the fast inward current, but the slow and the fluctuational components were preserved (albeit reduced as compared with the summer). In this work, the frogs were kept at a constant temperature throughout the year, but the dark-light cycle was not controlled. It was noticed later that this seasonal variation of the response pattern may be reduced (but not eliminated) if the animals are kept on a constant dark-light cycle;[67] the pattern of the response also varies with the batch of frogs, the source, etc. Yet, the reduction or disappearance of the fast component and the diminution of the other components during the winter months seems to be common to all batches. The reason for such behavior is unknown. It seems to be a manifestation of some kind of biological clock, and it is tempting to speculate that it may be under hormonal control: seasonal fluctuations of levels of various hormones indeed take place in these frogs.[15] Interestingly, the morphology of several endoplasmic reticulum-related structures (such as the connections between the plasma membrane and the endoplasmic reticulum) are also under hormonal control.[21,23] These structures have been implicated in the control of intracellular Ca^{2+}.[30,41] However, the only direct hormonal effect reported thus far was a disappearance of the muscarinic response during progesterone-induced maturation.[57,106] The fast inward current is more vulnerable than the slow one and starts to decrease earlier following the exposure to progesterone; eventually, at about GVBD, the oocytes lose all ACh responsiveness.[106] Such extreme regulation is undoubtedly a result of the profound changes that these cells undergo during maturation (see Sections II.B and V.E) and provides little information about the seasonal variation described previously. The role for progesterone as the natural regulator of ACh responsiveness cannot be rejected, though, because the effects of subthreshold (with respect to maturation induction) doses were not tested.

A response to ACh is followed by a period of refractoriness, or "desensitization", during which a subsequent application of ACh evokes a smaller response.[9,69,100] The duration of the desensitization period appears to depend on the dose of ACh and the duration of its application and possibly on the amplitude of the response;[67,69,100] the dependence of refractoriness on each of these factors has not been quantified. In most follicle-enclosed oocytes, the slow inward current component of the response cannot be evoked repetitively even if the intervals between the applications of ACh are very long, i.e., on the order of 1 hr.[67] The reason for such an irreversible diminution is not clear; in oocytes of some frogs this component is stable and can be evoked repetitively many times. The outward current com-

ponent is usually more stable; the most reproducible one is the fast inward current (D1) which can be elicited repetitively for hours, provided that ACh is applied at appropriate intervals. The molecular basis of desensitization is not known. In part, it probably arises from the Ca-dependent inactivation of the Cl^- channel (see Section X). However, the period of refractoriness that follows the response to ACh is longer than that following a response to elevated Ca^{2+} concentration in the presence of A23187.[374] Thus, a mechanism such as agonist-induced desensitization of the receptor or of another participant of the biochemical cascade initiated by the agonist must be considered.[246]

B. Ionic Mechanism

In their pioneering work, Kusano et al.[9] suggested that the main carrier of the current during the slow component of the response to ACh is Cl^-. This conclusion was, in general, supported by later studies. Dascal and Landau[100] demonstrated that the reversal potential of the fast (transient) component of the ACh response depends on $(Cl)_{out}$ as predicted by the Nernst equation, but not on extracellular Na^+, K^+, and Mg^{2+}. Thus, Cl^- is the main and probably the only ion that carries the current underlying the fast component. In this and the following studies of this group, the measurements of the reversal potential were performed using the ramp method. This method is based on application of a ramp-like change of holding potential to the membrane. The voltage and current monitors of the voltage-clamp device are connected to the X- and Y-axes, respectively, of a plotter. Application of the voltage ramp results in an automatically drawn current-voltage curve. If such a curve is obtained just before the application of the agonist and then again at the plateau of the response, the reversal pontential of the response is the voltage at which the two curves intersect. This method allows one to measure the reverse potential of a response during a single application of the agonist. Furthermore, if the response is long lasting, the ramp can be applied repetitively, and the changes of membrane conductance and in reversal potential can be followed as the response proceeds.

The value of the reversal potential of the fast component was -22 to -24 mV (range: -15 to -31 mV in Ringer's solution),[67,100] which is close to the Cl^- equilibrium potential (-14 to -19 mV[57] or -24 mV[64]). On the other hand, the reversal potential of the slow component measured by the ramp procedure or by a similar method exploited by Kusano et al. was always more negative (-32 to -34 mV).[57,67] At the same time, if voltage clamp was not applied, the membrane potential during the slow phase of the response reached about -20 mV. Kusano et al.[57] suggested that the slow response is a Cl^- current and that the deviation of the value of reversal potential, as measured during voltage clamp, from Cl^- equilibrium potential is due to the technical difficulties encountered in the use of such a method. This apparent discrepancy was explained by Dascal et al.,[67] who showed that the slope of the dependence of the reversal potential on $(Cl)_{out}$ was less than predicted by the Nernst equation, and it was also dependent on $(K)_{out}$. Furthermore, in some cells, the slow inward current was followed by an outward current "tail" when the washout of ACh began. These findings were interpreted as suggesting that, even in the cells that display only a (dominant) inward current response, there is a concomitant overlapping outward current, probably carried by K^+. Indeed, an intracellular injection of the K^+ channel blocker, TEA (extracellular TEA blocks muscarinic receptors[247]), enhanced the slow inward current component of the ACh response and rendered its reversal potential strictly Cl^- dependent; it also blocked the outward current "tails". Measurements of the reversal potential changes during the development of the slow phase of the response to ACh indicated that the outward current develops and fades (upon ACh washout) more slowly than the slow inward current component; therefore, the reversal potential of the outward current could be measured during the "tails" following ACh washout. This reversal potential was about -100 mV and depended on $(K)_{out}$ as predicted by the Nernst equation, confirming the assumption that it was a K^+ current.

The effects of changes of the concentrations of various ions on the fluctuational component of the muscarinic response have not been studied so carefully; however, their amplitude depends on $(Cl)_{out}$ [9,57] and they always reverse at a membrane potential identical to the reversal potential of the transient component.[67] Therefore, they probably are carried by Cl^-, too. Interestingly, the fluctuational response is inhibited by intracellular TEA,[67] a fact that cannot be explained by the presence of intracellular ACh receptors, because intracellular injection of ACh does not evoke any responses.[9,57] Extracellular TEA does not block spontaneous fluctuations.[374] If TEA blocks the Cl^- channels that carry this current, then they are different from the channels through which flow Cl^- currents underlying the fast and the slow response components because the latter two are not inhibited by the injection of TEA.[67] If, on the other hand, TEA acts on the molecular mechanism underlying the fluctuational response, then it must be different from that of the other two inward current components, although all ACh-evoked Cl^- currents appear to be mediated by changes in $a(Ca)_{in}$ (see following). In summary, ACh evokes an increase in K^+ and Cl^- conductances; the latter is usually dominant and can be divided into two components, a transient one and a slow, long-lasting one. An additional component of this complex response is fluctuation of the membrane potential (current), most probably carried by Cl^-.

C. Pharmacology

All components of the response of the oocyte to ACh are inhibited by atropine but not by nicotinic antagonists.[9,57,69,100] The same response can be elicited by various muscarinic and mixed muscarinic-nicotinic agonists, such as muscarine, oxotremorine, metacholine, pilocarpine, carbachol, etc.[57,69] Thus, the receptor present in the membrane of the oocyte is cholinergic muscarinic.

The dose-response relationships of different ACh response components have been studied separately.[100,248,249]

Dascal and Landau[100] characterized the dose-response characteristics of the fast inward current component. In most cells ("low-sensitivity cells"), the fast response displayed a sigmoidal dose-response curve; the slope of the Hill plot was about 2.5. The data could not be adequately fitted by equations, suggesting a cooperativity in binding (three molecules of ACh bind to a single receptor in order to produce the response), but could be fitted well under the assumption that three ACh-receptor complexes cooperate in the production of the final response. Noncooperative ACh-muscarinic receptor interaction is also suggested by binding studies in various preparations.[250] It should be noted that a more complicated model, involving a cooperative interaction at a more distant point in the chain of presumptive biochemical reactions mediating the response, could probably provide as good a fit; thus, the model presented in the work of Dascal and Landau can probably be considered just an empirical approximation to the (unknown) real situation. A similar model (with a cooperativity of four) has been used to describe the dependence of presynaptic transmitter release on Ca^{2+}.[251,252] The similarity between the two models and the fact that, like transmitter release, the muscarinic response of the oocyte is Ca dependent (see following) may be a coincidence, but may also indicate a similarity of mechanism. Using this model, a dissociation constant (K_d) of about 0.2 μM for ACh was calculated.

In many cells ("high sensitivity"), sensitivity to ACh was higher and the Hill plot had a slope between 1.5 and 2.5. The dose-response curve had a "shoulder" and could be fitted by a model assuming two binding sites for ACh with different affinities (dissociation constants: 0.03 and 0.3 μM).[100] A cooperativity of three ACh-receptor complexes was presumed for the response resulting from occupation of each site. Since K_d of the lower affinity site was very close to that found in the normal ("low sensitivity") cells, it was assumed that in "low-sensitivity" cells the proportion of the sites with higher affinity was lower, and it could not be detected. The dissociation constants of the two presumptive sites are close to

those found in the binding studies for the "superhigh" and "high" affinity muscarinic binding sites in rat brain,[250] and Dascal and Landau[100] suggested that a similar explanation may apply to the complex dose-response curve of the oocyte; clearly, other explanations are possible. No binding studies have been done yet in the oocytes; such studies may provide the answer whether there are two different muscarinic-binding sites (that may represent two states of a single receptor).[253]

The dose-response characteristics of the slower inward response were studied in cells in which this response was reproducible.[248,249] It was found that they were identical to those found for the faster inward current in "low-sensitivity" cells, with a Hill slope of 3 and a K_d of 0.3 μM. This finding strengthens the hypothesis that the two inward current components of ACh response are subserved by the same molecular mechanism (see the next section).

