J. Physiol. (1984), **352**, *pp.* 551–574
With 13 *text-figures*
Printed in Great Britain

XENOPUS OOCYTE RESTING POTENTIAL, MUSCARINIC RESPONSES AND THE ROLE OF CALCIUM AND GUANOSINE 3′,5′-CYCLIC MONOPHOSPHATE

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(*Received 16 June 1983*)

SUMMARY

1. Resting potential (r.p.) and muscarinic response mechanisms were studied in *Xenopus laevis* oocytes using the voltage-clamp technique.

2. Insertion of micro-electrodes into the oocyte produced a 'shunt' membrane conductance which partially sealed after a few minutes.

3. The oocyte resting potential (measured with a single intracellular electrode) ranged from −40 to −60 mV. Ouabain and low K^+ solution depolarized both follicles and denuded oocytes. The electrogenic Na^+–K^+ pump was more active in the latter.

4. In the presence of ouabain, the r.p. agreed with the constant field theory. α (P_{Na^+}/P_{K^+}) was 0·12 in follicles and 0·24 in denuded oocytes. β (P_{Cl^-}/P_{K^+}) was 0·4 in both. At $[Na^+]_o$ lower than 70 mM, the r.p. deviated considerably from the constant field predictions. The relatively large value of α indicated the major role of Na^+ in oocyte r.p. determination.

5. The oocyte muscarinic response was separated into four distinct components: the fast depolarizing Cl^- current, 'D_1'; the slow depolarizing Cl^- current, 'D_2'; the slow hyperpolarizing K^+ current, 'H'; and the large membrane Cl^- current fluctuation, 'F'.

6. The H response reversal potential showed a Nernst relationship to $[K^+]$ and was selectively blocked by intracellular injection of tetraethylammonium (TEA). The D_1 and D_2 reversal potential showed a Nernst relationship to $[Cl^-]$.

7. In Ca^{2+}-deficient, EGTA-containing medium, D_2 and F were abolished and D_1 and H were reduced. Verapamil inhibited all responses. Increasing $[Ca^{2+}]_o$ caused a significant increase in D_1, D_2 and F response amplitudes.

8. Intracellular injection of 0·6–10 pmol guanosine 3′,5′-cyclic monophosphate, induced a large outward K^+ current, similar to the muscarinic H response.

INTRODUCTION

Recently, the importance of *Xenopus* oocytes as a model for studying membrane mechanisms underlying cellular response to neurotransmitters has become increasingly

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obvious. Specific membrane responses to cholinergic (Kusano, Miledi & Stinnakre, 1977, 1982; Dascal & Landau, 1980, 1982), purinergic (Lotan, Dascal, Cohen & Lass, 1982) and adrenergic (Kusano *et al.* 1982) agonists were extensively studied.

The *Xenopus* oocyte resting membrane characteristics were studied by several authors (Moreau, Guerrier & Doree, 1976; Belle, Ozon & Stinnakre, 1977; Wallace & Steinhardt, 1977; O'Connor, Robinson & Smith, 1977; Robinson, 1979; Kado, Marcher & Ozon, 1981; Lee & Steinhardt, 1981; Baud, Kado & Marcher, 1982; Miledi, 1982). As a further step in characterizing this important experimental model, we aimed to study, quantitatively, the ionic mechanisms underlying the oocyte resting potential (r.p.). Na^+ ions were shown to play an important role in determining r.p. This was not considered in the aforementioned studies. The relative permeabilities of Na^+, K^+ and Cl^- were calculated and the role of the electrogenic Na^+–K^+ pump was re-evaluated. Results obtained from intact follicles were compared to those obtained in denuded oocytes.

The ionic mechanism of oocyte muscarinic response was analysed in terms of four distinct components: the 'fast' depolarizing current, 'D_1', and the slow depolarizing current, 'D_2' (both mediated by Cl^-); the slow hyperpolarizing current, 'H' (mediated by K^+), and the membrane current fluctuation 'F' (mediated by Cl^-). Also, the possible role of guanosine cyclic 3′,5′-monophosphate (cyclic GMP) in oocyte muscarinic response was investigated by intracellular injection of the cyclic nucleotide.

METHODS

Preparation

Groups of twenty-five mature female frogs, *Xenopus laevis*, were obtained once in every 6–9 months from the South African Snake Farm (Fish Hoek). The frogs were kept in a tank and fed twice a week with diced liver. In the preliminary experiments (summer 1979) the frogs were anaesthetized by hypothermia (Dumont, 1972). Later on, a 0·13–0·17% solution of tricaine methanesulphonate (Brown, 1970) was used. Small incisions were made on the frog's abdomen, and portions of the ovary removed and placed in frog Ringer, Merriam or Steinberg solution (solutions 1–3, Table 1). The incisions were closed by silk sutures; post-operative animals were returned to the tank. Up to ten consecutive dissections could be performed on a single frog before it was killed. Some frogs were used again several months later, when all the incisions on the abdomen healed; these animals' oocytes exhibited normal responses to acetylcholine (ACh). In all experiments, fully grown (stages 5 and 6, see Dumont, 1972) oocytes were used, as follicles or as denuded oocytes. *Follicle* is an oocyte surrounded by its outer 'envelope' as it appears in the intact ovary. The envelope consists of four layers (from the oocyte's membrane to the exterior): the vitellogenic membrane, a thin non-cellular layer; a monolayer of follicular cells; a non-cellular fibrous layer (theca) with unevenly distributed fibroblasts; a monolayer of epithelial cells, by means of which the oocyte is connected to the wall of the ovary (see Dumont, 1972; Dumont & Brummett, 1978). A *denuded oocyte* is the oocyte free of the outer cellular layers and of the theca. Follicles were singled out from the pieces of ovary by cutting the connexion of the epithelial layer to the ovary wall with microscissors. Denuded oocytes were obtained by defolliculating the oocytes as described below.

Defolliculation of oocytes

The cells surrounding the oocyte can be removed (i.e. the oocyte 'denuded') mechanically, by forceps (Masui, 1967) or by the use of collagenase (Eppig & Dumont, 1972; Moreau *et al.* 1976). Single follicles or small pieces of the ovary were placed for 2·5 h at room temperature in plastic tubes containing collagenase (Sigma, type 1A; 2 mg/ml), dissolved in Ringer solution. The tubes were shaken or vortexed periodically (every 5–15 min) for 0·5–1 min. Some of the oocytes treated in this way were damaged and the cell membrane was ruptured. The undamaged oocytes were placed for at least 30 min in normal Ringer solution before the experiment started. This treatment was

TABLE 1. The composition of solutions used in the *Xenopus* oocyte study

No.	Solution	Constituents (mM) Na^+	K^+	Ca^{2+}	Mg^{2+}	$Tris^+$	Cl^-	SO_4^{2-}	Sucrose	Osmolarity*
1	Normal Ringer	116	2	1·8	1	5	128·6	—	—	254·4
2	Steinberg†	58	1	1·8	1	5	68·6	—	—	136·4
3	Merriam†	90	1	0·74	0·82	5	97·58	0·82	—	195·86
4	OR-2†	82·5	2·5	1	1	5	94	—	—	188
5	Low Na^+ (Tris)	—	2	1·8	1	121	128·6	—	—	254·4
6	Low Na^+ (Mg^{2+})	—	2	1·8	59	5	128·6	—	55	254·4
7	High K^+	93	25	1·8	1	5	128·6	—	—	254·4
8	Low K^+ (Tris)	93	—	1·8	1	30	128·6	—	—	254·4
9	Low K^+ (Mg^{2+})	93	—	1·8	13·5	5	128·6	—	12·5	254·4
10	Low Cl^-	116	2	1·8	1	5	12·6	58	58	254·4
11	High Mg^{2+}	70	2	1·8	56	5	80·6	55	—	269·4
12	Low Mg^{2+}	70	2	1·8	1	5	80·6	—	108	269·4
13	Normal Ca^{2+}	116	2	1·8	17·2	5	161	—	—	303
14	High Ca^{2+}	116	2	18	1	5	161	—	—	303
15	Normal Mg^{2+}	116	2	1·8	1	5	128·6	—	18	272·4
16	High Mg^{2+}	116	2	1·8	10	5	128·6	9	—	272·4

* Assuming an activity coefficient of 1 for all constituent ions.
† Modified from: 2, Robinson, 1979; 3, Merriam, 1971; 4, Wallace, Jared, Dumont & Sega, 1973.

shown by histological methods (I. Lotan, personal communication) to remove all epithelial and, in most oocytes, all follicular cells, while the vitellogenic membrane remained intact. In some experiments, denuded oocytes which exhibited electrophysiological responses were studied histologically to verify the complete removal of theca and follicular cells.

Solutions

The experiments were performed at room temperature, 20–24 °C. The solutions had a pH of 7·4–7·6; all solutions used in a single experiment had the same pH, within ±0·02. A list of solutions is given in Table 1. Whenever the effect of changes of any ion concentration was studied, the concentrations of other ions, and the total concentration of particles (the osmolarity of the solution), were kept constant. The only exception was allowed when the effects of changes in $[Mg^{2+}]$ (external concentration) on r.p. (solutions 11 and 12) or on the reversal potential of D_1 (solutions 15 and 16) were studied. Here, the changes in $[Mg^{2+}]$ were accompanied by parallel changes in $[SO_4^{2-}]$.

