Muscarinic receptor heterogeneity in follicle-enclosed *Xenopus* oocytes

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1. Ionic current responses elicited by acetylcholine (ACh) in follicle-enclosed *Xenopus* oocytes (follicles) were studied using the two-electrode voltage-clamp technique. ACh generated a *fast* chloride current (F_{in}) and inhibited K^+ currents gated by cAMP ($I_{K,cAMP}$) following receptor activation by adenosine, follicle-stimulating hormone or noradrenaline. These previously described cholinergic responses were confirmed to be of the muscarinic type, and were independently generated among follicles from different frogs.

2. Inhibition of $I_{K,cAMP}$ was about 100 times more sensitive to ACh than F_{in} activation; the half-maximal effective concentrations (EC_{50}) were 6·6 ± 0·4 and 784 ± 4 nM, respectively.

3. Both responses were blocked by several muscarinic receptor antagonists. Using the respective EC_{50} concentrations of ACh as standard, the antagonist 4-diphenylacetoxy-*N*-methylpiperidine methiodide blocked the two effects with very different potencies. F_{in} was blocked with a half-maximal inhibitory concentration (IC_{50}) of 2·4 ± 0·07 nM, whilst the IC_{50} for $I_{K,cAMP}$ inhibition was 5·9 ± 0·2 μM.

4. Oxotremorine, a muscarinic agonist, preferentially stimulated $I_{K,cAMP}$ inhibition (EC_{50} = 15·8 ± 1·4 μM), whilst F_{in} was only weakly activated. In contrast, oxotremorine inhibited F_{in} generated by ACh with an IC_{50} of 2·3 ± 0·7 μM.

5. F_{in} elicited via purinergic receptor stimulation was not affected by oxotremorine, indicating that the inhibition produced was specific to the muscarinic receptor, and suggesting that muscarinic actions do not exert a strong effect on follicular cell–oocyte coupling.

6. Using reverse transcription-PCR, transcripts of a previously cloned muscarinic receptor from *Xenopus* (XlmR) were amplified from the RNA of both the isolated follicular cells and the oocyte. The pharmacological and molecular characteristics suggest that XlmR is involved in $I_{K,cAMP}$ inhibition.

7. In conclusion, follicular cells possess two different muscarinic receptors, one resembling the M_2 (or M_4) subtype and the other the M_3 subtype. These receptors are coupled to distinct membrane mechanisms leading to independent regulation of two membrane conductances.

Follicle-enclosed *Xenopus* oocytes (follicles) are endowed with cholinergic receptors which, when stimulated by acetylcholine (ACh), generate various ionic current responses (Kusano *et al.* 1977, 1982; Dascal *et al.* 1985; Arellano & Miledi, 1993; for a review see Arellano *et al.* 1996). At least five different responses to ACh have been characterized. One response arises in the membrane of the oocyte itself, and results from the opening of Ca^{2+}-dependent Cl^- channels (e.g. Miledi, 1982; Miledi *et al.* 1989). The other four types of cholinergic response have their origin in the membrane of the follicular cells and can be monitored via electrodes inserted into the oocyte, because of its strong electrical coupling with the enveloping follicular cells (Browne & Werner, 1984; van den Hoef *et al.* 1984; Woodward & Miledi, 1987; Arellano & Miledi, 1993, 1995). For the present study, we focused on two cholinergic current components that are the most ubiquitous responses in follicles. One is the *fast* inward Cl^- current (F_{in}; Arellano & Miledi, 1993), which can also be activated by purinergic agents (e.g. ATP, UTP). F_{in} activation by ACh cross-inhibits that generated by ATP, and vice versa, strongly suggesting that the transmitters act on a common channel-activation pathway (Arellano *et al.* 1998). The second cholinergic component studied here is the ACh-induced inhibition of K^+ currents ($I_{K,cAMP}$) that are activated by an increase in cAMP synthesis in the follicular cells stimulated by various compounds, including follicle-stimulating hormone (FSH), noradrenaline, adenosine (ADE) or dopamine (Kusano *et al.* 1977, 1982; Van Renterghem *et al.* 1984, 1985; Dascal *et al.* 1985; Stinnakre & Van Renterghem, 1986; Woodward & Miledi, 1987; Greenfield *et al.* 1990). An important feature of the cholinergic inhibition of $I_{K,cAMP}$ is that when that K^+ current is generated by superfusion of a membrane-permeant cAMP analogue, or by intra-oocyte injection of cAMP, ACh is still able to inhibit the current completely, thus suggesting that the cholinergic action takes place downstream of cAMP synthesis (Dascal *et al.* 1985; Miledi & Woodward, 1989).