Dascal and Cohen[249] studied the pharmacological characteristics of the outward current response in oocytes held at a membrane potential equal to the reversal potential of the Cl^- current. Under such conditions, no **net** Cl^- current was flowing through the membrane, and the amplutide of the outward current could be accurately measured. In contrast with the inward currents, the outward current component of the ACh response showed no signs of cooperativity: the slope of the Hill plot was very close to unity, and the dose-response curve could be well fitted by the Michaelis-Menten equation. On the other hand, the calculated dissociation constant of this response was 0.4 μM — practically identical to those found for the Cl^- current responses. These findings may indicate that all three major components of the muscarinic response in the oocytes result from the binding of ACh to the same **receptor**, but different molecular mechanisms are coupling between the receptor and Cl^- and K^+ channels. Another possible interpretation is mediation of Cl^- and K^+ currents by the binding of ACh to two functionally (and possibly structurally) different subtypes of muscarinic receptors with similar dissociation constants. And, of course, there always exists a possibility that the value of dissociation constant calculated from the data obtained in an electrophysiological experiment does not correspond to the actual K_d. This may happen if there are "spare receptors"[255] or if the final response (opening of a channel) is coupled to the receptor indirectly (through a chain of biochemical events).[256] While the latter appears to be true at least for ACh-evoked Cl^- currents (see following), the former is probably not the case in the oocytes. The existence of "spare receptors" is usually manifested in the ability of a submaximal concentration of an agonist (i.e., less than 100% occupancy of receptors) to produce the maximal physiological response,[257] whereas in the oocytes injection of Ca^{2+} or IP_3 may elicit larger Cl^- currents than even a supramaximal dose of ACh (see References 110 and 362; see the next section).

In conclusion, although some interesting data on the pharmacology of the muscarinic response of oocytes have accumulated, further work, especially binding studies, are necessary if one desires to understand the meaning of these findings.

D. The ACh-Evoked Cl^- Current is Mediated by Mobilization of Intracellular Ca^{2+}

The involvement of Ca^{2+} in the muscarinic response in the oocytes has been studied by the Tel-Aviv University group. A role for Ca^{2+} was suggested by the early finding that the slow and the fluctuational Cl^- currents were suppressed in Ca-free, EGTA-containing medium;[69] the fast Cl^- and the K^+ currents were diminished.[67] Elevation of $(Ca)_{out}$ potentiated all Cl^- components of ACh response; the slow component was more sensitive to this treatment than the fast one.[67] The Ca-channel blocker, verapamil, and the calmodulin antagonist, trigluoperazine, suppressed the muscarinic responses only at concentrations generally thought too high to be associated with specific effects.[67,109]

Dascal et al.[109] found that in Ca-depleted, EGTA-containing solution, the oocytes quickly deteriorate, vitiating investigations on the role of extracellular Ca^{2+}. However, if external Mg^{2+} concentration is elevated to 20 mM, the cells remain stable for long periods. All

components of the muscarinic response still appear in this Ca-depleted, high-Mg^{2+} solution, or in a similar solution containing a high (18 m*M*) concentration of an even more potent Ca-channel blocker, Mn^{2+}, or 20 μ*M* nifedipine. These findings suggested that none of the response components was mediated by an influx of Ca^{2+} and that the full suppression of the slow and fluctuational Cl^- responses in Ca-depleted solution could result from the deterioration of the cells. On the other hand, all Cl^- response components were fully suppressed by intracellular injection of the Ca-chelator, EGTA. The interpretation of these results by Dascal et al.[109] was that ACh-evoked Cl^- currents were the result of mobilization of Ca^{2+} from intracellular stores, a mechanism similar to that proposed for muscarinic, α-adrenergic, and dopaminergic responses in various preparations.[258-262] This hypothesis was supported by the following findings:

1. Intracellular injection of Ca^{2+} evoked a two-component Cl^- current response accompanied by Cl^- current fluctuations.[35,109]
2. Repetitive applications of ACh in a Ca-depleted, high-Mg^{2+} (or high-Mn^{2+}) solution resulted in a progressive decrease in the amplitudes of Cl^- currents. This was interpreted as the consequence of the depletion of intracellular Ca^{2+} stores. Indeed, a temporary exposure to normal Ca^{2+} solution (''Ca window'') partially restored the fast and slow Cl^- responses, presumably due to partial repletion of the Ca^{2+} store.[109]
3. Exposure to ACh evoked release of $^{45}Ca^{2+}$ from preloaded oocytes. This was interpreted as an indication of an increased $a(Ca)_{in}$,[109] although an alternative mechanism, such as activation of a Ca^{2+} extrusion mechanism, cannot be excluded. Two components of this efflux could be sometimes discerned, although the termporal resolution of the method was not high enough for decisive discrimination.
4. Intracellular injection of the Ca-releasing messenger, IP_3, evoked a two-component Cl^- current closely resembling the ACh response.[110] Application of ACh resulted in an increased polyphosphoinositide turnover, reflected in the increase of intracellular concentrations of IP_3 and products of its degradation.[110] Injection of IP_3 into oocytes preloaded with $^{45}Ca^{2+}$ resulted in an increased Ca^{2+} efflux.[263]
5. Intracellular injection of GTP-γ-S, a nonhydrolizable analog of GTP, evoked slow Cl^- current accompanied by inward current fluctuations.[245] These responses were inhibited by intracellularly injected EGTA. GTP-γ-S may be expected to activate any G-protein, including the one coupled to phospholipase C and thus lead to Ca-mobilization (see Section XI.A).

Taken together, these findings strongly support the hypothesis that the ACh-evoked Cl^- current responses are mediated by mobilization of Ca^{2+} from intracellular store or stores, according to the general scheme presented in Section XI.A. The differences in sensitivities of the fast and the slow response components to Ca depletion[109] and to elevated $(Ca)_{out}$,[67] as well as the two-component appearance of the response to IP_3 injection,[110] suggest that the fast and the slow Cl^- components of ACh response may result from the release of Ca^{2+} from two functionally different internal stores.[109] This idea is supported by the recent findings by Gillo et al.[114] that depletion of intracellular Ca^{2+} stores by the exposure to low doses of the ionophore A23187 (in a Ca-free solution) selectively inhibits the fast component of ACh response. High doses inhibit the slow component, too. Thus, it can be inferred that the two stores are different not only functionally but also structurally. An alternative is a Ca-dependent Ca^{2+} release as the mechanism of the second, slow response[362] or a Ca-dependent activation of phospholipase C that would bring about an additional ''wave'' of IP_3 production and Ca^{2+} release. Two-component responses were observed upon IP_3 injection into *Xenopus* eggs, and the interpretation of the results included the various possibilities mentioned here.[47] Similarly, the fast component of the response may reflect an increase in free Ca^{2+} in the

submembrane layer of the cytoplasm, while the slow one would be in the subcortical layer (or the "bulk" of cytoplasm), as proposed for the eggs undergoing activation (Section VIII.A).

The mechanism of the fluctuational response is obscure. Although it is evident that it is Ca dependent and that most probably Ca^{2+} comes from intracellular stores, it is the fluctuational character of this response component that is mysterious. Kusano et al.[57] have speculated that a fluctuation of the concentration of a second messenger substance (we may say now, Ca^{2+} or IP_3) may underlie this phenomenon; the molecular events underlying such fluctuations may be important and deserve further study. One important question is whether each individual fluctuation is confined to a small region of the oocyte, perhaps because "packets" of Ca are released locally as found in neurons.[364]

E. Is Cyclic GMP Involved in the Mediation of the K^+ Current Evoked by ACh?

In 1982, Dascal and Landau[100] showed that bath application of cyclic GMP (cGMP) and its analogs, 8-bromo-cGMP and dibutiryl cGMP, evoked a small transient Cl^- current followed by a small, long-lasting outward current. The Cl^- current elicited by cGMP never achieved a magnitude comparable to that evoked by ACh, and no reproducible dose-dependence could be demonstrated. The authors have speculated that cGMP may mimic the muscarinic response either directly (i.e., if cGMP is the actual second messenger for ACh response) or indirectly, e.g., by the activation of a cGMP-dependent PDE.[264] Later experiments[67] unequivocally proved that cGMP is not the second messenger of the ACh-evoked Cl^- response: intra-oocyte injection of cGMP evoked outward (K^+) current, and an inward current was observed only at nonphysiological doses of cGMP. We now think that the Cl^- current elicited by extracellular application of cGMP and its analogs might be due to a nonspecific effect, such as displacement of membrane-bound Ca^{2+}, etc., although the PDE mechanism cannot be rejected at the moment.

The outward current evoked by the intracellular injection of cGMP behaved quite differently. It appeared at cGMP doses as low as 0.6 pmol/oocyte (corresponding to an increase of 60% above the resting level, assuming homogeneous distribution of the injected cGMP inside the cell), although, in many cells, a threshold response was observed at 2 to 3 pmol/oocyte.[67,265] At higher cGMP concentrations, the outward current reached amplitudes that exceeded the maximal ACh response in the same cells. These facts were interpreted as an indication of a role for cGMP as the second messenger of the ACh-evoked K^+ current.[67] Of course, the fulfillment of this single criterion is not enough to state that a certain substance is the second messenger participating in mediation of a neurotransmitter response.[159] Dascal et al.[265] performed a series of experiments aimed at further testing the cGMP hypothesis. ACh elevated the intracellular cGMP level, with a time course roughly corresponding to that of the ACh-evoked K^+ current, to about 30% above the basal level (i.e., about 0.3 pmol/oocyte) at saturating concentrations of ACh. Although this increase is smaller than the dose of cGMP necessary for even a threshold response, one could argue that the elevation of cGMP produced by ACh is confined to the vicinity of the plasma membrane, and the actual increase in cGMP level there might be much bigger than 30%. K^+ current elicited by ACh was partially suppressed by the depletion of external Ca^{2+} and potentiated by an increase in $(Ca)_{out}$. This is in line with the notion that, in many preparations, the ACh-evoked increase in cGMP level is Ca mediated (see Section XI.A).

Several results, though, contradicted the cGMP hypothesis:

1. Theophylline and isobutylmethylxanthine, which inhibit various types of cyclic nucleotide PDEs, potentiated the outward current elicited by cGMP injection, but reduced that evoked by ACh, contrary to the prediction of the cGMP hypothesis. However, both agents had a similar inhibitory effect on the Cl^- current responses evoked by

ACh. It is possible, therefore, that the effect was nonspecific, e.g., an interference with the muscarinic receptor or with a Ca^{2+} store; the latter is rather probable, in view of the well-known Ca-releasing action of methylxanthines, especially caffeine.[266-268] Another possibility is a direct inhibition of phospholipase C (functionally, a PDE) by these PDE inhibitors.