Intermediate concentrations of any ion under study were obtained by mixing two parental solutions (Table 2). Again, the mixing kept the osmolarity and the concentrations of all ions constant, except the ion under study and the ion which substituted for it.

In most cases, the experiments were performed in Ringer solution, and all data (unless stated otherwise) refer to this solution.

Electrophysiology

Electrophysiological experiments were performed on single oocytes (follicles or denuded). An oocyte was placed in a 1 ml capacity bath with a paraffin-covered bottom. A hemispheric hole about 0·7 mm in diameter was made in the paraffin layer, and the oocyte was partially immersed into the hole. Since the hole diameter was smaller than that of the oocyte (1·1–1·3 mm), the major part of the oocyte was exposed to the bathing medium. Throughout the experiment, the bath was constantly perfused with physiological solution at a rate of 5–15 ml/min. The 'dead time' of the perfusion system varied in different experiments between 3 and 15 s; the rate of flow was always constant during a single experiment. Bath application of the substances under study was done by flowing the solution containing the substance without changing the rate of flow. The extracellular and intracellular injection of ACh, and the intracellular injection of tetraethylammonium (TEA), were performed ionophoretically. The intracellular injection of cyclic GMP was done by pressure. For measurement of membrane potential and for current injection, conventional 3 M-KCl-

filled electrodes, having a resistance of 3–10 MΩ, were used. The cell was impaled by one, two or three electrodes, according to the design of the experiment. The voltage-recording electrode was connected through a cathode follower to the lower beam of a dual beam oscilloscope (Tektronic 565 or Tektronix 5103N) and, in parallel, to the input of the voltage-clamp device. The clamp apparatus was built in our laboratory and employed high voltage (120 V) operational amplifiers. Whenever a voltage-clamp experiment was designed, the oocyte was impaled by a second, 'current' electrode, and the membrane potential was set constant, routinely at −50 mV, by the use of a conventional voltage-clamp technique. The bath was connected to the ground Ag/AgCl electrode through a Ringer/agar bridge; in experiments involving changes in bathing chloride concentration,

TABLE 2. The method for obtaining intermediate concentrations of ions by mixing the parental solutions

	Parental solutions	
Ion under study	Option 1	Option 2
Na^+	1 and 5	1 and 6
K^+	7 and 8	7 and 9
Cl^-	1 and 10	—
Mg^{2+}	11 and 12	15 and 16

3 M-KCl/agar bridges were employed. At the end of the experiment (i.e. the recording in an oocyte), the potential drift of the voltage electrode was checked. As a rule, the drift was less than ±2 mV; otherwise the results were discarded. The membrane current was displayed on the upper beam of the oscilloscope. The output of the lower beam amplifier (membrane voltage) was connected to the X input of the X–Y recorder (Hewlett-Packard 7035B) and to the lower pen of a two-channel chart recorder (YEW 3022). The output of the upper oscilloscope beam amplifier (membrane current) was connected to the Y input of the X–Y plotter and to the upper pen of the chart recorder. To study the voltage–current relationship and the ACh response reversal potential, a ramp method was used in most cases. The output of the Wavetek VCG 112 oscillator was connected to the command input of the voltage-clamp device, and a ramp-like change of the holding potential was superimposed on the 'steady' holding potential. The ramp duration was 0·8–1 s when D_1 was studied. Otherwise it was 4–5 s. The resulting changes in the membrane current and potential were recorded simultaneously on the X–Y plotter. Thus, the voltage–current (V–I) curve was plotted automatically on a millimetric paper.

Reversal potential (V_r) of the membrane responses to ACh and other substances were measured in most cases using the same ramp method. A ramp was applied before the application of the substance (resting V–I curve) and at the plateau of the response (unless stated otherwise). The reversal potential was determined from the crossover point of the two curves, i.e. the membrane potential at which the application of the substance does not cause a net change in the membrane current (see Fig. 7). Short ramps lasting 0·8–1 s were employed since the D_1 plateau normally lasts for 1–4 s. The validity of these measurements was tested as shown in Fig. 7. In all tests ($n = 8$), the conventional method and the ramp method gave the same V_r values, within ±1 mV. The input resistance of the membrane, R_{in}, was calculated from the slope of the linear V–I curve at −50 mV.

Intracellular injection of substances

The injections were performed by a third micropipette, while the oocyte membrane was clamped by the two other electrodes. Both ionophoretic and pressure injections were performed.

Ionophoretical injection of TEA. This was done by a glass micropipette, filled with 2 M-TEA, having a resistance of 90–100 MΩ, which was connected to a constant current unit (Grass CCU1). The second output of the current unit was connected to the bathing solution indirectly, with an agar/Ringer bridge. This bridge was independent of the first one, which connected the bathing solution to the common ground. The injected current was monitored with a second oscilloscope (Tektroniks 565).

Pressure injection. Injection of cyclic GMP and GMP (sodium salts, Sigma) was achieved using broken pipettes with tips of 2–4 μm filled with 0·02–0·1 M-cyclic GMP or -GMP (pH = 6·4). The

distal end of the injection pipette was connected by a nylon pipe to a balloon containing 95% O_2–5% CO_2 gas mixture through a plastic valve. The valve was opened manually for the desired period of time. When the access of the gas from the balloon was turned off, the distal end of the pipette became connected to the open air. This adjustment prevented the residing of extra pressure in the pipette after turning the valve off. Before the insertion of the injection pipette into the cell the pipette was placed under a microscope, its tip immersed in a drop of paraffin oil, and the diameter of the fluid drop emerging from the pipette under a given pressure in a given period of time was measured. Thus, the volume of the drop (usually less than 0·4% of the oocyte volume) and the amount of the substance to be injected into the cell were calculated. Volume measurements were repeated, as a rule, after the withdrawal of the pipette from the cell at the end of the experiment. In a number of experiments the injection pipettes were blocked, probably by the yolk. This could be detected in the course of the experiment from the decreased amplitudes of the responses at repetitive injections, and verified by testing the pipette under the microscope as described above. The results of such experiments were discarded.

RESULTS

(*a*) *Sealing of oocyte membrane during intracellular recording*

It was consistently noticed that the amphibian oocyte r.p. slowly 'repolarized' to more negative values, reaching a steady value within a few minutes after penetration (Maeno, 1959; Dick & McLaughlin, 1969; Wallace & Steinhardt, 1977; Kusano *et al.* 1982).

This phenomenon was further studied by sequential penetration and withdrawal of up to three intracellular electrodes and parallel measurements of r.p. and the oocyte input impedance (R_{in}) (see Methods).

One such experiment is shown in Fig. 1*A*. After penetration with the first electrode, the immediate r.p. was −25 mV and it stabilized to −40 mV. Further penetrations by the second and third electrodes caused immediate depolarization and partial recovery. Withdrawal of the electrodes caused immediate large depolarization and a rapid recovery. No voltage gradient was detected between the intracellular electrodes. Occasionally, R_{in} was determined and was found to exhibit a linear relationship to r.p. (Fig. 1*B*). The magnitude of the instantaneous depolarization (following penetration) varied widely (3–30 mV) and was observed both in follicles and denuded oocytes in Ringer, Steinberg, Merriam and OR-2 solutions (see Table 1) at any time (up to 7 h) after dissection from the ovary. Exposure to ouabain (10^{-4} M), which completely blocked the electrogenic Na^+–K^+ pump (see below), did not abolish the instantaneous depolarization and the subsequent recovery. These results were analysed by assuming that the electrode insertion produces a shunt resistance R_s, having an electromotive force E_s, defined by Goldman's equation for a 'wide', non-specific channel where $P_{Na^+} = P_{K^+} = P_{Cl^-}$. If the *true* membrane potential (E_m) and input impedance (R_m) are estimated from the *measured* r.p. and R_{in}, the following relationship must hold:

$$r.p. = \frac{R_{in}}{R_m}(E_m - E_s) + E_s. \quad (1)$$

Indeed, a strict linear relationship was found between r.p. and R_{in} in five oocytes (two follicles and three denuded; see Fig. 1*B*). Extrapolation of the lines obtained in five experiments to $R_{in} = 0$ gave an estimate of E_s of $-11{\cdot}6 \pm 2{\cdot}7$ mV (mean ± S.D.).

This value was correlated with the theoretical E_s, calculated from Goldman's equation with $P_{Na^+} = P_{K^+} = P_{Cl^-}$:

$$E_s = 58 \lg \frac{(a^o_{Na^+} = 87) + (a^o_{K^+} = 1{\cdot}5) + (a^i_{Cl^-} = 33{\cdot}2)}{(a^i_{Na^+} = 20) + (a^i_{K^+} = 88) + (a^o_{Cl^-} = 96)} = -13{\cdot}1 \text{ mV} \quad (2)$$

(a denotes the activity (mM) of a given ion).