The follicle responses elicited by ACh are of the muscarinic type (Kusano *et al.* 1977, 1982; Arellano & Miledi, 1993). Studies aimed at determining the pharmacological and molecular profile of the receptors involved have all focused on the receptors responsible for activation of the typical oscillatory current due to opening of Ca^{2+}-dependent Cl^- channels in the oocyte membrane (Kusano *et al.* 1982; Van Wezenbeek *et al.* 1988; Davidson *et al.* 1991), with little attention paid to the other response types.

Currently, little is known about the molecular nature of the muscarinic receptors mediating the follicular cell-based cholinergic responses, or about the membrane mechanisms being activated. It is not known how many receptor subtypes are involved in generating the multiple components of the cholinergic response of the follicle, knowledge that would help to elucidate their role in follicular cell–oocyte physiology. However, one muscarinic receptor (XlmR) from *Xenopus laevis* oocytes has been cloned and characterized. Based on its amino acid sequence, XlmR is homologous to the human m4 subtype (Herrera *et al.* 1994, 1997).

Although a specific physiological role has not yet been clearly shown for any of the muscarinic responses, it appears that cholinergic actions on *Xenopus* follicles modulate important events, such as the maturation induced by progesterone, where ACh accelerates the process (Dascal *et al.* 1984). Also, it has been suggested that muscarinic activation of osmolarity-dependent Cl^- currents plays a role in follicle volume regulation (Arellano & Miledi, 1993). Moreover, knowledge of the pharmacological and physiological membrane mechanisms activated in *Xenopus* follicles may serve as a model towards a comprehensive understanding of muscarinic actions in other cellular systems, especially in ovarian systems of other species, including human, where muscarinic effects have also been shown (Eusebi *et al.* 1984).

METHODS

Cell preparation

Xenopus laevis frogs were obtained from Xenopus I (Ann Arbor, MI, USA) and Xenopus Express (Homosassa, FL, USA). Three to four ovarian lobules were surgically removed under sterile conditions from frogs anaesthetized with 1 g l^{-1} tricaine, and rendered hypothermic. After surgery, the frogs were allowed to recover consciousness. No further oocytes were taken for at least 2 months. After the final taking of oocytes, the anaesthetized frogs were killed by decerebration and pithing. The procedure was approved by the institutional animal use committees. The lobules were placed in sterile modified Barth's medium (containing (mM): 88 NaCl, 0·2 KCl, 2·4 $NaHCO_3$, 0·33 $Ca(NO_3)_2$, 0·41 $CaCl_2$, 0·82 $MgSO_4$, 0·88 KH_2PO_4, 2·7 Na_2HPO_4, pH 7·4; with 70 μg ml^{-1} gentamicin).

Follicle-enclosed oocytes (stage VI; Dumont, 1972) were removed from the ovary by peeling away the inner ovarian epithelium, together with the thecal blood vessels, with sharp watchmaker's forceps. This procedure leaves the follicular cell basement membrane, thus providing protection and a natural environment for the follicular cells. Moreover, removal of the epithelium facilitates electrode insertion, improves the stability of electrophysiological recording and simplifies the interpretation of results by eliminating the possible participation of the epithelium or other surrounding thecal tissues in the responses (Arellano *et al.* 1998). These 'epithelium-removed' follicles were incubated (18–20 °C) in sterile Barth's medium supplemented with glucose (5 mM) and fetal bovine serum (0·1–0·2%). Under these conditions, follicular cell–oocyte electrical coupling and follicular responses can be maintained for more than 10 days.