2. ACh, when applied at the plateau of cGMP-evoked outward current, strongly **suppressed** it, an effect that cannot be explained by the cGMP hypothesis. Rather, this resembles the effect of ACh on the response to injection of cAMP or to application of adenosine.[269]
3. Inhibition of cAMP- and adenosine-evoked K^+ currents in follicle-enclosed oocytes by ACh can be mimicked by C-kinase activators, phorbol esters[269] (see Section XIII.E). The response to cGMP was completely inhibited by phorbol esters, too, whereas the ACh-evoked K^+ current was **potentiated** by the same treatment.[265] This specific discrimination between ACh and cGMP response is the strongest argument against the cGMP hypothesis.

In all, it appears that cGMP is not the second messenger that mediates the ACh-evoked K^+ current. Moreover, the similarity between the responses to intracellularly injected cGMP and cAMP implies that cGMP simply "mimics" the cAMP effect. Indeed, the threshold dose of cAMP is at least ten times lower than that of cGMP;[270] at such high concentrations, cGMP may activate nonspecifically the cAMP-dependent protein kinase.[271,272] What is (if any) the second messenger of the ACh-evoked K^+ current? We do not know at present. One possibility is that it may be Ca^{2+}; in support of this idea, the K^+ current response is Ca sensitive. Although injections of IP_3 and Ca^{2+} produced no detectable outward current component, it just could be obscured by the dominant inward currents. Indeed, a clear K^+ current component can be detected in the A23187-evoked response.[360]

F. Biological Role

It is not clear what the functional role of muscarinic receptors is in the oocytes. A hint may come from the fact that muscarinic agonists promote progesterone-induced maturation.[106] The oocytes are extremely sensitive to ACh in this respect; doses as low as 1 n*M* (a level that may be found in blood) are already able to facilitate the maturation process.[273] Endogenous ACh is found in oocytes in about the same concentrations as found in muscle.[274] The source of this endogenous ACh is not clear, and any variations of its level have not been reported; one cannot disregard the possibility that this endogenous ACh may provide a means of fine regulation of the rate of maturation. This guess is supported by the fact that atropine slows the rate of progesterone-induced maturation.[273] However, this hypothesis lacks additional support, e.g., an indication of changes of ACh blood levels under different conditions, etc. Moreover, adenosine[365] and insulin[275,276] also promote, or even initiate, maturation.

A role for ACh in fertilization can be discarded both on the basis of disappearance of muscarinic responsiveness after maturation (see Section XII.A) and on the basis of the finding of Robbins and Molenaar[274] that neither atropine nor ACh had any effect on the process of fertilization.

It is possible that ACh plays some role in the maintenance of the vectorial Ca-dependent Cl^- current flowing through the oocytes at rest (Section VI.A). The idea fits nicely with the polarity of the oocyte with respect to ACh response, the disappearance of both standing Cl^- current and ACh responsiveness in the course of maturation, and the pronounced Ca-dependence of Cl^- currents in both cases. It would be interesting to see whether atropine has any effect on the standing Cl^- current at rest.

XIII. β-ADRENERGIC AND PURINERGIC RESPONSES

A. Pattern and General Characteristics

Kusano et al.[9] demonstrated that epinephrine, serotonin, and dopamine evoked a membrane hyperpolarization in follicle-enclosed oocytes. It was found later that the responses to serotonin were somewhat erratic and that oocytes of most frogs did not display serotonin-evoked hyperpolarization.[277] In a low percentage of frogs, some oocytes developed a small fluctuational response to serotonin.[245] Later, Lotan et al.[68] demonstrated a similar hyperpolarizing response to adenosine and other purinergic agonists; in some cells, it was preceded by a transient inward (depolarizing) current. Similarly, the responses to epinephrine and dopamine were mostly hyperpolarizations, but in some cells, small depolarizations were observed.[57] The hyperpolarizing responses to epinephrine[57] and to adenosine[68] desensitized rather quickly and were followed by a period of refractoriness. Like the muscarinic response, catecholaminergic responses appeared to be stronger in the animal hemisphere and disappeared in the course of maturation;[57] no data of this kind on purinergic responses are available. The similarity between adrenergic and purinergic responses in the oocytes is further emphasized by the identity of ionic mechanisms and second messenger systems (see following); for these reasons, the two kinds of responses will be discussed together.

B. Ionic Mechanism

Kusano et al.[57] have characterized some electrophysiological properties of the responses to epinephrine and dopamine. The reversal potential of the adrenergic response was about -80 mV, while the K^+ equilibrium potential, calculated from intracellular activity measured with a K^+-sensitive electrode, was -100 mV. Thus, the reversal potential was more positive than predicted for a perfect K^+ electrode; it changed about 50 mV per tenfold change in $(K)_{out}$. Both facts suggested that the outward current evoked by epinephrine is carried mostly by K^+, but that another ionic conductance was involved. Indeed, sometimes a biphasic current flow was seen by these authors when the membrane potential was held inside positive; they did not characterize this phenomenon further. Later, Van Renterghem et al.[278] repeated these experiments and found that the outward current evoked by isoprenaline reversed at about -95 mV, again suggesting that K^+ was the dominant charge carrier.

Purinergic response was characterized by Lotan et al.[270] The transient inward current component of the response reversed around -22 mV; the response depended on extracellular Cl^- as predicted by the Nernst equation, suggesting that this current was carried by Cl^-. The reversal potential of the dominant (outward current) component of the response to adenosine was about -102 mV in normal Ringer and changed with $(K)_{out}$ as predicted by the Nernst equation. Furthermore, this current was suppressed by extracellularly applied TEA (10 to 40 m*M*). Thus, it was concluded that the adenosine-evoked outward current was carried by K^+. The inward current component was more pronounced when, instead of adenosine, ADP or ATP was used to evoke the response. The reversal potential of the outward current was more positive than with adenosine, and in some cells, at a holding potential of -50 mV, a slow **inward** current appeared instead of outward.[279] In these cells, the outward current usually could be seen as a "tail" upon the washout of the agonist. When the changes of the reversal potential of ATP response were followed during a single application of the agonist using the ramp method, the reversal potential became more negative as the response developed and even more negative during the first minute or two after ATP washout, until the response became undetectably small. These observations suggested that, in addition to the transient Cl^- current and the slow K^+ current, ATP elicited an additional slow inward current with kinetics of development and washout somewhat faster than those of the K^+ current response.[279] This picture resembled very much the muscarinic response (Section XII); the similarity was further confirmed by the finding that, when the slow outward

current was inhibited by TEA or theophylline (see following), the emerging slow inward current had a reversal potential of −25 mV, which changed by 66 mV for a tenfold change in $(Cl)_{out}$.[279] This is somewhat higher than predicted by the Nernst equation; the reason for such behavior is not known. None of the response components were inhibited by atropine, arguing against the idea that purinergic agonists might act by releasing ACh.[68,279]

This section may be summarized as follows. In follicle-enclosed oocytes, purinergic agonists evoke a response that consists of three main components similar to those evoked by ACh: fast and slow Cl^- currents, and a slow K^+ current. In contrast to ACh, the outward current is usually dominant, and when adenosine is used to evoke the response, the slow Cl^- current is detected only rarely. Epinephrine and dopamine apparently evoke a similar response, with a dominant K^+ component; the ionic mechanism of the inward current component has not been studied.

C. Pharmacology

The response to epinephrine was blocked by β- but not α-adrenergic blockers, and could be evoked by the β-adrenergic agonist isoproterenol but not by the α-adrenergic agonist phenylephrine, suggesting that the response was mediated by a β-adrenergic receptor. The dose-response relationship of epinephrine was sigmoid (suggesting some kind of cooperativity);[57] a more thorough study has not yet been done.

The pharmacology of the purinergic response has been studied by Lotan et al.[68,270,279] The K^+ current was blocked by theophylline at concentrations well below those used to inhibit PDEs. The potency sequence of the different purinergic agonists for this current was adenosine = AMP > ADP = ATP. These facts indicated that the receptor that mediated this response belonged to the P1 purine receptor class.[280] The agonist potency sequence within the P1 class was 5′-*N*-ethylcarboxamideadenosine (NECA) > adenosine > N^6-phenylisopropyladenosine,[270] suggesting that the receptor pertains to the R_a subclass.[281] The dose-response characteristic of the outward current evoked by adenosine or NECA displayed a Hill slope of at least 3; it could be fitted by a model similar to that applied to the ACh-evoked Cl^- current (Section XII.C), i.e., assuming that at least three receptor-bound agonist molecules are required to produce the final response.

In contrast to the muscarinic response, where no evidence exists that the various ACh-evoked currents are mediated by different receptor subtypes, the K^+ and Cl^- response components of the purinergic response undoubtly result from activation of distinct purinoceptors. Neither fast nor slow Cl^- current responses to adenosine or ATP were inhibited by theophilline, suggesting that a receptor other than P1 was involved in the mediation of these currents.[68,279] The agonist potency sequence was opposite to that of the K^+ response,[279] corresponding to the P2 type.[280] Unfortunately, no specific blockers for this receptor are known. The noncompetitive P2 blocker, quinidine sulfate, did not inhibit the Cl^- components of ATP response;[279] however, the selectivity of this agent is doubtful.[282,283] Thus, the receptor mediating the Cl^- responses can be tentatively identified as the P2 receptor.