$a^i_{K^+}$ was calculated from the purinergic H response reversal potential ($-102{\cdot}7$ mV; Lotan *et al.* 1982). $a^i_{Cl^-}$ was calculated from the cholinergic D_1 response reversal

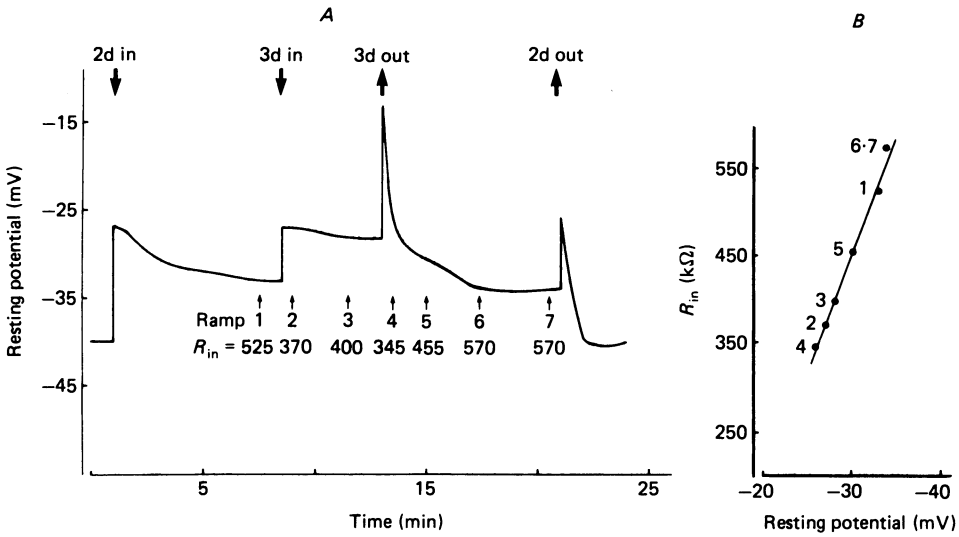

Fig. 1. Sequential insertion and withdrawal of three intracellular electrodes in *Xenopus* oocyte. *A*, time course of the immediate depolarization and the partial recovery, when the second and the third electrodes were inserted or pulled out (indicated by the arrows at the top). The cell was occasionally voltage clamped for a short period and a ramp command voltage was applied in order to measure R_{in} (small arrows 1–7 under the trace). *B*, a plot of the oocyte input resistance (R_{in}) *vs.* the resting potential at several points along the experiment shown in *A*.

potential ($-24{\cdot}2$ mV; see below). $a^i_{K^+}$ and $a^i_{Cl^-}$ were probably unchanged during prolonged clamping since the reversal potentials of the muscarinic responses were stable. $a^i_{Na^+}$ was taken from direct measurements of Kusano *et al.* (1982). Extracellular activities were calculated with activity coefficient of 0·75 for all monovalent ions in normal Ringer solution (Dick & McLaughlin, 1969).

It was thus concluded that a shunt resistance is produced by pricking the oocyte membrane with an intracellular electrode, as shown by the linear relationship of r.p. and R_{in} (Fig. 1 *B*). Also, the E_s value obtained by extrapolation ($-11{\cdot}6$ mV), was similar to the theoretical E_s, calculated from the 'non-specific' Goldman's equation. Oocytes never depolarized to more than -10 mV, even after many insertions and withdrawals of intracellular electrodes. The rapid recovery following withdrawal of electrodes probably indicated a rapid sealing of the oocyte membrane.

(*b*) *The steady-state r.p. of Xenopus oocytes*

Estimates of r.p. were obtained in cells which showed a clear 'repolarization' following insertion of the intracellular electrodes. Steady depolarization following insertion was assumed to result from membrane damage, and the cell was discarded. Also, since several oocytes obtained from the same frog tended to have the same r.p., one or two oocytes were obtained from each frog, and a large number of animals was screened for r.p.

TABLE 3. The characteristics of resting oocyte membrane

	Follicles						
	r.p. (mV)			*r.p. vs.* lg[K^+] slope at 20 mM Tris$^+$ subst. for K^+		*r.p. vs.* lg[Na^+] slope at 93 mM	
	Control	+Ouabain	R_{in} (MΩ)	Control	+Ouabain	Tris$^+$ subst. for Na^+	Mg^{2+} subst. for Na^+
Mean	−47·6	−41·5	0·60	20·5	23·9	21·6	20·8
S.D.	8·8	8·8	0·25	4·2	5·4	9·1	5·4
*n**	30	17	16	10	8	5	5
*N**	14	8	10	4	3	3	4

	Denuded oocytes						
	r.p. (mV)			*r.p. vs.* lg[K^+] slope at 20 mM			*r.p. vs.* lg[Na^+] slope at 93 mM
	Control	+Ouabain	R_{in} (MΩ)	Tris$^+$ subst. for K^+	Mg^{2+} subst. for K^+	Tris subst. for K^+ +ouabain	Mg^{2+} subst. for Na^+
Mean	−46·8	−32·8	0·57	19·4	21·0	19·9	31·2
S.D.	11·6	3·8	0·36	5·8	6·9	1·6	10·5
*n**	26	6	12	7	3	4	5
*N**	12	3	7	4	2	3	4

* n = number of oocytes, N = number of frogs.

In our series, r.p. was −47·6±8·8 mV (mean±S.D., $N = 14$ frogs, $n = 20$ oocytes) and R_{in} was 0·6±0·25 MΩ (mean±S.D., $N = 10$, $n = 16$) in follicles; r.p. was −46·6±11·6 mV (mean±S.D., $N = 12$, $n = 26$) and R_{in} was 0·57±0·36 MΩ ($N = 7$, $n = 12$) in denuded oocytes (see Table 3). Thus, no significant difference in r.p. and R_{in} was found between intact follicles and denuded oocytes.

(*c*) *Effects of ouabain* (10^{-4} M)

Short-term effects. Ouabain depolarized *both* follicles and denuded oocytes within 2–8 min after application (Fig. 2). The ouabain-induced depolarization was 6·1 mV ($n = 17$) in follicles and 14·0 mV ($n = 6$) in denuded oocytes (see Table 3 for details). R_{in}, measured in the same cell before and after ouabain, was unchanged in short-term exposures (up to 30 min). The ouabain effect was difficult to reverse, and only partial repolarization was observed even after prolonged washings (30–60 min).

Long-term effects. R.p., which was relatively stable for up to 30 min in ouabain, gradually deteriorated over a period of several hours. Here, the r.p. was low, the

repolarization following intracellular penetration was small, and R_{in} increased. In a 5 h experiment with several groups of oocytes, r.p. ($-53{\cdot}5+3{\cdot}0$ mV, $n = 4$) was not affected by the 5 h incubation in normal Ringer solution. The repolarization following penetration averaged $21{\cdot}0+2{\cdot}9$ mV. A second group was incubated in ouabain for 40 min, r.p. was $-33{\cdot}0+4{\cdot}0$ mV ($n = 4$), and the repolarization was $16{\cdot}2+6{\cdot}1$ mV. A third group was incubated for 3 h in ouabain; r.p. was $-24{\cdot}8+4{\cdot}9$ mV ($n = 6$) and the repolarization was only $6{\cdot}3+3{\cdot}8$ mV. R_{in} after 3 h in ouabain was $1{\cdot}15+0{\cdot}42$ MΩ compared to the control group in Ringer solution ($R_{in} = 0{\cdot}60\pm0{\cdot}09$ MΩ, n = 4). This increase in R_{in} after prolonged incubation in ouabain may result from a decrease in $[K^+]_i$ and was not further studied.

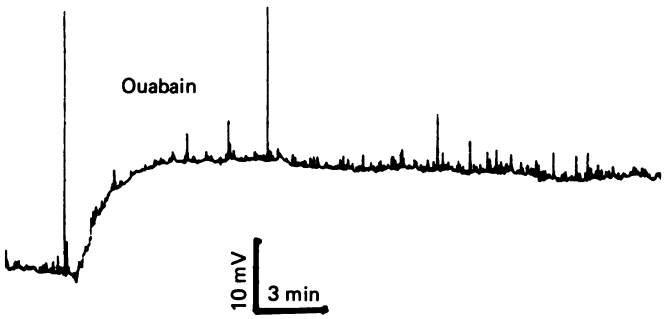

Fig. 2. Depolarization in a follicle induced by ouabain (10^{-4} M). Resting potential = −50 mV.

(d) The effect of the $[K^+]_o$ *on oocyte r.p.*

Fig. 3 shows the effect of changing $[K^+]$ on the oocyte r.p. Data from the same cell are shown, with or without ouabain (10^{-4} M). As observed by others (Wallace & Steinhardt, 1977), the electrogenic Na^+–K^+ pump was inhibited at low $[K^+]$ and the membrane was hyperpolarized when $[K^+]$ was increased from 2 to 4 mM. This hyperpolarization was abolished by ouabain in the same cell. At high $[K^+]$ (>15 mM), the pump was presumably saturated, and a final slope of 19·5 or 20·5 mV per 10-fold increase in $[K^+]$ was observed with or without ouabain, respectively. Similar results were obtained in eight follicles (slope = $23{\cdot}9\pm5{\cdot}4$) and four denuded oocytes (slope = $19{\cdot}9\pm1{\cdot}6$) (see Table 3). The experimental data correlated well with the theoretical r.p. (continuous line in Fig. 3), which was calculated from Goldman's equation (see below), assuming that the electrogenic pump is inhibited.