For follicular cell isolation, the follicle-enclosed oocytes (stage VI) were dissected from the ovary as above except that the external layers were removed together with the basal membrane of the follicular cells. In this way, the follicular cell layer was exposed and remained attached to the vitelline envelope (cf. plate I, Miledi & Woodward, 1989), thus allowing isolation of the follicular cells by enzymatic treatment. For this purpose, 200–1000 of these 'unzipped' follicles were incubated (5 min) in Hanks' balanced salt solution containing 0·05% trypsin and 0·5 mM EDTA, then gently washed in Barth's medium containing 10% fetal bovine serum. The follicular cells were dislodged from the oocyte by repeatedly drawing the treated follicles into a polished Pasteur pipette. The oocytes were discarded and the dislodged follicular cells were recovered from the supernatant and maintained at −80 °C prior to RNA purification. Using this method we obtained a homogeneous population of follicular cells, corresponding to those that are in immediate contact with the oocyte (R. Reyes, S. Alshihabi, R. O. Arellano & R. Miledi, unpublished observations).

Defolliculated oocytes were prepared by treatment with collagenase (0·5–1 mg ml^{-1}) at room temperature for 40–50 min in normal frog Ringer (NR) solution containing (mM): 115 NaCl, 2 KCl, 1·8 $CaCl_2$, 5 Hepes, pH 7·0. After washing of the oocytes, all their remaining external envelopes, except for the vitelline layer, were removed using fine forceps. The defolliculated oocytes maintained in Barth's medium were then used for purification of RNA (see below), or for electrophysiological recording.

Electrophysiological techniques

Follicular electrical responses were monitored using a two-microelectrode voltage clamp (Miledi, 1982). Unless otherwise stated, the follicles were voltage clamped at −60 mV for studies of F_{in}, and at −40 mV for $I_{K,cAMP}$, for better differentiation of the responses. Follicles were bathed in NR solution, and drugs were applied by superfusion (10 ml min^{-1}, chamber volume of 0·4 ml). Current–voltage relationships were obtained by changing the membrane potential from +20 to −120 mV, in 20 mV steps of 80 ms, before (control membrane current) and during the peak of the current response activated by the different drugs. For each voltage step, the control membrane currents were subtracted from those obtained during drug-elicited currents, and these values were plotted, as in Fig. 1*A*.

Intra-oocyte injections of EGTA were made by pneumatic pressure ejection from micropipettes; the injection solution (100 mM EGTA) was made up in 5 mM Hepes, pH adjusted to 7·0 with KOH. A similar injection apparatus was used for extracellular applications of a brief pulse jet of ACh (100 μM in NR solution) from a pipette positioned close (*ca* 50 μm) to the follicle (Arellano *et al.* 1998).

Reverse transcription-PCR (RT-PCR) and hybridization analysis

Amplification of *XlmR* (Herrera *et al.* 1994) transcripts from total RNA purified from isolated follicular cells or from defolliculated oocytes was performed using the RT-PCR technique. Total RNA was purified using the guanidinium isothiocyanate method (Chomczynski & Sacchi, 1987). First strand cDNA was synthesized using SuperScript II RNase H^- reverse transcriptase with oligo-dT (100 pM) and random hexamers (10 pM) as primers in a total volume of 25 μl (1 h, 42 °C). Aliquots of 2 μl were then amplified by a step cycle (94 °C for 1 min; 55 °C for 1 min; and 72 °C for 1 min), for thirty cycles. In control reactions the reverse transcriptase was omitted. The primers designed to amplify 430 bp of the *XlmR* cDNA (bases 18 to 449) were based on the sequence reported by Herrera *et al.* (1994) and were as follows: forward, 5′-CTT CTT GCT GGG TAA GTC AGT G-3′; and reverse, 5′-GGA- AAA CGA CAC TTG GGA AAA T-3′. The RT-PCR products were then resolved in 1 % agarose gels, and were further analysed by Southern blotting, using standard procedures (Sambrook *et al.* 1989). DNA was transferred to Hybond N membrane (Amersham, NJ, USA), and the *XlmR* cDNA, generously supplied by Dr Juan Olate (Laboratorio de Genética Molecular, Facultad de Ciencias Biológicas, Universidad de Concepción, Chile), was used as a specific hybridization probe.