D. cAMP Mediates the K^+ Currents Induced by Purinergic and β-Adrenergic Agonists

The slow time course of the purinergic response led Lotan et al.[68] to the suggestion that it might be mediated by a second messenger. This hypothesis was supported in a later report of the same group.[270] Additional support came also from independent work by Stinnakre and Van Renterghem.[284] The evidence that cAMP serves as the second messenger of an adenosine-evoked K^+ current response in follicle-enclosed *Xenopus* oocytes is as follows:

1. The receptor that mediates this purinergic response is of the R_a type,[270] known to elevate cAMP levels in other tissues.[281]
2. Exposure to adenosine leads to a relatively small (28% of control) but significant

increase in the intracellular cAMP level. In this study, the basal level of cAMP was 1.4 pmol/oocyte; thus, adenosine "adds" about 0.4 pmol/oocyte. A peak increase is observed as early as 3 to 5 sec after application of adenosine, i.e., it **precedes** the electrophysiological response. Furthermore, after reaching a peak, cAMP begins to level off slowly to about 10% above control in 1.5 to 2 min. This pattern corresponds to the desensititization of the adenosine-evoked K^+ current.[270]

3. Externally applied methylxanthines antagonize the purinergic effect at the receptor level (previously described); however, intracellular injection of these PDE inhibitors **potentiates** adenosine-evoked K^+ current.[270]
4. Intracellular injection of cAMP elicits a large outward current.[270] The reversal potential of this current is close to -100 mV and changes by 48 mV per tenfold change in $(K)_{out}$. These results suggest that this current is carried mostly (but probably not solely) by K^+. A similar outward current is evoked by a PDE-resistable cAMP analog 8-bromo-cAMP,[100,278] by the adenylate cyclase activator forskolin,[269,270] and, in some cells, by PDE inhibitors.[269,270] Threshold outward current responses are obtained with cAMP doses as low as 0.1 pmol/oocyte, which is less than the increase in cAMP level induced by adenosine.
5. At low concentrations (that do not produce a large K^+ current), forskolin strongly potentiates the response to adenosine.[284] This may possibly be due to forskolin-induced enhancement of the interaction between G_s and C subunits of adenylate cyclase.[285] The response to adenosine was also enhanced by isoproterenol, another adenylate cyclase activator.[284] This phenomenon suggests that there is a receptor-independent positive cooperative interaction at the level of adenylate cyclase or at a more distant step; otherwise, a simple additivity or even an attenuation would be expected. It is tempting to speculate that this cooperative interaction is the same one that underlies the observed cooperativity of the dose-response relationships for adenosine and (probably) epinephrine (see the previous section).
6. Intracellularly injected protein kinase inhibitors (types II and III) suppress the response to adenosine in a dose-dependent fashion,[270] suggesting that, as one would expect, the effect of cAMP is mediated by activation of protein kinase A.
7. At relatively large doses of cAMP (that evoke outward currents larger than 50 nA), the responses to cAMP and adenosine are less than additive, suggesting a common pathway. Furthermore, at such doses the intracellularly injected cAMP attenuates both the response to a subsequent injection of an identical amount of cAMP and the response to adenosine. The mechanism of this cAMP-mediated refractoriness is not clear, but cAMP-induced inhibiton of its own and of adenosine responses suggests similarity of a biochemical basis of these responses.

Adenosine partially suppresses the response to a subsequent injection of cAMP only at very high concentrations and only in some (but not in all) oocytes.[270] One possible explanation for this apparent contradiction is that the cAMP-dependent refractoriness may result from an increase of cAMP level in a cellular compartment to which the access of the injected cAMP is easier than that of cAMP produced as the result of adenosine action. Indeed, in the experiments of Lotan et al.,[270] cAMP was injected relatively deep into the cytosol, whereas the adenylate cyclase-mediated increase in cAMP levels would be expected to be confined mostly to the submembrane layer of the cytoplasm. Of course, other explanations are possible, but there is no point in further speculation in view of the lack of relevant experimental evidence.

Mediation of the β-adrenergic response by cAMP has been proposed by Van Renterghem et al.[278] on the basis of the well-known adenylate cyclase-activating effect of this receptor. They have supported this hypothesis by demonstrating that forskolin and 8-bromo-cAMP

evoke a K^+ current and that the response to isoproterenol is potentiated by PDE inhibitors and by forskolin. Although these results do not fulfill all the criteria that are necessary for an unequivocal demonstration of the involvement of cAMP in mediation of the response,[159] they render this hypothesis highly probable. Interestingly, a long-lasting exposure to the PDE inhibitor isobutylmethylxanthine **reduced** the response to isoproterenol,[278] possibly due to a "desensitization" similar to that generated by intracellularly injected cAMP.

E. Inhibition by ACh of the K^+ Current Elicited by cAMP or by Purinergic or β-Adrenergic Agonists

ACh is known to inhibit adenylate cyclase activity (either basal or activated by a stimulating agent; Section XI.A). Therefore, it could be expected to inhibit the responses to adenosine and to norepinephrine. Indeed, such an effect has been demonstrated independently by Van Renterghem et al.[278] and by Dascal et al.[269] ACh inhibited the K^+ current evoked by isoproterenol[278] and by adenosine.[269,284] Inhibition was observed either when ACh was applied together with the cAMP-elevating agonist or before the agonist; the inhibitory effect of ACh lasted for more than 1 hr.[284] ACh-induced inhibition of the K^+ current was not due to the development of a Cl^- current because (1) the inhibition was observed in cells that did not develop a Cl^- current in response to ACh;[284] (2) it was observed in cells that were voltage clamped at a holding potential equal to the reversal potential of the Cl^- current response measured in the same cell;[269] (3) it was accompanied by a decrease in the conductance to a level observed with ACh alone.[269] Thus, ACh actually causes a cessation of the current through the K^+ channels opened by adenosine or isoproterenol.

Our observations show that, rather surprisingly, the inhibition by ACh of adenylate cyclase is apparently not involved in the effect described previously because ACh is able to suppress (with equal efficiency) the response to intracellularly injected cAMP, a procedure that bypasses the adenylate cyclase activation step.[269] It also does not appear to result from activation (e.g., through a Ca^{2+}-dependent mechanism) of PDE: inhibitors of PDE do not prevent ACh from suppressing the cAMP responses; moreover, in oocytes that respond by an outward current to application of isobutylmethylxanthine, the latter is inhibited by ACh, too.[269]

It is not clear if the effect of ACh is Ca dependent. Intracellular injection of EGTA sometimes potentiated the adenosine response.[284] The effect of ACh on adenosine and isoproterenol responses could be mimicked by application of the divalent cation ionophore A23187[278,284] but not by intracellular injection of Ca^{2+} or IP_3.[269] The ionophore also inhibits the cAMP response.[362] This discrepancy may be explained by assuming that the site of Ca^{2+} action is the vicinity of the plasma membrane, where the ionophore is expected to elevate the level of Ca^{2+} preferentially, while intracellular injection of Ca^{2+} and IP_3 may elevate mostly the Ca^{2+} level deeper in the cytoplasm.[366] We cannot reject this possibility; however, the amounts of the injected Ca^{2+} and IP_3 were enough to evoke substantial Cl^- currents in the same cells in which they did not affect the ACh-induced attenuation of adenosine response,[269] suggesting that submembrane Ca^{2+} was elevated enough to activate Cl^- channels.

Another way to explain the inability of injected Ca^{2+} and IP_3 to mimic the inhibitory effect of ACh is to assume that the cAMP-activated K^+ channels are situated in the follicular cells (see below). Here, one could suspect that intra-oocyte-injected (or released by IP_3) Ca^{2+} does not reach follicular cells, while A23187 allows for Ca^{2+} entry into these cells.

Finally, it is possible that the inhibitory effect of ACh truly does not require an elevation in $a(Ca)_{in}$ and that the effect of A23187 is Ca independent, but rather is due to some unknown effect of this compound on the membrane.

Besides elevating intracellular Ca^{2+} activity, the ACh-dependent activation of phospholipase C may be expected to lead to activation of C-kinase. We have, therefore, tested the possibility that this process might be involved in the inhibitory effect of ACh on cAMP-

evoked K^+ current,[269] using the C-kinase-activating β-phorbol esters phorbol dibutyrate (PDBu) and phorbol myristate acetate (PMA). Neither of these agents evoked any membrane currents by itself, suggesting that this pathway is not involved in mediation of ACh-evoked Cl^- or K^+ currents. PDBu inhibited very quickly the K^+ current evoked by either externally applied adenosine or intracellularly injected cAMP; a 100% inhibition developed within 1 min at 1 μ*M* PDBu. Slower effects were obtained with lower concentrations of the ester. PMA inhibited K^+ current with a slower time course but at lower concentrations than PDBu. The potency of both agents in suppressing adenosine and cAMP responses was very similar to that reported in various tissues with respect to C-kinase activation;[194] a 50% effect with PMA was attained at less than 5 n*M*, and with PDBu at about 20 n*M*. The inactive analog of βPMA, αPMA, did not mimic ACh effect.[367] Thus, it appears that activation of C-kinase is involved in the inhibitory action of ACh.

It is not known whether the targets of action of Ca^{2+} (assuming that the effect of ionophore is a specific one) and of phorbol esters are the same protein(s). Activation of C-kinase itself by diacylglycerol is Ca dependent (Section XI.A); Ca alone may activate C-kinase at high concentrations.[286] Ca^{2+} may also act through activation of a Ca-dependent protein kinase and phosphorylation of a protein, the same or different from that phosphorylated by C-kinase. There is a possibility that both Ca^{2+} and phorbol esters exert their effect on gap junctions between the oocyte and the follicular cells; this will be discussed in detail in the following section.

F. Site of Action of cAMP and cAMP-Elevating Agents

As already mentioned, the β-adrenergic response is severely diminished or disappears in oocytes that are defolliculated manually or by collagenase.[57,278] The same is true for the purinergic response in most instances.[86,374] Although a residual response to adenosine has been observed in oocytes that were shown to lack any follicular cells in a histological examination,[68] one cannot exclude the possibility that some follicular cells remained attached to the surface of the oocyte and were "missed" in the slices that were tested. It was later observed that the denuded oocytes also lost (partially or completely) the response to intracellularly injected cAMP and to forskolin.[374]

These observations can be interpreted in three different ways:

1. It has been proposed that β-adrenergic and purinergic receptors and K^+ channels may be located in follicular cells; due to the presence of gap junctions, the follicular cells and the oocyte are electrically coupled, and any current flowing through the follicular cell membranes will also be recorded in the oocyte.[57,278,366]
2. It is possible that some processes following receptor activation (e.g., activation of adenylate cyclase) take place in the oocyte, while the later ones (including the activation of the K^+ channel) happen in the follicle cells; the connection between these events could be provided by the diffusion of a small molecule (e.g., cAMP) through the gap junctions.
3. The process of defolliculation (either mechanically or by collagenase) may damage the cAMP-dependent K^+ channel, much in the same way as this process disturbs other functional properties of the membrane of the oocyte (see Sections II.A and V.B), especially if these channels are close to the contacts between the membrane of the oocyte and that of follicular cells: this region may be damaged more than the others when the follicular cells "fall apart".