(e) The effect of $[Na^+]$ *on oocyte r.p.*

Decreasing $[Na^+]$ caused a marked hyperpolarization in follicles and denuded oocytes (Fig. 4*A*). Ouabain had no effect on the hyperpolarization observed at low $[Na^+]$. At $[Na^+] > 70$ mM, the slope of r.p. *vs.* log$[Na^+]$ was 18 mV per 10-fold change in $[Na^+]$ (Fig. 4*B*). Similar results were obtained in ten follicles ($21{\cdot}2\pm7{\cdot}1$, data with $Tris^+$ or Mg^{2+} brought together). In five denuded oocytes, the slope was $31{\cdot}2\pm10{\cdot}5$ (Table 3). At low $[Na^+]$, the data deviated considerably from the theoretical r.p.,

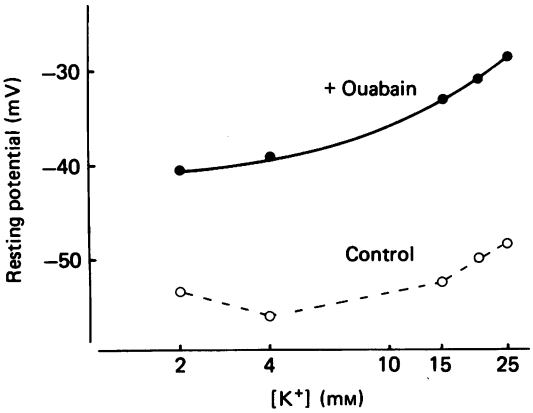

Fig. 3. A plot of a denuded oocyte resting potential (r.p.) against log[K^+]. $Tris^+$ substituted for K^+ (solutions 7 and 8, Tables 1 and 2). Two plots in the same cell are shown, with and without ouabain (10^{-4} M). At low [K^+], the electrogenic pump was partially inhibited, and therefore the cell was hyperpolarized when [K^+] was increased from 2 to 4 mM. The hyperpolarization was abolished by ouabain. The continuous line is the theoretical resting potential, calculated from Goldman's equation with $\alpha = 0{\cdot}115$ and $\beta = 0{\cdot}62$.

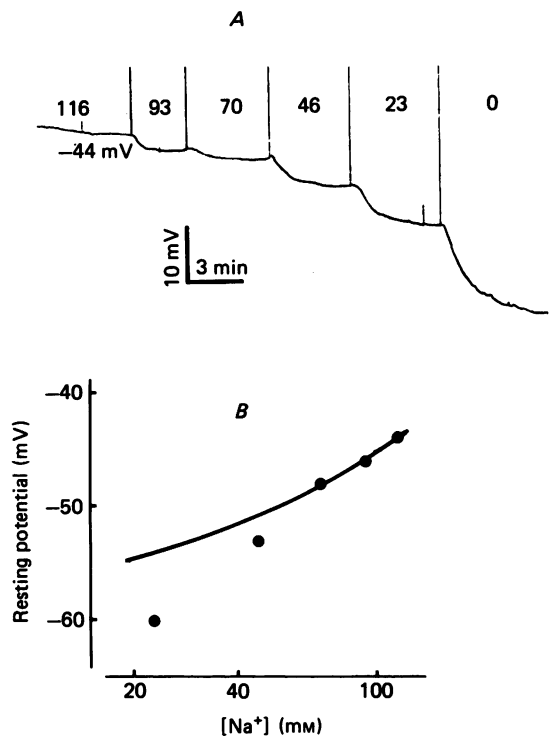

Fig. 4. Dependence of a follicle resting potential on [Na^+]. *A*, hyperpolarization of oocyte membrane at low [Na^+] (solutions 1 and 5, Tables 1 and 2). The vertical lines denote application of a new solution and the numbers above the trace show the [Na^+] in mM. *B*, results shown in *A*, replotted as resting potential *vs.* log[Na^+]. The continuous line was drawn using Goldman's equation with $\alpha = 0{\cdot}12$ and $\beta = 0{\cdot}4$, assuming a 2·5 mV contribution to the resting potential by the pump at all Na^+ concentrations.

calculated from Goldman's equation (see below). In many cells (follicles and denuded oocytes), a *two step* hyperpolarization was observed following complete removal of Na^+ ions (Fig. 5). The initial hyperpolarization was complete within 0·5–2 min. This was followed by a much slower hyperpolarization, accompanied by membrane voltage fluctuations, which resembled the *F* response to ACh (see Kusano *et al.* 1977, 1982; Dascal & Landau, 1980, 1982).

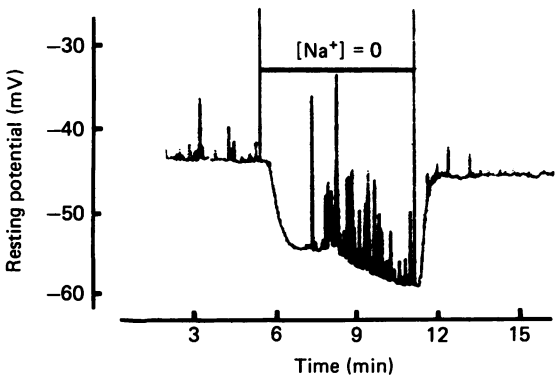

Fig. 5. A two-step hyperpolarization in Na^+-free Ringer solution.

(f) Calculation of the permeability ratios $\alpha = P_{Na^+}/P_{K^+}$ *and* $\beta = P_{Cl^-}/P_{K^+}$

α and β were calculated from Goldman's equation, assuming that the electrogenic pump is abolished by ouabain:

$$r.p. = 58 \lg \frac{a^o_{K+} + \alpha a^o_{Na+} + \beta a^i_{Cl-}}{a^i_{K+} + \alpha a^i_{Na+} + \beta a^o_{Cl-}}. \tag{3}$$

The theoretical slopes of r.p. *vs.* $\lg a^o_{K+}$ and $\lg a^o_{Na+}$ are given by:

$$\frac{\partial r.p.}{\partial \lg a^o_{K+}} = \frac{58\, a^o_{K+}}{a^o_{K+} + \alpha a^o_{Na+} + \beta a^i_{Cl-}}, \tag{4}$$

$$\frac{\partial r.p.}{\partial \lg a^o_{Na+}} = \frac{58\, \alpha a^o_{Na+}}{a^o_{K+} + \alpha a^o_{Na+} + \beta a^i_{Cl-}}. \tag{5}$$

The slopes defined by eqns. (4) and (5) were measured as shown in sections (*d*) and (*e*). The ionic activities were estimated as shown in section (*a*). α was 0·12 in follicles and 0·24 in denuded oocytes; β was 0·40 in follicles and 0·39 in denuded oocytes.

Although the estimates for α and β may be influenced by the incomplete sealing (section (*a*)), we believe that α and β represent true values since the slopes defined by eqns. (4) and (5) were measured in single-electrode experiments where the shunt is minimal. The α value is supported by Na^+ flux studies (O'Connor *et al.* 1977).

(g) Four muscarinic responses in Xenopus oocytes

The oocyte muscarinic responses were analysed in terms of four distinct components, which could be most clearly detected in voltage-clamp recordings (Fig. 6): an early,

relatively fast inward current, D_1, followed by a late, slow inward current, D_2, and a long-lasting outward current, H. In many cells, however, H was not seen until the wash-out of ACh started, when it appeared as an outward current 'tail' following the rapid decay of D_2. Large current fluctuations, F, were usually superimposed on D_2 and H. In the winter, the sensitivity of the oocytes to ACh usually decreased, D_1 appeared rarely and in some cases, no response was observed, even at 10^{-4} M-ACh

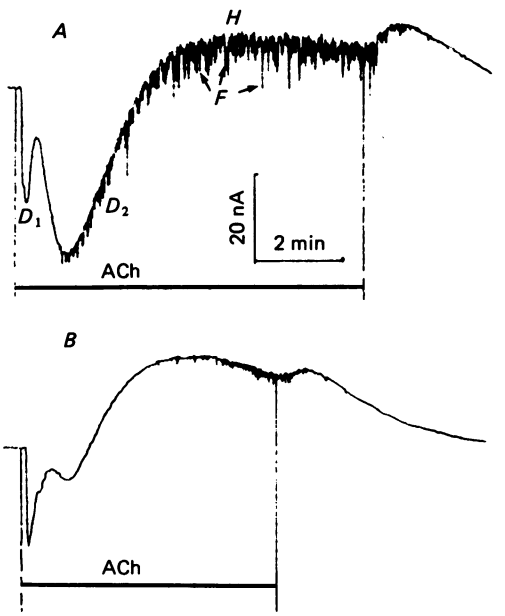

Fig. 6. Four muscarinic responses in a voltage-clamped *Xenopus* oocyte. Holding potential = −60 mV. *A*, first application of 10^{-6} M-ACh in a follicle. *B*, second application, 22 min after the beginning of ACh wash-out in *A*. D_2 is diminished in the second application and H is more pronounced. Note a tail of the H response following wash-out of ACh.

(Dascal & Landau, 1980). The seasonal variations were partially eliminated when the frogs were kept at a 15/9 h light/dark cycle. In oocytes of some frogs, all responses were fully preserved after collagenase treatment. However, in oocytes of many frogs, the responses were diminished or disappeared altogether after defolliculation. The results varied among various frogs and depended on the batch of collagenase used. D_1 and H were always more stable than D_2 and F.

Whenever D_1 was present, it appeared at the lowest ACh concentration, sometimes as low as 10^{-10} M, but usually around 10^{-8} M (see Dascal & Landau, 1982). H appeared at the same or slightly higher concentration; however, as the concentration was increased further (usually above 10^{-7} M), D_2 appeared and, in most cells, obscured the H component. The threshold for F was extremely variable, being sometimes lower than that of D_2, and sometimes as high as 10^{-4} M.