Substances

follicle-stimulating hormone was purchased from Calbiochem (La Jolla, CA, USA). Pilocarpine hydrochloride, oxotremorine sesquifumarate, 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP), pirenzepine dihydrochloride (PZP), metoctramine tetrahydrochloride (MTT), tropicamide (TPC) and pertussis toxin were obtained from RBI (Natick, MA, USA). Hanks' balanced salt solution, SuperScript II RNase H^- reverse transcriptase, primers, and oligo-dT were from Gibco BRL (Gaithersburg, MD, USA). Random hexamers were from Boehringer (Mannheim, Germany). All other compounds, such as collagenase Type I, ATP, ACh, carbachol, nicotine, noradrenaline, ADE, dopamine, atropine, tubocurarine, tricaine, trypsin and salts, were from Sigma Chemical Co. (St Louis, MO, USA).

RESULTS

Dose–response relationships of follicular cholinergic responses

The follicles used in this study had a mean resting potential in NR solution of −43 ± 6 mV (234 follicles, 27 frogs) (all data given as means ± S.E.M.), and a mean input resistance of 0·62 ± 0·14 MΩ and, as reported previously, ACh-activated current responses made up of several components whose amplitudes varied greatly among follicles from different frogs (Kusano *et al.* 1977, 1982; Arellano & Miledi, 1993). The two cholinergic responses studied here, F_{in} activation and $I_{K,cAMP}$ inhibition, were generated in *ca* 90 % of the donors. For all frogs, both cholinergic responses were routinely confirmed to be of follicular cell origin, since oocyte defolliculation ($n = 2$–5 per donor) completely eliminated F_{in} and $I_{K,cAMP}$ (Fig. 1*B* and *C*). Moreover, the ACh responses were confirmed to be potently blocked by atropine (1 μM; 5 follicles, 2 frogs), and were not affected by intra-oocyte ($n = 9$, 4 frogs) injections of EGTA, indicating that both currents were fundamentally Ca^{2+} independent, as has been shown previously (Dascal *et al.* 1985; Miledi & Woodward, 1989; Arellano & Miledi, 1993). Some other important characteristics of F_{in} and $I_{K,cAMP}$ were confirmed in this study, for comparison with previous work. Briefly, F_{in} was follicular cell-based current with a reversal potential of −21·6 ± 3 mV (Fig. 1*A*), and with an onset delay of *ca* 400 ms, measured by application of a brief jet of ACh (100 μM) from a micropipette positioned 50 μm from the follicle (5 follicles, 2 frogs). F_{in} inactivated (50 % from the peak) in approximately 10–25 s (e.g. Fig. 1*B*), and was not dependent on the external osmolarity (15 follicles, 6 frogs). F_{in} elicited by ACh was inhibited (50 %) by overnight incubation with pertussis toxin (5 μg ml^{-1}), suggesting a G protein-mediated receptor channel pathway (7 follicles, 2 frogs). All of these characteristics were consistent with those of F_{in} responses elicited by ATP (Arellano *et al.* 1998). F_{in} activation by ATP cross-inhibited F_{in} activated by ACh, suggesting that both agonists acted on a common channel-activation pathway.

On the other hand, $I_{K,cAMP}$ had a mean reversal potential of −98 ± 2 mV (Fig. 1*A*), and the inhibitory effect of ACh on $I_{K,cAMP}$ had the same characteristics as those described before (Dascal *et al.* 1985; Van Renterghem *et al.* 1985; Stinnakre & Van Renterghem, 1986; Woodward & Miledi, 1987) (Fig. 1*C*). For example, inhibition of $I_{K,cAMP}$ was associated with a decrease in membrane conductance (Fig. 1*A*), was Ca^{2+} independent as mentioned above, and was mimicked by 4β-phorbol 12,13-dibutyrate (1 μM; 3 follicles, 2 frogs).