At present, there is no direct proof that any of the three possibilities is true. We shall now consider briefly the evidence for and against the involvement of gap junctions and follicular cells in purinergic and β-adrenergic responses.

1. The defolliculation-induced loss of a membrane response to an agonist does not necessarily mean that the response does not take place in the oocyte. The ACh-induced Cl^- current disappears in many instances following defolliculation, although there is no argument about the location of all constituents of its mechanism in the oocyte (Section XII). It appears that in this case the entity that is damaged is the muscarinic receptor, whereas the Ca^{2+} release mechanism and the Ca-dependent Cl^- channel are not touched: indeed, even the cells that lost all ACh responsiveness produced large Cl^- currents when injected with IP_3.[374] The muscarinic response that was "lost" following collagenase treatment reappears sometimes after a 24-hr incubation (Section XII), whereas the purinergic response does not;[374] however, the *de novo* synthesis of the damaged K^- channels may be very slow compared with that of the muscarinic receptor. The damage can be produced by the treatment leading to defolliculation (collagenase, EDTA) even before the actual removal of the follicular cells. For instance, we have observed a complete disappearance of the response to adenosine in many oocytes after a 0.5- to 1-hr treatment with collagenase, while all outer layers remained attached rather firmly.
2. Denuded oocytes still respond by an elevation of cAMP level to forskolin[231] and cholera toxin,[233] whereas neither forskolin nor intracellularly injected cAMP evoke a K^+ current. Thus, defolliculation does not impair the ability of the oocyte to produce cAMP, and from the disappearance of a cAMP-mediated K^+ current one can only infer that the K^+ channel does not activate any more.
3. Adenosine elevates cAMP levels in denuded oocytes to the same extent as in follicles.[365] This finding strengthens the conclusion of the previous paragraph, that it is probably the K^+ channel that is damaged or removed by defolliculation. It also proves that a purinergic receptor in the membrane of the oocyte is preserved following defolliculation by collagenase. Of course, it does not disprove the possibility that there is an additional purinergic receptor in follicular cells.
4. Injection of protein kinase inhibitors into the oocyte suppressed the response to adenosine (see Reference 270; Section XIII.D). A $\geq$70% inhibition was obtained at an intra-oocyte concentration of about 1 μM; at concentrations as low as 0.1 μM, a 20 to 30% inhibition was observed. Injection of similar amounts of bovine serum albumin had no effect on the purinergic response. These inhibitors are proteins with a molecular weight >20 kdaltons, which by no means can be suspected to diffuse from the oocyte to the follicular cells if the connections are normal gap junctions. Therefore, the site of action of these inhibitors was the oocyte itself, and if their effect in the experiments described previously were specific, the target would be the cAMP-dependent protein kinase (A-kinase). This argument supports the notion that (at least the primary) site of action of cAMP is the oocyte itself.
5. The Ca- and phorbol ester-induced inhibition of adenosine- and cAMP-evoked K^+ currents supports the gap junction hypothesis.[278,366] Both Ca^{2+} [287,288] and C-kinase[289] may cause the closure of these junctions. Moreover, cAMP may either up-regulate or decrease the permeability of cell-to-cell junctions.[288-291] This is important because (if indeed the gap junctions are involved in cAMP response) at rest all cell-to-cell junctions should be closed. Otherwise (if they are open), phorbol esters could close them, and this will reduce the total conductance of the oocyte at rest; this was not observed.[269]

Thus, a possible scenario of the effect of cAMP may be — cAMP-dependent phosphorylation opens the gap junction channel → cAMP diffuses into the follicle cell → cAMP-dependent phosphorylation leads to the opening of a K^+ channel.

The ACh-evoked K^+ current, like the purinergic response, disappears after defolliculation; moreover, it does not reappear again even in the cells that regain their Cl^- response to ACh.

Such behavior is nicely explained by the gap junction hypothesis, i.e., either the ACh receptor responsible for this current or the channel itself is situated in the follicular cell. Following this interpretation further, one would expect that phorbol esters and high Ca^{2+} will inhibit this response; however, the opposite is true: the ACh-evoked K^+ current is potentiated by both treatments.[265] This observation does not argue directly against the gap junction hypothesis concerning the cAMP-dependent K^+ channel, but calls for a critical approach in interpretation of such data.

To summarize, none of the evidence presented above argues directly against any one of the three hypotheses concerning the involvement of follicular cells in the purinergic response of oocytes. The same probably holds for the β-adrenergic response, although there is even less data on this. It is clear that the disappearance (or, as happens in some cases, attenuation) of the response following defolliculation does not by itself prove that the receptors or other constituents of the cascade which are involved in production of the response are situated in the follicular cells. Much of the evidence presented favors (at least indirectly) the notion that at least the purinergic receptor and cAMP-regulating machinery are in the oocytes. The K^+ channels may be located in follicular cells, and in this case, cAMP should have a double function: opening the cell-to-cell channels and the K^+ channels. We look forward to a solution of this puzzle by direct experiments.

XIV. EXPRESSION OF EXOGENOUS MEMBRANE PROTEINS IN mRNA-INJECTED OOCYTES: GENERAL CONSIDERATIONS

It was first demonstrated by Gurdon and colleagues that, when injected with foreign mRNA, *Xenopus* oocytes synthesize the exogenous proteins encoded by it.[10,292] Several other cell types are able to do the same, but the oocyte is certainly the most popular preparation, probably due to its large size and high efficiency in expressing the message.[8] Some of the newly synthesized exogenous proteins have been identified by their characteristics, such as antibody specificity, substrate specificity, cellular localization, etc. The various aspects of the complex process of expression of exogenous proteins by mRNA-injected oocytes have been extensively reviewed,[6-8,293-295] and only several points concerning the expression of membrane proteins are touched upon here. The study of the exogenous neurotransimtter- and voltage-regulated channels is the subject of the following sections. In addition to these channels, the oocytes have recently been shown to express a gap-junction channel.[22]

The exogenous membrane proteins, including the neurotransmitter- and voltage-activated ion channels, are synthesized, processed, and inserted into the membrane of the oocyte. The injection procedure per se, or some unknown species of the injected heterologous poly(A)$^+$ RNA, does not cause an enhancement of **transcription** of the endogenous messages because the exogenous receptors and channels can be expressed in enucleated or actinomycin D-treated oocytes.[277,296-298] However, when one wants to use the oocytes as a model system for the study of these channels, several problems have to be faced.

Fully grown oocytes, in addition to the normally processed endogenous mRNA, contain a large amount of unprocessed "interspersed" poly(A)$^+$ RNA.[299,300] It is not known what factors prevent this RNA from being expressed or what proteins it may encode. There is a chance that the injected exogenous heterologous mRNA somehow enhances the expression of this RNA, similar to the effect produced by adenovirus RNA[301] or poly(A)$^+$ RNA from human carcinoma cells.[8] This may provoke the appearance of **endogenous** ion channels that are not present in the membrane of noninjected oocytes. (At the extreme, it could be an endogenous protein characteristic of a specific adult tissue.) Several facts argue against this possibility.

First, the injection of foreign RNA, in fact, **decreases** the rate of synthesis of endogenous membrane proteins because it competes with the endogenous mRNA for polysomes.[302,303]

Second, poly(A)$^+$ RNAs from the brains of young rats and from chick embryos cause the appearance of voltage-dependent Na^+ and K^+ channels and of GABA and kainate receptors (see following), but only the rat brain RNA gives rise to ACh and serotonin responses. Also, the desensitization properties of the responses to GABA[304] and the kinetic properties of K^+ currents[368] from the two sources are different. Such examples with other RNA preparations are numerous. It is hard to believe that the heterologous poly(A)$^+$ RNA from different sources contains messages that specifically enhance the translation of some, but not all, endogenous RNAs of the oocyte.

Third, different fractions of size-fractionated heterologous mRNA cause the appearance of different receptors and channels.[305-307] Here, if one sticks to the "endogenous translation enhancement" hypothesis, one would have to suggest that the various fractions of exogenous RNA contain different specific "enhancing factors". Would not it be more logical to accept the view that the injected RNA codes directly for the newly synthesized proteins?

Fourth, it has been shown in several instances that the new receptor or channel differs from its analog which **does** appear in *Xenopus* oocyte not injected with exogenous RNA (as with the voltage-operated Ca^{2+} channel; Section XVI.B). Unfortunately, the properties of the receptors and channels of the adult or larval *Xenopus* tissues are not well characterized to allow a comparison with the (presumably) exogenous ones expressed in the oocytes.

The evidence presented so far argues in favor of a direct synthesis of exogenous membranal proteins on the template of their RNAs. The only way to make certain that this is so in each particular case is to look at the expression of membrane proteins in oocytes injected with homologous mRNA, synthesized from a cloned cDNA coding for just the protein under study. This, indeed, has been done in the case of nicotinic receptor-channel protein,[308] voltage-sensitive Na^+ channel,[309] and brain muscarinic receptor.[310] However, with most other receptor and channels that were reported to appear in mRNA-injected oocytes, cDNAs are not available yet; obviously, more and more will be cloned in the near future.

Another source of uncertainty is the use of different batches of oocytes and different preparations of RNA. These factors contribute to the diversity of results, especially when one tries to quantify them. The oocytes from different donors may differ significantly in the levels of internal second messengers and in resting membrane properties; both factors may affect the properties of the exogenous channels. This is an inevitable drawback of the method, and one has to be aware of it when trying to draw any generalized conclusions. The only way to avoid mistakes that may be introduced by such variability is to compare as many batches of oocytes and RNA as possible.