In ionophoretic experiments, a delay of 1–2 s, a time-to-peak of about 2 s and a duration of a few seconds were observed for the D_1 response. In prolonged bath

application of ACh, the D_2 and H responses lasted for several minutes. The oocytes were refractory to a repeated bath application of ACh for 5–25 min. The duration of the refractory period was dependent on the ACh concentration and the duration of the previous application.

In most frogs, prolonged washing of the oocyte (up to 1 h) failed to restore the control amplitude of the D_2 and F responses. The D_1 and H responses were reproducible even after several ACh bath applications (Fig. 6).

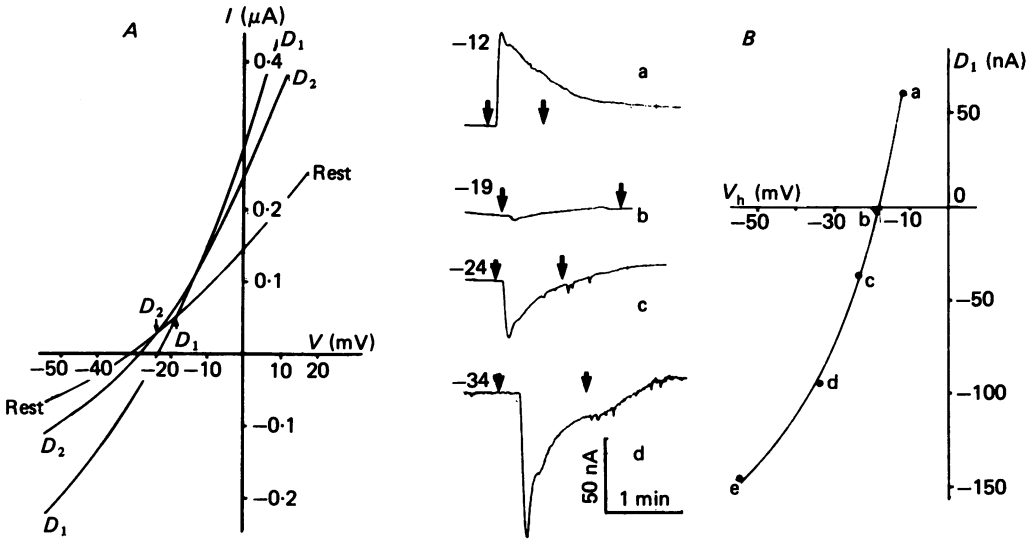

Fig. 7. *A*, voltage–current relationship obtained automatically by a ramp command voltage and an *X–Y* plotter during one application of 10^{-6} M-ACh at a holding potential of −55 mV. 0·8 s ramps were produced during rest and during the D_1 and D_2 responses. $V_r(D_1)$ was −18·5 mV, and $V_r(D_2)$ was −24 mV. *B*, reversal of the D_1 response at a holding potential of *ca.* −19 mV, in the same cell as in *A*. The conventional method shown in *B* correlated well with the ramp method which is more suitable to slow membrane ionic currents.

(*h*) *The fast depolarizing current* (D_1)

D_1 was studied by using the ramp method, as shown in Fig. 7*A*. This method allowed us to determine the reversal potential (V_r) during a *single* ACh application. V_r measurement by the conventional multiple ACh application method is shown in Fig. 7*B*. The D_1 reversal potential ($V_r(D_1)$) was shown to be −18·5 mV by the two methods in the same cell. The mean value of $V_r(D_1)$ was $-24{\cdot}2 \pm 3{\cdot}8$ mV (mean ± S.D., $N = 19$, $n = 53$, range: −15 to −31·5). The variations in $V_r(D_1)$ were observed in oocytes obtained from different donors, and different batches of frogs, while oocytes of the same frog were fairly homogenous (within 2 mV). $V_r(D_1)$ showed a strict Nernst relationship to $[Cl^-]$ (Fig. 8*C*; see also Dascal & Landau, 1982). The D_1 response amplitude and $V_r(D_1)$ were *not* affected by a 10-fold increase in $[K^+]$ (solutions 6 and 7, Table 1, three cells), a 10-fold increase in $[Mg^{2+}]$ (solutions 15 and 16, two cells), or a 2-fold decrease in $[Na^+]$ (solutions 1 and 5, four cells). Thus it appeared that the D_1 response resulted from the efflux of Cl^- ions.

(*i*) *The slow depolarizing current* (D_2)

The reversal potential of D_2 ($V_r(D_2)$), as measured by the ramp method, was $-32{\cdot}1 \pm 6{\cdot}1$ mV (mean ± S.D., $N = 10$, $n = 32$; see Fig. 8*A*), and was always more negative than $V_r(D_1)$. The slope of $V_r(D_2)$ *vs.* log [Cl^-] deviated considerably from the Nernst relationship (Fig. 8*C*). However, when the *H* response was blocked by intracellular ionophoresis of TEA (see below), the $V_r(D_2)$ became less negative (Fig.

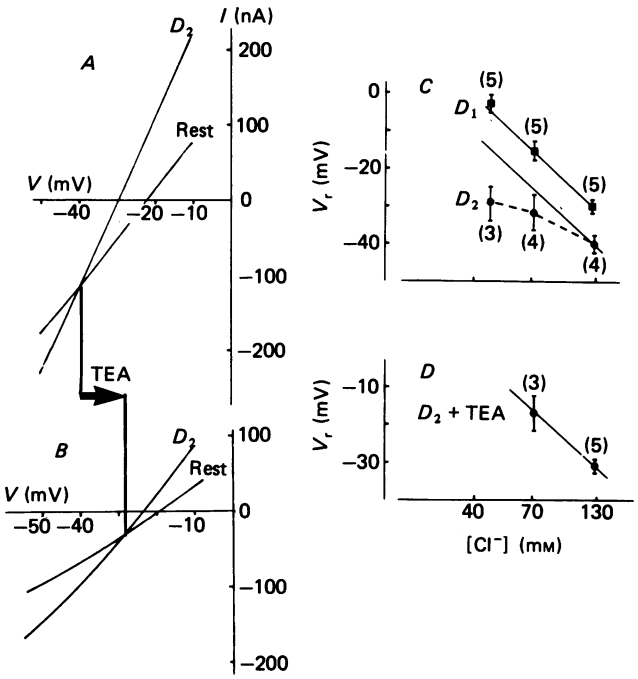

Fig. 8. The dependence of $V_r(D_2)$ on [Cl^-] and the effect of intracellular TEA injection. *A*, control *V–I* characteristics of D_2; *B*, after a 35 min injection of TEA. $V_r(D_2)$ was -39 mV in *A* and -29 mV in *B*. *C*, the dependence of $V_r(D_1)$ and $V_r(D_2)$ on [Cl^-] in oocytes of the same frog. Each point represents mean ± S.D., with number of cells in parentheses. The continuous straight lines have a slope of 58 mV per a 10-fold change in [Cl^-]. *D*, the dependence of $V_r(D_2)$ on [Cl^-] in TEA-injected cells of the same frog.

8*B*) and showed a Nernst relationship to [Cl^-] (Fig. 8*D*). It was thus concluded that the slow depolarizing current results from an outward movement of Cl^- ions during prolonged bath application of ACh. The D_2 response was 'contaminated' by the *H* response, and therefore, its V_r was more negative than $V_r(D_1)$ and the Cl^- equilibrium potential.

(*j*) *The slow hyperpolarizing current* (*H*)

Fig. 9*A* shows voltage-clamp recording in an oocyte in which the *H* response was dominant. When V_r was estimated by the ramp method, it became more negative as the response developed, being -50 mV initially, -80 mV after 9 min in ACh and even more negative (-99 mV) 2 min after wash-out of ACh (Fig. 9*B*). A plot of $V_r(H)$

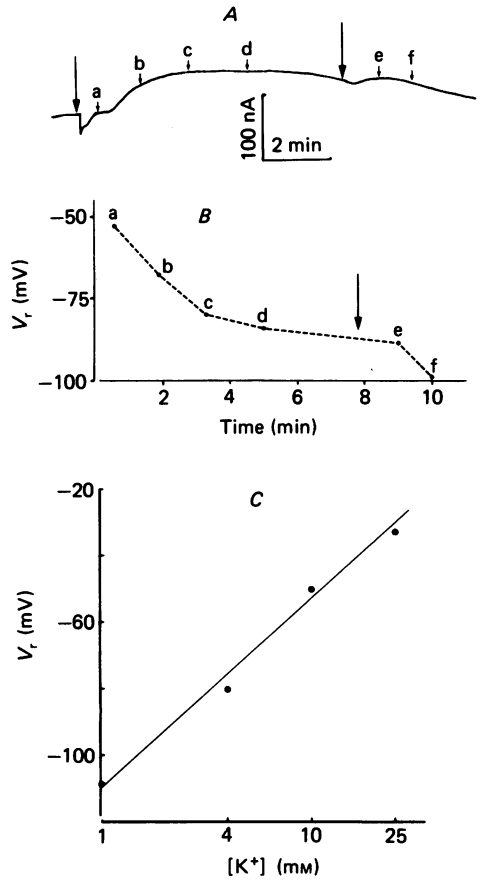

Fig. 9. *A*, a long-lasting *H* response, following the D_1 response. ACh concentration was 10^{-5} M. Note a tail of the outward current after ACh wash-out. Ramps were applied at points a–f. *B*, the *H*-current reversal potential, $V_r(H)$, at points a–f. *C*, Nernst relationship of the $V_r(H)$ at the tail of the *H* response *vs.* log [K^+]. The straight line has a slope of 58 mV per 10-fold change in [K^+].

after ACh wash-out *vs.* log [K^+] (Fig. 9*C*) showed a clear Nernst relationship (two oocytes, two frogs). These results indicated that the *H* response resulted from an outward movement of K^+ ions, which was partially masked by an outward movement of Cl^- ions (D_2 response). This was further studied by intracellular ionophoresis of TEA, which is known to block K^+ channels (Hagiwara & Saito, 1959; Hille, 1967; Imaizumi & Watanabe, 1981; Hermann & Gorman, 1981).