Thus, in full accord with previous studies, the two ACh responses studied here shared certain characteristics: (1) both were abolished by defolliculation, (2) both were blocked by atropine, a non-specific muscarinic antagonist, and (3) both cholinergic actions were Ca^{2+} independent. Despite these common general characteristics, some other properties suggested that they were elicited independently. For example, the amplitudes of the two cholinergic actions in follicles from different frogs were not correlated. In follicles ($n = 12$) from one particular frog, ACh (10 μM) elicited a strong inhibition (95 ± 3 %) of $I_{K,cAMP}$ activated by FSH (0·25 μg ml^{-1}) or ADE (10 μM), while in the same follicles ACh (10–100 μM) elicited weak F_{in} responses of 48 ± 15 nA. Similar results were obtained in follicles from two other frogs. In contrast, in follicles ($n = 11$) from another frog, ACh inhibited $I_{K,cAMP}$ by only 63 ± 5 % while it generated robust F_{in} of 947 ± 152 nA; a similar relationship was also found in follicles from two other donors. Also, in both follicles and oocytes where endogenous acetylcholinesterase (Moya *et al.* 1991) was specifically blocked, the amplitudes of the muscarinic currents were still highly variable (R. Miledi & R. O. Arellano, unpublished observations), suggesting that this characteristic was not due to the activity of the enzyme. Thus, as has been amply shown for several different types of receptor in *Xenopus* follicles (Arellano *et al.* 1996), the amplitudes of the two responses to ACh studied here were independently expressed among follicles from different frogs.

The latter indirect evidence for independence of the two cholinergic responses was then investigated in more detail. Dose–response curves were constructed for both ACh effects (Fig. 1*B–D*) in 12–16 follicles from 5 frogs; the F_{in} amplitude and percentage of $I_{\mathrm{K,cAMP}}$ inhibition were normalized and plotted as in Fig. 1*D*. In these experiments, follicles from two frogs were tested for ACh-generated inhibition of $I_{\mathrm{K,cAMP}}$ (elicited by 0·25 μg ml^{-1} FSH) when the K^+ current was at its maximum (Fig. 1*C*). In follicles from another frog, curves were generated by superfusing ACh for approximately 1 min before and during stimulation by FSH (same concentration) or ADE (e.g. Fig. 2*B*). Both stimulation protocols gave the same results. The follicular ACh responses were dose dependent, with $I_{\mathrm{K,cAMP}}$ inhibition being much more sensitive to ACh than F_{in} activation. The half-maximal effective concentration (EC_{50}) for $I_{\mathrm{K,cAMP}}$ inhibition was