Although the oocytes usually faithfully translate the foreign mRNAs, the post-translational modification steps (glycosylation, phosphorylation, transport, etc.) that may be tissue specific have been shown to proceed incorrectly in some cases.[8] Thus, the final product, the membrane protein, a receptor, or a channel in our case, may differ in some respects from that in the native tissue. Therefore, a thorough comparison (whenever possible) of the features of neurotransmitter receptors and ion channels expressed in the oocytes, with those of the nascent ones, is imperative whenever the oocyte expression system is exploited for the study of structure or function of these proteins.

In conclusion, together with the obvious advantages and even uniqueness of the oocyte as a model for the study of "implanted" receptors and channels, one has to be aware of the disadvantages and uncertainties described above and treat the obtained results carefully and critically. Once this is done, the full strength of this approach can be revealed.

XV. NEUROTRANSMITTER RESPONSES THAT APPEAR IN mRNA-INJECTED OOCYTES

A. Nicotinic Cholinergic Receptor-Channel

As often happens in neurobiology, the nicotinic synapse has provided a model for new

techniques and ideas. In this case, the nicotonic ACh receptor was the first receptor or ion channel to be expressed in *Xenopus* oocytes. The pioneering work was performed by Sumikawa et al.,[11] who showed that microinjected mRNA from *Torpedo* produced ACh receptors (as judged by α-bungarotoxin-binding activity) in the oocyte. A short time later, Miledi joined the collaboration and showed that agonist-induced conductances appeared as well.[12] mRNA from innervated or denervated cat muscle also induced ACh receptors. A series of studies showed the expected single-channel conductance and duration, pharmacology, voltage dependence, and other characteristics.[312,313]

The four subunits of the nicotinic ACh receptor have been cloned and sequenced from several species. The laboratory of Numa in Kyoto took the lead in utilizing the *Xenopus* oocyte to express these cloned genes. The first report (Mishina et al.[308]) employed transfection of *Cos* monkey cells with the cloned *Torpedo* genes in an SV40 vector. The mRNA, encoding each of the four subunits, was isolated from these cells and injected into oocytes. The oocytes gave clear responses to ACh and also bound α-bungarotoxin (about 1 fmol/oocyte). All four subunit RNAs were required for robust responses, although very small responses were observed in oocytes without γ-subunits and even smaller but detectable responses were observed without δ-subunit RNA (these small responses from δ-less receptors have been observed consistently in later studies but still not explored quantitatively). Only the α RNA was required for α-bungarotoxin binding, as expected from the data of Tzartos and Changeux.[355]

Two groups exploited the new technique of in vitro RNA synthesis employing the specific RNA polymerase from the bacteriophage SP6.[314-316] For this procedure, the cDNA for each subunit was cloned into a vector downstream from the SP6 promoter; the polymerase and a cap analog were then used to synthesize microgram quantities of the capped message. Oocytes injected with this synthetic mRNA showed responses to ACh and bound α-bungarotoxin.[13,317]

Thus the stage was set for quantitative studies to show that ACh receptors expressed in oocytes display the hallmarks of receptor function as studied at nicotinic synapses. White et al.[317] found that SP6 synthesized *Torpedo* RNA directed ACh receptors with a nonlinear dose-response relation; the Hill coefficient for this relation (the slope on double-logarithmic coordinates at low agonist concentration) was very near 2. Furthermore, prolonged exposure to the agonist caused desensitization. These two characteristics, observed with all known nicotinic ACh receptors, inspired confidence that *Xenopus* oocytes were correctly assembling receptors when provided simply with RNA for all four subunits.

Additional questions concern the homologies and functional relations among the four subunits. Several publications have explored this problem. In their SP6 study, White et al.[317] showed that the δ-subunit from mouse could substitute for that from *Torpedo*, but not for any of the other subunits. Furthermore, responses were larger with the mouse δ-subunit, despite equivalent numbers of surface α-bungarotoxin-binding sites. In an outstanding paper, Sakmann et al.[24] performed similar but more extensive experiments. They had access to the complete sets of four clones for both *Torpedo* and calf AChR. Single-channel and whole-cell clamping showed that all calf receptors expressed in oocytes differed from *Torpedo* receptors in two ways: the calf receptors were open roughly tenfold longer and the channel duration depended on voltage. Single calf subunits also produced functional replacements for the other *Torpedo* subunits; but the calf δ-subunit alone produced the calf-like single-channel duration and voltage sensitivity. Other single calf-for-*Torpedo* subunit substitutions produced much smaller effects on channel duration. Many of these observations have now been confirmed with *Torpedo*-mouse subunit hybrids.[369] Unfortunately, results were not reported with reciprocal hybrids (a single *Torpedo* subunit in an otherwise all-calf receptor), but this experiment did give rise to the working hypothesis that the δ-subunit is responsible for the voltage-dependent conformational change that closes the open channel.

Another type of subunit hybrid experiment was made possible by the discovery in a fetal calf cDNA library of the ε-subunit which is quite homologous to the γ-subunit. The new subunit could also substitute for the *Torpedo* subunit in the oocyte expressions system. Recent Northern blot analysis showed expression of the ε-subunit only in adult muscle and the γ-subunit only in fetal muscle. Furthermore, the αβγδ injections and αβεδ injections produce, respectively, AChR channels with the characteristics of fetal and adult bovine muscle.[321] Thus the possibility arises that this subunit accounts for the difference in channel duration and channel conductance between fetal and adult muscle.

Site-directed mutagenesis is of course the next level of resolution in analyzing structure-function relations at the ACh receptor. In fact, the first publication on this topic antedates the subunit hybrid experiments described above; the α-subunit was used[13] and responses were assessed with voltage recording and iontophoretic application of ACh. The starting point was the theoretical analysis of Finer-Moore and Stroud[322] and of Guy,[323] who predicted a total of five transmembrane α-helices, including one with an amphipathic character, in each of the subunits. Mishina et al. therefore introduced deletions within each of the putative transmembrane regions, including the postulated amphipathic region, and each of these deletions abolished ACh responses. In contrast, there was no major effect of deletions within two putative cytoplasmic regions following the third and fifth helices. These data give support to the theoretical models proposed by Finer-Moore and Stroud[322] and by Guy.[323] However, a potential source of ambiguity is that the deletion technique (restriction cleavage, *Bal31* exonuclease digestion, and relegation with the *SacI* linker CGAGCTCG) resulted in the incorporation an extra pair of cys residues in many cases. Some of the deletion mutants completely abolished α-bungarotoxin-binding activity; for those which did not, the interpetation is unclear because the binding measurement was to lysates rather than to surface receptors.

Mishina et al.[13] also utilized oligonucleotide-directed mutagenesis[324] to introduce single amino acid changes into the receptor in the putative ACh-binding region near the amino terminus. When asn_{141} was changed to asp, there was no α-bungarotoxin-binding and no response, underlining the importance of the presumed glycosylation at this residue. There were no ACh responses following cys-to-ser mutation at any of the sites 128, 142, 192, or 193, although the same mutation at cys_{222} had only minimal effects. These data tend to support the importance of disulfide bonds within this region. In particular, Kao et al.[325] have shown that the affinity ligand MBTA is tethered to cys 192 and 193. However, it should be noted that these individual amino acid mutations could have resulted in the production of ACh receptors with an unpaired cys residue; the resulting cysteic acid moiety might conceivably have produced nonspecific effects on assembly, agonist binding, and α-bungarotoxin binding.

B. GABA and Glycine Receptors

Heterologous mRNA from chick optic lobe,[296] rat brain,[304,326] and human fetal cortex[298] renders the oocytes responsive to GABA. The response is an inward current that desensitizes after reaching a peak. The reversal potential is −25 mV,[296,304] close to Cl equilibrium potential in the oocytes (Section III.C). Unlike the fluctuational Cl^- responses evoked by serotonin, ACh, and glutamate (see following), the current evoked by GABA is smooth[327] and not inhibited by intracellular injection of EGTA.[245,327] The desensitization is less pronounced in chick RNA-injected than in rat RNA-injected oocytes.[304,326] Pharmacology of the response resembles that of GABA responses in nerve cells;[328] it can be evoked by muscimol and inhibited by strychnine (reversibly), bicuculline, penicillin, and picrotoxin, and selectively enhanced by barbiturates and benzodiazepines.[296,298,304,326] The slope of the log dose-log response curve is greater than unity, suggesting that binding of more than a single GABA molecule is needed to open the channel;[296] a more thorough characterization has not been done yet.

Noise analysis of GABA-evoked channel activity in chick brain mRNA-noninjected oocytes yielded values of 25 msec and 4 pS for mean open time and conductance, respectively;[296] a few successful patch clamp recordings suggested two populations of GABA-dependent channels with the average values of 16 msec and 29 pS.[313] The reason for the discrepancy between noise analysis and patch clamp data is not clear; the number of observations was limited and the two populations of channels were not clearly described. Thus, the single-channel properties of the GABA-activated channels expressed in the oocytes await further study.

GABA and glycine have been hypothesized to activate a common channel;[329] potentially, this problem can be studied in the oocytes. Glycine responses appear in oocytes injected with mRNA from human brain,[248] rat brain,[304] and bovine retina.[327] Like the response to GABA, it is a smooth inward current, unaffected by intracellularly injected EGTA,[330] with a reversal potential of about -25 mV.[304,327] The dose-response relationship for glycine displays a Hill slope of about 3. This suggests that at least three glycine molecules have to bind to the receptor in order to activate the channel.[298] The response to glycine is inhibited by strychnine and picrotoxin, like the response to GABA, but it is more sensitive to bicuculline, and the inhibition by strychnine is almost irreversible.[304,298] Unlike the GABA response, the glycine-evoked current is not affected by barbiturates.[298] So far, the ionic selectivity and the pharmacology of glycine response are in general agreement with those of the neurones.[331]

The steady-state I-V curve of the glycine response in oocytes injected with fetal human cortex mRNA showed a negative rectification region at membrane potentials more negative than -70 mV. During voltage steps to such negative potentials (during glycine application), a relaxation of the current was observed. The instantaneous I-V curve just after voltage steps was linear in the whole range tested (down to -160 mV). These results suggested that the probability of a channel to open is lower at negative potentials.[298] The mean time constant of current relaxation (presumably corresponding to mean channel lifetime[332-334]) was 164 msec at -80 mV and 91 msec at -160 mV,[298] much longer than in mouse spinal neurones.[335] It is not clear whether this difference can be attributed to voltage-clamp insufficiencies, to species differences, or to the incorrect expression of glycine-activated channel protein by the oocytes.