When applied externally, TEA was shown to block selectively the adenosine-induced K^+ current at a concentration of 10–30 mM (Lotan *et al.* 1982). The adenosine-induced Cl^- current was not affected by TEA. Here, however, externally applied TEA blocked the cholinergic responses, possibly due to its quaternary ammonium configuration (Cannon & Long, 1967). Therefore, TEA was applied internally, by ionophoresis from a third electrode filled with TEA. Ionophoretic current of 150–200 nA was applied for 20–30 min. Following the intracellular injection of TEA, the D_1 response was

unchanged, $V_r(D_2)$ became less negative (Fig. 8*B*, see above), and the *H* response was abolished (Fig. 10).

(*k*) *Membrane current fluctuations* (*F*)

Spontaneous membrane current fluctuations were manifested as inwardly directed single or multiple current peaks, returning to the base line as fast as they rose. They could appear in response to electrode penetration, a change in osmotic pressure, elimination of Na^+ and ACh application. The ACh-induced fluctuations (*F*) were

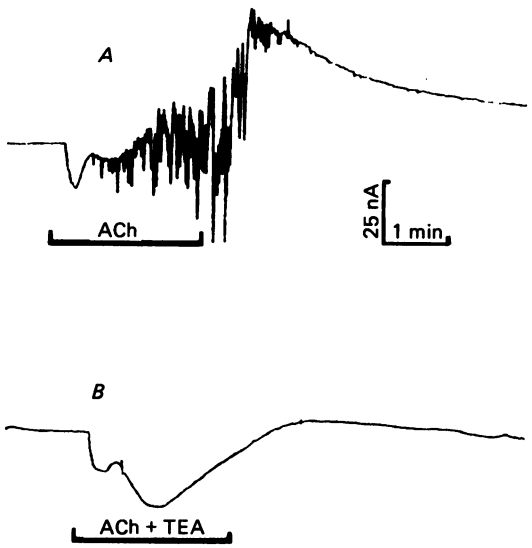

Fig. 10. The effect of TEA injection on the *H* and the *F* response. Holding potential = −40 mV, ACh concentration was 10^{-6} M. *A*, control response; *B*, after a 30 min injection (150 nA) of TEA.

initially small and inwardly directed. However, as the response developed and the amplitude of *F* increased, the current overshot the base line and became bi-directional. The *F* response was reversed at membrane potentials equal to $V_r(D_1)$ measured in the same cell (twenty-three oocytes), indicating that a Cl^- current is rhythmically activated when the *F* response is present (Kusano *et al.* 1982). *F* disappeared after TEA injection (see Fig. 10).

(*l*) *Oocyte muscarinic response and* [Ca^{2+}]

D_2 and *F* were fully inhibited after a 10–15 min pre-treatment with Ca^{2+}-free Ringer solution containing 10^{-4} M-EGTA, while D_1 and *H* were diminished (five cells). Interestingly, replacing the Ca^{2+}-free, EGTA-containing medium by normal Ringer solution resulted in the appearance of an inward current resembling the D_2 response, and in some cells *F*-like current fluctuations also appeared.

A 10-fold increase in [Ca^{2+}] caused a significant increase in D_1 and D_2, without any change in $V_r(D_1)$ (Fig. 11). An increase in the amplitude of *F* response was also observed in some cells. In five experiments of the type shown in Fig. 11, D_1 amplitude

in high-Ca^{2+} solution increased by $143 \pm 87\%$, and D_2 amplitude by $338 \pm 131\%$ (mean $\pm$ S.D.). Again, D_1 appears to be less sensitive to Ca^{2+} changes than D_2. It was also noticed that replacing the normal Ringer by high-Ca^{2+} solution caused a D_2-like current, sometimes accompanied by F-like current peaks.

When ACh was applied after a 10–20 min pre-treatment with 10^{-5}–10^{-4} M-verapamil, a Ca^{2+}-channel blocker (Fleckenstein, 1977), the amplitude

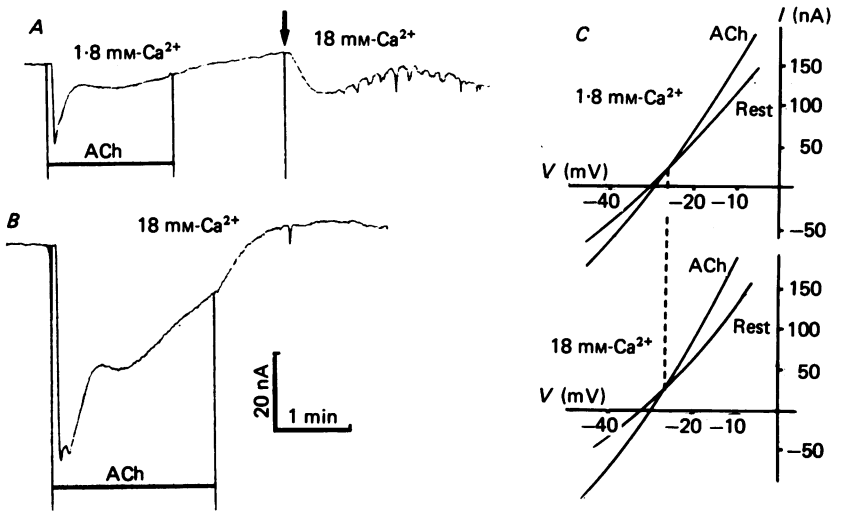

Fig. 11. The effect of increasing $[Ca^{2+}]$ on D_1 and D_2 in the same oocyte. The holding potential was -50 mV. *A*, the response to 10^{-5} M-ACh in normal Ca^{2+} solution (solution 13, see Table 1) with 1·8 mM-Ca^{2+} and 17·2 mM-Mg^{2+}. ACh was washed out with normal Ca^{2+} solution, which was then replaced by the high Ca^{2+} solution (solution 14) containing 18 mM-Ca^{2+} and 1 mM-Mg^{2+} (denoted by the downward arrow above the trace). *B*, the response to 10^{-5} M-ACh in the same cell in high Ca^{2+} solution. The record was made after the stabilization of the resting membrane current in the high Ca^{2+} solution, about 20 min after the end of the first ACh application shown in *A*. *C*, $V_r(D_1)$ in normal Ca^{2+} and in high Ca^{2+} solutions.

of all the muscarinic responses was decreased. Full inhibition of the responses occurred sometimes at $5 \cdot 10^{-5}$–10^{-4} M-verapamil, but in many oocytes, a concentration of 1 mM was necessary (Fig. 12). The H response was, as a rule, less sensitive to verapamil than D_2; however, a clear correlation between the type of response and its sensitivity to verapamil could not be made.

(*m*) *Intracellular injection of cyclic GMP*

When a few picomoles of cyclic GMP were injected by pressure, hyperpolarizing (outward current) response was always observed in follicles (twenty-five cells, nine frogs) and denuded oocytes (five cells, two frogs). The hyperpolarizing response was usually much stronger in follicles. A typical response of a follicle to injection of about 5 pmol cyclic GMP is shown in Fig 13. The response appeared after a delay which lasted 5–120 s, and had a time-to-peak of several tens of seconds. After reaching a plateau, the response gradually decayed until a new and more positive steady level

of holding current was achieved (Fig. 13*A*), which resulted from an increase in R_{in} (Fig. 13*C*; compare *V–I* curves nos. 1 and 3). The second intracellular injection of 5 pmol cyclic GMP is shown in Fig. 13*B*. Here, the response decayed to the base-line holding current, and no further change in resting R_{in} was observed in repeated applications of cyclic GMP. The reversal potential of the cyclic GMP-induced outward current was from −85 to −96 mV (five cells; see Fig. 13*C* and *D*). The outward current induced by cyclic GMP injection in the range 1–10 pmol was clearly dose-dependent (not shown).