C. The Response to Kainate

In oocytes injected with mRNA from human fetal cortex,[336] rat brain,[297] and bovine retina,[327] a response to kainate appears that is never observed in noninjected oocytes. It is a smooth inward current that, unlike the smooth GABA and glycine responses, does not desensitize. The slope of log dose-log response curve is 1.6.[327] The reversal potential of the current evoked by kainate is between 0 and -10 mV; it is unaffected by changes of $(Cl)_{out}$, but shifted to about -20 mV by halving $(Na)_{out}$.[297,327,336] It appears, therefore, that this current is carried by both Na^+ and K^+ through a nonspecific cation channel. It is not known yet to what extent this channel is permeable to Ca^{2+}; the response to kainate is strongly inhibited by Mn^{2+}, but this may be due to an inhibition of Na^+ influx.[327] The steady-state current-voltage relationship of this response is linear down to -100 mV, but shows strong rectification at positive potentials.[297,327] The response to kainate is independent of intracellular Ca^{2+}. Although preliminary, this characterization of the response to kainate reveals several unique properties, the study of which may throw light on the function of kainate in the nervous system. Further use of the oocyte expression system may also lead to identification of the molecular components of the protein or proteins constituting the kainate-activated receptor and channel.

D. The Response to Serotonin

Although the "native" denuded, noninjected oocytes sometimes respond to application of serotonin with a small fluctuational current,[245] the vast majority are unresponsive to this neurotransmitter. In contrast, a large response to serotonin appears in oocytes injected with mRNA from rat brain,[277] human fetal brain,[336] mouse brain,[370] but not chick embryo brain.[307] The response was first described by Gundersen et al.[277] as fluctuations appearing after a long delay; later, it was noticed that a smooth current underlies these fluctuations.[330] The fluctuations ceased at hyperpolarizing voltages; they had a reversal potential between -20 and -24 mV, as expected for a Cl^- current.[277,336] The fluctuational response to serotonin is suppressed by LSD and methysergide, but weakly by cyproheptadine and ketanserine,[336,337] suggesting that the receptor is of the S_1 type.[338] Our recent results suggest that it is the $5HT_{1c}$ subtype of serotonin receptor and that the choroid plexus contributes most of the mRNA encoding this response.[307]

Dascal et al.[245] found that, with well-prepared rat brain mRNA, it takes about 36 to 48 hr for full serotonin responsiveness to develop (when the oocytes are incubated at room temperature). The response to a saturating concentration of serotonin (about 10 μM) consists of a large (up to 20 μA at a holding potential of -60 mV) transient peak followed by a much smaller (50 to 150 nA), long-lasting, smooth inward current, accompanied in many cases by inward current fluctuations. The first peak appears to be the result of a "summation" of many fluctuations: the fluctuational response is seen when a saturating dose of serotonin is applied a relatively short time (<24 hr) after mRNA injection or when a threshold (2 to 10 nM) concentration of the transmitter is applied in an oocyte with fully developed serotonin responsiveness. In both cases, it can be assumed that the number of serotonin-activated receptors is low. As the time passes after RNA injection, the serotonin-evoked fluctuations become larger and appear synchronously at the beginning of the response.

In all, the response to serotonin strongly resembles the "native" oocyte response to ACh (but it is not due to activation of a muscarinic receptor because it is not inhibited by atropine) and may be suspected to be mediated by a similar biochemical mechanism. The similarity is further emphasized by the recent finding by Parker et al.[330] that the injection of EGTA in amounts that were sufficient to suppress $I_{Cl(Ca)}$ abolished the fluctuations evoked by serotonin. The authors reported that the smooth serotonin-evoked inward current was not suppressed and that the changes in conductance that underlie this current are an increase in Cl^- conductance and a decrease in another (possibly K^+) conductance (exact values of reversal potential were not reported). Dascal and co-workers confirmed these results; however, at large (0.5 to 1 nmol/oocyte) doses of injected EGTA, the smooth Cl^- current component of serotonin response was also abolished.[245,360] Thus, serotonin-evoked Cl^- currents are Ca dependent. Like ACh in the "native" oocytes, serotonin in rat brain RNA-injected oocytes causes a significant increase of Ca^{2+} efflux.[371] ACh- and serotonin-evoked responses exhibited a clear cross-desensitization, again suggesting convergence onto a common pathway.[245] Involvement of a G-protein (as in the case of ACh; see Section XII.D) is further supported by the fact that PTX, which was shown in many instances to inhibit hormone-stimulated polyphosphoinositide turnover,[200,339-342] inhibited the response to serotonin by about 50%.[245]

The evidence presented thus far suggests that, as for the endogenous muscarinic response, the chain of events initiated by serotonin is receptor-mediated activation of a G-protein → activation of phospholipase C → elevation of the levels of IP_3 and diacylglycerol → release of Ca^{2+} from intracellular stores → opening of Ca-dependent Cl^- channels. All the links of this chain, except the serotonin receptor, are already present in the oocyte **before** the injection of exogenous mRNA. Thus, one may hypothesize that brain RNA-directed synthesis of serotonin receptors may be enough to give rise to the observed responses. In support of this hypothesis, injection of size-fractionated rat brain mRNA indicated that the response to

serotonin is directed by a single fraction containing 5 to 6 kbase mRNA.[307] Activation of serotonin receptors in the choroid plexus leads to increased polyphosphoinositide turnover;[343,344,372] thus, coupling of a newly synthesized receptor to a preexisting G-protein-phospholipase C system will not be surprising. It is also possible, of course, that the synthesis of several other enzymes participating in this chain is also directed by the exogenous mRNA. Whether the synthesis of the receptor protein alone is enough to render the oocyte responsive to serotonin is an open question that may possibly be answered once the cDNA coding for the receptor is available.

The response to serotonin is inhibited by high doses of theophylline and papaverine (the explanation may be similar to that proposed for the effect of PDE inhibitors on the endogenous ACh response; see Section XII.E), by high (1 *mM*) concentrations of adenosine, dopamine, and epinephrine,[277,337] and by reasonably low (50 μM) concentrations of prostaglandins E_2 and D_2.[337] The latter effects are unexplained; any speculations will be superfluous in view of the diversity of cellular processes that may be affected by these agents. An additional interesting feature is the enhancement by serotonin of the hyperpolarization-evoked, Ca-dependent transient Cl^- current (see Section VI.D); the molecular mechanism of this phenomenon is not known.

Although it is too early to draw definite conclusions as to the relevance of the serotonin-evoked events observed in mRNA-injected oocytes to the processes that may take place in the choroid plexus, it seems safe enough to say that at least some of the observed events may be inherent to the oocytes. Therefore, at present the oocyte should be considered a good model for the study the serotonin receptor itself or its coupling to second messenger events; it would be incautious to speculate on electrical processes that this transmitter may produce in brain cells based on the experiments in mRNA-injected oocytes.

E. Other Neurotransmitter Responses

Injection of rat brain mRNA renders the oocytes responsive to several other neurotransmitters. Among them are glutamate and other agonists of excitatory amino acid receptors,[297,304] norepinephrine, and dopamine.[345] The response to ACh is also greatly enhanced and appears in oocytes of frogs that have been unresponsive to ACh or lost their responsiveness as a result of collagenase treatment.[127,346] Gundersen et al.[297] have shown that glutamate (and several other amino acids such as malate and quisqualate) produces a two-component response consisting of (1) a smooth inward current with a reversal potential of about -8 mV and (2) inward current fluctuations with a reversal potential of -24 mV. The fluctuations were not affected by changes in extracellular Na^+ concentrations and showed a rectification at hyperpolarizing membrane potentials similar to that displayed by ACh-evoked fluctuations. The glutamate-evoked fluctuational response thus seems to be carried by Cl^-.[297] The ionic nature of the smooth currents has not been studied. Sumikawa et al.[306] found that, with mRNA fractionated on sucrose gradient, the smooth and the fluctuational components of glutamate response appear separately in oocytes injected with different fractions of RNA. Thus, the two response components may be mediated by different receptors.

Sumikawa et al.[345] also demonstrated that a response similar to that evoked by glutamate can be elicited by norepinephrine and dopamine in oocytes injected with mRNA from rat brain. The smooth component of the response to norepinephrine had a reversal potential of about 0 mV; the fluctuations reversed between -20 and -30 mV. While no conclusions can be drawn concerning the similarity of the smooth component to "smooth" currents evoked by other transmitters described previously, the fluctuational response is almost certainly identical to that evoked by ACh (in noninjected or RNA-injected oocytes), serotonin, or glutamate (in rat brain mRNA-injected oocytes). The pharmacology of this component is rather unclear. The β-adrenergic antagonist, propranolol, alone elicited a smooth inward current in the oocytes (whether injected or uninjected with mRNA). This current was most

probably the result of a decrease in membrane conductance to K^+. The mechanism of this effect is absolutely obscure. Propranolol also blocked the fluctuational response to norepinephrine; the latter (and the similar response evoked by dopamine) was blocked by the serotonergic antagonist methysergide. The significance of these findings, as well as the selectivity of the effects of the various antagonists tested, is not clear. A more extensive pharmacological study will be necessary to clarify whether the responses to the various agonists mentioned above are not mediated by a single receptor, and indeed if there are separate receptors, what types and subtypes of the catecholamine receptors underlie these responses.

Beyond the uncertainties concerning the pharmacology of the various putative receptors expressed in the oocytes following the injection of rat (and, for many of the transmitters, human fetal) mRNA, there is one striking feature that is rather clear: several neurotransmitters, many of them acting on pharmacologically distinct receptors, elicit a practically identical response. The list incudes serotonin, ACh, glutamate, norepinephrine, and dopamine. The response is inward current fluctuations carried by Cl^-, similar to those evoked by muscarinic agonists in noninjected follicular oocytes. Another feature in common is that all these transmitters also elicit a "smooth" inward current; however, on the basis of present data, it is impossible to state that in all cases the same membrane conductance is affected. Finally, all these agonists enhance the hyperpolarization-evoked Ca-dependent Cl^- current.[297,330,345] The response to at least two of the transmitters listed, i.e., serotonin and ACh, is partially inhibited by PTX[245,346] and dependent on internal Ca^{2+} (see the previous section). These facts suggest that all agents mentioned previously may activate a common system leading to the same membrane events.[345] We propose that they all activate, through different receptors, the G-protein-dependent phospholipase C, leading to Ca^{2+} release from intracellular stores, just as in the case of endogenous muscarinic response (see Sections XII.D and XV.D). In fact, as with the serotonin receptor (Section XV.D), one can imagine that the mRNA-directed synthesis of a receptor protein is the only event necessary in order for a given neurotransmitter responsiveness to appear; the newly synthesized receptor would then couple to the system preexisting in the oocytes. Furthermore, it may even be possible that a receptor that is usually coupled to a different kind of G-protein in the source tissue would interact with the phospholipase C-coupled G-protein if the latter is dominant in the membrane of the oocyte. Such coupling to "wrong" G-proteins has been demonstrated for β-adrenergic receptors in an artificial reconstitution system.[347] Thus, when interpreting the data obtained in the RNA-injected oocytes, one should be careful not only with respect to the type of ionic conductance that a certain transmitter may affect in the source tissue (brain), but also with respect to the second messenger system to which it may be coupled.

XVI. VOLTAGE-DEPENDENT CHANNELS THAT APPEAR IN mRNA-INJECTED OOCYTES

A. The Voltage-Dependent Na Channels

Messenger RNA extracted from rat brain, cat muscle, *Electrophorus electricus* electric organ, or embryonic human brain induces voltage-dependent Na channels when injected into *Xenopus* oocytes.[336,348] The resulting currents have many characteristics of normal Na channels from the source tissue, such as inactivation after a few milliseconds, blockade by nanomolar concentrations of tetrodotoxin, and single-channel conductances of 20 pS.[26] Interestingly, even though denervated mammalian muscle develops tetrodotoxin-resistant Na channels, the Na channels induced by RNA from such muscle are still blocked roughly 50% by 10 n*M* tetrodotoxin.[348] Analysis of the waveform of the voltage-clamp currents suggested that both rat brain and cat muscle induce at least two populations of Na channels.[348]

Several groups have reported partial purification of the RNA encoding these voltage-

sensitive Na channels. Sumikawa et al.[306,345] used a sucrose-gradient fractionation and found that the Na channels were induced by a single fraction near the bottom of the gradient, suggesting that no low molecular weight RNAs are needed to form a functional Na channel. A similar sucrose-gradient fractionation was reported by Goldin et al.[350] In the latter study, the gradient fractions were also subjected to agarose gel electrophoresis; hybridization with a labeled poly-T probe showed that no low molecular weight RNA was detectable in the high molecular weight fractions that induced Na channels.

Sequence data have been obtained from cDNA clones for electrically excitable Na channels from *E. electricus* electroplaques and from rat brain,[352,373] where there are three distinct Na channel messages. Goldin et al.[350] studied the hybridization of a clone encoding one of these rat brain channels to the sucrose gradient fractions. Only the active fractions contained message which hybridized to the clones. These data all suggest strongly that only a single message class, corresponding to the 250-kdalton α-subunit, is needed to induce functional Na channels; the 30- to 40-kdalton β-subunits would either be unnecessary or endogenous to the oocyte. The most decisive experiments, however, involved highly purified mRNA for the α-subunit, either hybrid selected using partial length clones[350] or in vitro synthesized using full-length clones and the SP6 promoter/polymerase system.[309] This RNA still induced Na channels in the oocyte. A recent study[349] reveals though an important difference between Na channels directed by size-fractioned high molecular weight rat brain mRNA and those directed by the heterologous mRNA or mRNA pooled from all fractions. The former had a slower inactivation than the latter as revealed by "big patch" and single-channel recordings. These results imply that the small subunits may play a role in determination and/or modulation of channel kinetics.

Thus the oocyte expression system has provided a clear answer to a question involving structure-function relations at Na channels; only the α-subunit need be specified by endogenous mRNA for expression, assembly, and function of Na channels. This result agrees with bilayer reconstitution experiments.[353] It remains possible, of course, that the oocyte itself can synthesize β-subunits or analogous proteins that serve the same role. In the future, oocyte expression will doubtless be used in site-directed mutagenesis experiments to explore more details of the function of the channel protein.

B. The Voltage-Dependent Ca Currents

Our experiments[111,354] show that in oocytes injected with mRNA from rat heart, brain, or skeletal muscle $I_{Cl(Ca)}$ is significantly larger than in noninjected oocytes, suggesting that the underlying Ca^{2+} influx is enhanced. Indeed, in high-Ba^{2+}, Cl^--free solution (see Section VI.C), Na-independent inward currents that are blocked by low doses of Cd^{2+} are observed with RNA from all three tissues. It is assumed that these currents represent the influx of Ba^{2+} through the voltage-dependent Ca^{2+} channels. In oocytes injected with heart RNA, two components of the inward Ba^{2+} current (I_{Ba}) can be distinguished: a transient one, that decays in less than 0.5 sec and a long-lasting one that does not appreciably inactivate even if the cell is kept depolarized for several seconds.

In our experiments, the slow component of I_{Ba} in heart RNA-injected oocytes was the dominant one; in many cases, the fast component could not be detected at all. The slow component of I_{Ba} was selectively blocked by 0.1 to 10 μ*M* nifedipine, whereas the fast component was nifedipine resistant. Another way of separating the two components is by using the differences in their inactivation properties: at a membrane potential of −20 mV, the fast component is almost completely inactivated, whereas the slow one can still be elicited by stepping the voltage to more positive values. The two components also differ (although less strikingly) in their activation properties, the slow component being activated at more positive potentials. In all these characteristics, the two components resemble their counterparts in the real heart cells.[122,356] We have concluded, as for the Ca^{2+} currents in

the heart cells, that the two components of I_{Ba} in RNA-injected oocytes represent two distinct voltage-dependent Ca^{2+} channels. However, we cannot yet be sure that the fast component of I_{Ba} in RNA-injected oocytes is distinct from the endogenous current seen in noninjected oocytes, although there are several indications that they are different. First, in RNA-injected oocytes the fast component sometimes attained amplitudes as large as 100 nA, whereas in noninjected oocytes it did not exceed 25 nA. Second, the inactivation of the fast I_{Ba} in RNA-injected oocytes is only half-maximal at -40 mV, whereas in noninjected oocytes at this potential I_{Ba} is 90% inactivated (see Section VI.C).

Another hallmark of identity of the voltage-dependent Ca^{2+} current in heart and in the mRNa-injected oocytes is the regulation by neurotransmitters and second messengers: I_{Ba} in the oocytes is potentiated by β-adrenergic stimulation, by the adenylate–cylase activator forskolin, and by intracellularly injected cAMP; it is inhibited by ACh. These are the classical regulatory phenomena in the heart.[183,185] Interestingly, the slow component of I_{Ba} in brain RNA-injected oocytes is not nifedipine sensitive and is not potentiated by forskolin.[357] Since the phenomenology and the involvement of second messengers in regulation of Ca^{2+} channels in the brain is much less well understood than that of heart Ca^{2+} channels, the significance of these preliminary results is not clear. However, the differences between the slow Ba^{2+} currents originating from heart and brain raise the hope that the oocyte may be successfully used for a study of regulation of brain Ca^{2+} channels.

C. Voltage-Dependent K Channels

Two voltage-dependent K^+ conductances that appear in mRNA-injected oocytes have been reported. Gundersen et al.[358] have shown that the dominant current that develops in oocytes injected with RNA from the brain of the electric fish (*Torpedo*) is a slowly inactivating, Ca-independent K^+ current resembling the classical delayed rectifier. Noise analysis revealed an average single channel lifetime of 14 msec and conductance of 14 pS at -10 mV. The current was blocked by TEA and, in a use- and voltage-dependent fashion, by 3,4-diaminopyridine. Under some conditions, diaminopyridine seemed to promote the process of channel inactivation, turning the current into a transient one, resembling the A-current. (This may also be interpreted as a block of open channels.) We have sometimes observed a similar current (albeit much smaller) in oocytes injected with rat brain mRNA.[374]

The dominant voltage-activated K^+ current that appears in oocytes injected with whole or fractionated rat and chick brain mRNA is a transient one,[306,348] resembling the A-current. This current was only preliminarily characterized. It activates at voltages more positive than -50 mV, reverses at -100 mV, and is reduced by TEA and by La^{3+} and Mn^{2+}. The latter fact raises the possibility that there might be Ca-dependent and Ca-independent components of this current, as shown in other preparations.[359]

XVII. CONCLUSIONS

The following facts emerge from this review.

1. The oocyte is an excellent preparation for the study of ion channels, especially those regulated by second messengers.
2. The oocyte is presently the most convenient system to study RNA-induced exogenous channels by various kinds of electrophysiological approaches.
3. The oocyte offers the opportunity (not always exploited) to do biochemistry in few or single cells and to correlate between biochemistry and electrophysiology, both in terms of the timing and also nature of the process.
4. The oocyte has a well-characterized "background": membrane properties, intracellular ion activities, channels. Of course, there are open questions.

5. The endogenous channels are diverse, some unique, some probably common with other tissues. Further study in this direction is promising.
6. The endogenous neurotransmitter responses are mediated by second messengers. There has been rapid progress in research on these responses, which are now better understood than in many "classical" preparations, but again, there are open (exciting) questions.
7. The problem of cAMP-dependent K channels remains. Where they are?
8. RNA-induced channels open up a whole new world. The oocyte will be especially important as an assay for manipulations involving molecular biology.

ACKNOWLEDGMENTS

The author is grateful to Dr. H. A. Lester for writing several portions of this review, for revising the manuscript, and for many discussions. The author also thanks Professor R. Kado and Dr. L. A. Jaffe for reading the manuscript and for helpful remarks. During the preparation of this review, the author was supported by a Bantrell Fellowship, by NIH Grant GM-29836 at the California Institute of Technology, and by a Schreiber Foundation grant at Tel-Aviv University.

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