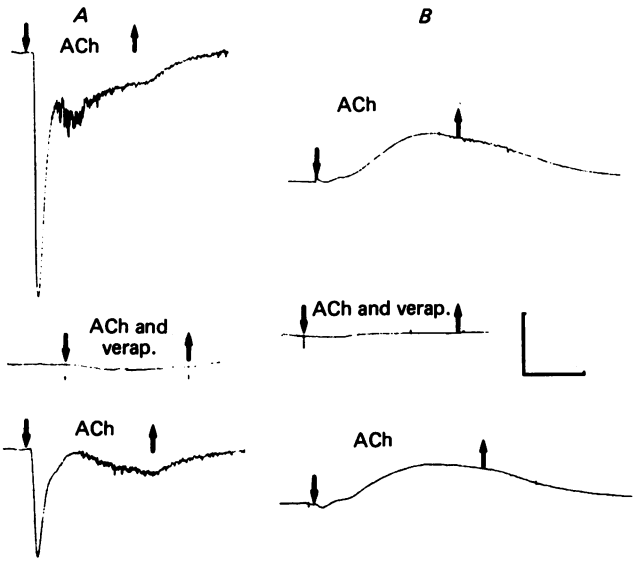

Fig. 12. The effect of 10^{-3} M-verapamil on the muscarinic responses in two oocytes of different frogs. In *A* (upper trace), the D_1 and D_2 responses were dominant. In *B* (upper trace), the *H* response was dominant. Verapamil (verap.) abolished all responses component (middle trace), and the effect was reversible (lower trace). In *A*, the ACh concentration was 10^{-6} M; calibration: horizontal 1 min, vertical 50 nA. In *B*, the ACh concentration was 10^{-5} M; calibration: 2 min, 50 nA.

Injection of greater amounts of cyclic GMP (ten to several tens of picomoles) caused outward currents with less negative V_r (−52 to −75 mV, four cells) or an inward current which followed the outward current. *F*-like current fluctuations having amplitudes up to 200 nA accompanied these responses. These fluctuations were reversed at about −20 mV (two cells). The inward current response was more pronounced in denuded oocytes.

DISCUSSION

Errors and discrepancies in the measurement of oocyte r.p.

An almost inevitable error arises from the insertion of intracellular electrodes, and the measured r.p. is probably an underestimation of the true resting potential. An instantaneous depolarization is observed immediately after the electrode insertion, followed by a slow recovery (Miledi, 1982; Kusano *et al.* 1982). As shown in the

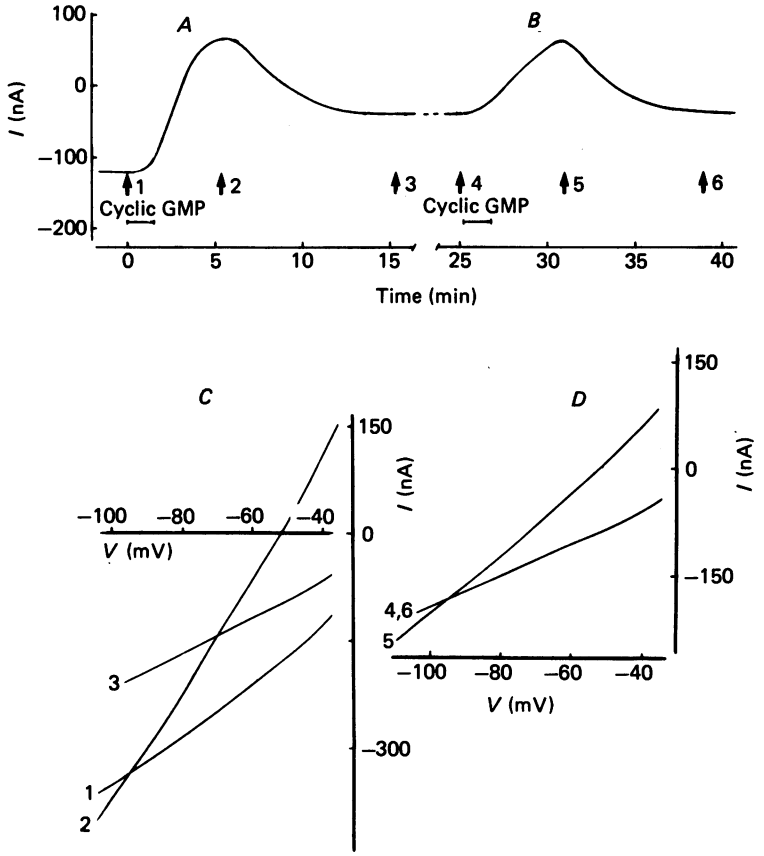

Fig. 13. The response of an oocyte to intracellular cyclic GMP injection. *A* and *B*, first and second injection of 5 pmol cyclic GMP. The arrows (1–6) denote the ramp applications. *C* and *D*, voltage–current characteristics of the first and second responses to cyclic GMP. The numbers near the *V–I* curves correspond to the numbers in *A* and *B*. Note that *V–I* curves 4 and 6 were identical.

Results, the electrode produces a significant 'shunt' conductance which seals over a period of at least a few minutes. The shunt resistance (R_s) was shown to represent a 'non-specific' conductor where $P_{Na^+} = P_{K^+} = P_{Cl^-}$. The instantaneous depolarization and subsequent repolarization persisted in the presence of ouabain (10^{-4} M), and thus a possible activation of the Na^+–K^+ pump (Wallace & Steinhardt, 1977) was ruled out.

The oocytes r.p.

Table 3 is a summary of oocyte membrane characteristics obtained in this study. Oocyte r.p. values represented in Table 3 generally agree with previous measurements in *Xenopus* oocytes (Moreau *et al.* 1976; Kusano *et al.* 1982) and in *Rana* follicles (Ziegler & Morrill, 1977). Nernst potentials of K^+ (−102·7 mV) and Cl^- (−24·2 mV) were estimated from the reversal potentials of the muscarinic D_1 response (this report) and the purinergic *H* response (Lotan *et al.* 1982) respectively. Nernst potential of Na^+ (about +45 mV) was estimated from direct measurements (Kusano *et al.* 1982).

None of these ions is at equilibrium at oocyte r.p. (−50 mV), and therefore, the Donnan approach to oocyte r.p. is not applicable. The steady-state constant field theory correlated well with the experimental data (except at very low [Na^+]) and estimates for $\alpha = P_{Na^+}/P_{K^+}$ and $\beta = P_{Cl^-}/P_{K^+}$ were obtained from Goldman's equation.

Major role of [Na^+] in oocyte r.p. determination

In classical excitable cells where α is small ($\alpha = 0{\cdot}01$ in skeletal muscle and nerve; see Hodgkin & Katz, 1949; Hodgkin & Horowitz, 1959), [Na^+] has little effect on the resting membrane potential. In the *Xenopus* oocyte, Na^+ ions are relatively permeable, with $\alpha = 0{\cdot}12$ as calculated from Goldman's equation. This value correlates well with $\alpha = 0{\cdot}1$ or $0{\cdot}18$ obtained from labelled Na^+ flux studies (Tupper & Maloff, 1973; O'Connor *et al.* 1977). In physiological conditions (where [Na^+] = 116 mM, $\alpha \cdot$ [Na^+] = 14 mM, and [K^+] = 2mM), it turns out that Na^+ is comparable to K^+ in oocyte r.p. determination. This fact was overlooked in previous electrophysiological studies (Wallace & Steinhardt, 1977; Ziegler & Morrill, 1977) where Na^+ was used as a substitute for K^+ (to keep osmolarity constant) when [K^+] effects on r.p. were studied. This procedure, based on the assumption that $P_{Na^+} \ll P_{K^+}$ would cause an over-estimation of $a^i_{K^+}$. At [Na^+] $\geqslant$ 70 mM the oocyte was hyperpolarized when [Na^+] decreased, as predicted by Goldman's equation. This hyperpolarization was not affected by ouabain. At very low [Na^+], the hyperpolarization was much larger than expected from Goldman's equation, and again, it was not affected by ouabain.

Oocyte Na^+–K^+ electrogenic pump

It is known that when the oocytes are denuded by the method of Masui (1967), i.e. after a 20–30 min pre-treatment with Ca^{2+}- and Mg^{2+}-free, EGTA-containing Ringer solution, r.p. is −70 to −80 mV; ouabain and K^+-free solution in these oocytes inhibits the electrogenic Na^+–K^+ pump and produces a marked depolarization of 30–50 mV (Vitto & Wallace, 1976; Wallace & Steinhardt, 1977; Ziegler & Morrill, 1977; Baud *et al.* 1982). However, in follicles ouabain was reported to have little (Ziegler & Morrill, 1977) or no effect (Wallace & Steinhardt, 1977). When the oocytes are denuded manually without EGTA pre-treatment, r.p. is closer to that observed in follicles (−50 mV, Kusano *et al.* 1982) and ouabain effectively inhibits the active K^+ influx (O'Connor *et al.* 1977).

Our data conclusively show that ouabain and K^+-deficient solution depolarize both follicles and collagenase-treated oocytes. The effect of ouabain was stronger in denuded oocytes. R.p. after a short-term treatment with 10^{-4} M ouabain was $-41{\cdot}7 \pm 8{\cdot}8$ mV in follicles and $-32{\cdot}8 \pm 3{\cdot}8$ mV in denuded oocytes (see Table 3). Thus, it appears that the pump is most active in oocytes denuded manually after a pre-treatment with EGTA, less active in oocytes denuded manually or by collagenase in normal Ringer solution, and even less active in follicles. The reason for a stronger pump activation in denuded oocytes may be an increase in $a^i_{Na^+}$. Thus in denuded oocyte, r.p. is similar to follicles r.p. since the negative change due to the increase in α and $a^i_{Na^+}$ is balanced by the increase in the electrogenic pump activity.

Ca^{2+} deprivation is known to increase P_{Na^+} (Tupper & Maloff, 1973). Following this treatment, $a^i_{Na^+}$ is significantly increased (Morrill, Ziegler & Zabrenetzky, 1977). A moderate increase in $a^i_{Na^+}$ was reported to occur in oocytes denuded manually or

by collagenase in normal Ringer solution (Frank & Horowitz, 1980; Kusano *et al.* 1982). Our data suggest that in collagenase-treated oocytes α is increased from 0·12 to 0·24; this would increase the passive Na^+ influx and thus activate the pump (see Thomas, 1978).

The four muscarinic responses in Xenopus oocytes

The oocyte muscarinic response described in this report was separated into four distinct components, based on differences in the time course, ionic mechanisms and pharmacological characteristics. Also, the possible role of cyclic GMP was demonstrated by intracellular injection of the cyclic nucleotide. All responses could be elicited by the muscarinic agonists oxotremorine and carbachol and were inhibited by atropine, which suggests that all responses are mediated through the binding of the agonists to cholinergic muscarinic receptors. This is in accord with the conclusion of Kusano *et al.* (1977, 1982) concerning D_2, H and F.

As shown in the Results, the ionic current underlying any one of the four response components, does not depend on the presence of any other component. The persistence of all response components in denuded oocytes rules out the possibility that some of them might be mediated through an interference of the follicular cells.

The main events caused by muscarinic agonists in *Xenopus* oocyte membrane are an increase in Cl^- conductance and a long-lasting increase in K^+ conductance. The increase in Cl^- conductance undoubtedly accounts for the D_1 and D_2 responses. These two Cl^- currents exhibit pronounced differences in time course, reproducibility on repetitive ACh applications, and Ca^{2+} sensitivity. It is not yet clear if both are mediated by the opening of the same type of Cl^- channels.

The use of the ramp method provided a convenient tool for the study of the muscarinic responses in a single ACh application. The reversal potential of D_1 ($V_r(D_1)$) *vs.* [Cl^-] behaves in accord with the predictions of the Nernst equation, although the mean value of $V_r(D_1)$, −24·2 mV, is more negative than the equilibrium potential for Cl^- calculated from direct measurements (−14·2 mV, Kusano *et al.* 1982). One of the reasons for this discrepancy may be the variation among oocytes of frogs from different batches. Thus, in our experiments $V_r(D_1)$ varied between −15 and −31·5 mV, while Kusano *et al.* (1982) reported a variation of $V_r(F)$, also a Cl^- current, between −11 and −25 mV.

$V_r(D_2)$ is usually more negative than $V_r(D_1)$ in the same cell, indicating that K^+ conductance is probably also increased during D_2. Kusano *et al.* (1982) have measured the V_r of the long-lasting depolarizing response and found a V_r of −34 mV, which is close to $V_r(D_2) = -32$ mV found by us. However, the authors do not provide an explanation for this value. Our data demonstrate that $V_r(D_2)$ becomes equal to $V_r(D_1)$ and exhibits a Nernst-like dependence on [Cl^-] when the K^+ current is suppressed by intracellularly injected TEA (Fig. 8). We interpret these results as demonstrating that in the majority of oocytes a Cl^- and a K^+ current (D_2 and H) occur simultaneously but utilize separate channels. D_2 rises faster than H and therefore $V_r(H)$ becomes more negative as the response develops (Fig. 9). Also, since the D_2 response ceases faster than H when ACh is washed out, the $V_r(H)$ measured at the tail of the H current exhibits purely Nernst-like dependence on K^+ (Fig. 9).

The F response is the result of Cl^- conductance fluctuations (see Kusano *et al.* 1982). These fluctuations are bidirectional, i.e. there are inward- and outward-going current

peaks with respect to the base line; both disappear when the holding potential is equal to $V_r(D_1)$. The disappearance of F response after TEA injection, suggests that K^+ channels opening may play a role in the origination of F.

The four muscarinic responses appear to be dependent on external Ca^{2+}. All responses are partially or fully suppressed in Ca^{2+}-free, EGTA-containing solution. All responses are inhibited by verapamil (Fig. 12). The concentrations of verapamil required for full inhibition of the responses ($5 \cdot 10^{-4}$–10^{-3} M) are much higher than those known to block Ca^{2+} channels in the heart and in the smooth muscles (see Fleckenstein, 1977) but of the same order as in the vertebrate neurones (Nachshen & Blaustein, 1979).

The increased amplitudes of D_1, D_2 and F at high [Ca^{2+}] favours the assumption that these responses are the result of Ca^{2+} entry from the outside solution into the cell. However, since we did not observe a Ca^{2+} current, which would be expected to precede D_1, and since $V_r(D_1)$ was not affected by changes in [Ca^{2+}], it follows that this Ca^{2+} current is negligible. The sensitivity of D_1 to Ca^{2+} is less than that of D_2 when [Ca^{2+}] is either reduced or increased (Fig. 11). Therefore, the mechanism of Ca^{2+} involvement in the initiation of D_1 and D_2 may be different. Thus, it can be assumed that D_1 is the result of a membrane-bound Ca^{2+} release, while D_2 is the result of Ca^{2+} influx. Such biphasic Ca^{2+} movements were suggested to be elicited by muscarinic and α-adrenergic receptors in other preparations (Putney, 1978; Poggioli & Putney, 1982). An exchangeable Ca^{2+} pool was supposed to exist in *Xenopus* oocyte membrane (see Baulieu, Godeau, Schorderet & Schorderet-Slatkine, 1978). Verapamil may inhibit both the influx of Ca^{2+} and the release of membrane-bound Ca^{2+}, since at high concentrations verapamil alone is able to release membrane bound Ca^{2+} (see Morrill, Ziegler & Kostellow, 1980) and thus may cause an exhaustion of the exchangeable Ca^{2+} pool.

Possible role of cyclic GMP in the mediation of muscarinic responses in the oocytes

Our results demonstrate that cyclic GMP induces conductance changes similar to those caused by the muscarinic agonists. Although cyclic GMP has been proposed as the second messenger for muscarinic slow membrane responses (Beam & Greengard, 1976; Greengard, 1976, 1978), there was no unequivocal proof that cyclic GMP can fully mimic the muscarinic response. Thus cyclic GMP was claimed to mimic the muscarinic response in rabbit superior cervical ganglion (McAfee & Greengard, 1972), in rat auricle (Nawrath, 1976), and in rat cerebral cortex (Stone, Taylor & Bloom, 1975). However, some studies contend these findings (Gallagher & Schinnick-Gallagher, 1977; Brooker, 1977; Nawrath, 1977; Busis, Weight & Smith, 1978; Dun, Kaibara & Karczmarek, 1978; Fleming, Giles & Lederer, 1981; Frey & McIsaak, 1981), while some provide only partial evidence for the involvement of cyclic GMP in the muscarinic membranal responses (Hashiguchi, Ushiyama, Kobayashi & Libet, 1978; Trautwein, Taniguchi & Noma, 1982). In *Xenopus* oocytes, externally applied cyclic GMP and its analogues induced a D_1-like transient Cl^- current and an outward current which was accompanied by a conductance increase. Both currents never achieved amplitudes comparable to those of ACh-induced D_1 and H. The ionic mechanism of the outward current was not studied (Dascal & Landau, 1982).

Intracellular injection of cyclic GMP, as described in this report, caused a very large, long-lasting outward current, which was the result of membrane conductance

increase and had a V_r in the range -85 to 96 mV, which is close to K^+ equilibrium potential (-102 mV; Lotan *et al.* 1982). Since the equilibrium potentials of all other ions are much more positive, it may be concluded that this response is primarily the result of K^+-conductance increase, like the muscarinic *H* response. Injection of higher doses of cyclic GMP caused an inward current superimposed on the plateau of the outward current. The ionic nature of this event was not studied; however, V_r of the total response (outward + superimposed inward current) was shifted to less negative values than the V_r of the *H*-like response elicited by the low doses of cyclic GMP. It is tempting to assume, by analogy with the muscarinic D_2 and *H*, that Cl^- conductance was increased; however, the involvement of Na^+ or Ca^{2+} cannot be excluded on the basis of the present data. Another type of response evoked by cyclic GMP were *F*-like membrane current fluctuations. This *F*-like response was reversed at membrane potentials close to -20 mV, suggesting that its ionic mechanism is identical to that of the muscarinic *F* response.

The responses to cyclic GMP were specific, since intracellularly applied GMP was ineffective, in the same conditions and in the same cells in which cyclic GMP elicited pronounced responses.

Our results may indicate that cyclic GMP acts as a second messenger in the chain of events leading to the muscarinic response. The level of cyclic GMP may be increased, like it is in other muscarinic preparations as a result of ACh-induced increase in $a^i_{Ca^{2+}}$ (Schultz, Hardman, Schultz, Baird & Sutherland, 1973; Richelson & El-Fakahany, 1981). This would explain the fact that Ca^{2+} deprivation and the Ca^{2+}-channel blocker verapamil inhibit the responses to ACh. Particularly, our data support the assumption that the *H* response is mediated through cyclic GMP level increases: both extra- and intracellularly applied cyclic GMP mimics it; intracellular injection of cyclic GMP is much more effective in eliciting the *H*-like response; *H*-like response to intracellular injection of cyclic GMP is clearly dose-dependent; the maximal *H*-like response is stronger than the maximal muscarinic *H* response (several hundred nanoamperes as compared to several tens). The latter can be easily explained by assuming that the maximal cyclic GMP level increase caused by ACh is smaller than that attained following injections of a large amount of cyclic GMP.

This work was supported by a research grant from the Recanati Foundation.

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