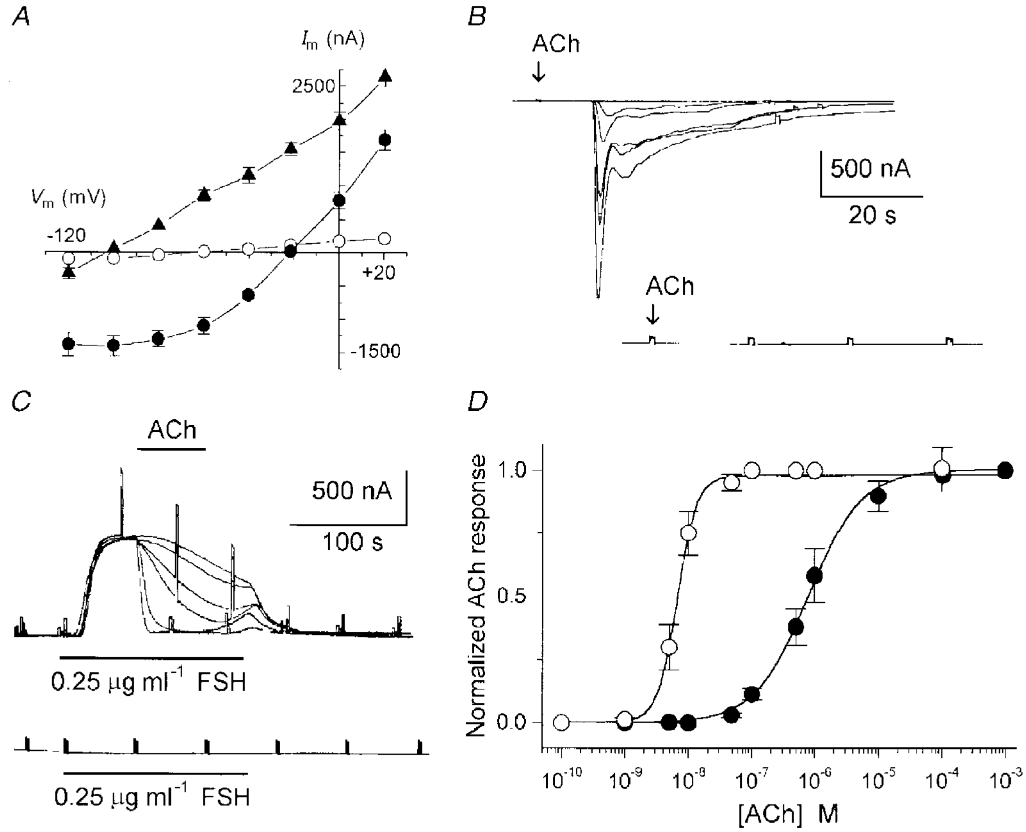

Figure 1. Current–voltage and dose–response relationships of follicular responses generated by ACh

A, membrane current–voltage relationships of F_{in} (●) and $I_{\mathrm{K,cAMP}}$ (▲) elicited by FSH (0·25 μg ml^{-1}), and during $I_{\mathrm{K,cAMP}}$ inhibition (○) by 0·1 μM ACh. Each point indicates the mean (± S.E.M.) from 4–7 follicles (3 frogs) tested in each condition. Reversal potentials for the currents were $-21{\cdot}6 \pm 3$, -98 ± 2 and -55 ± 9 mV, respectively. *B*, top traces: follicular F_{in} elicited by ACh applied consecutively at concentrations of 0, 0·05, 0·1, 0·5, 1 and 10 μM, with 10 min wash intervals in NR solution. The ACh applications started at the point indicated by the arrow and lasted about 80 s. The superimposed traces are from a single epithelium-removed follicle held at −60 mV. Bottom, 10 μM ACh application in a defolliculated oocyte from the same frog. *C*, top traces: inhibition of $I_{\mathrm{K,cAMP}}$ (generated by FSH) by increasing concentrations of ACh (0, 1, 5, 10, 50 and 100 nM, with 15 min wash intervals). Data from 1 follicle held at −40 mV. In this and subsequent records, the bars indicate the time of drug application, and voltage steps of +20 mV (2 s) were applied periodically to monitor membrane conductance. Bottom, FSH application in a defolliculated oocyte from the same frog. *D*, dose–response relationships for activation of F_{in} (●) and inhibition of $I_{\mathrm{K,cAMP}}$ (○) normalized with respect to the maximal response. Means (± S.E.M.) of 12–16 follicles from 5 frogs. Curves are fits to the equation:

$$I/I_{\mathrm{max}} = [(A_1 - A_2)/1 + ([\mathrm{ACh}]/\mathrm{EC}_{50})^{n_{\mathrm{H}}}] + A_2,$$

by the method of non-linear least squares fitting, where EC_{50} is the half-maximal effective concentration of ACh, n_{H} is the slope factor (Hill coefficient), A_1 and A_2 are the initial and final normalized I values, respectively, and [ACh] is the concentration of the neurotransmitter.

$6{\cdot}6 \pm 0{\cdot}4$ nM, while for F_{in} activation the EC_{50} was 784 ± 4 nM. A clear difference was also observed in the Hill slope coefficients (n_H) of the dose–response curves, which were approximately 3 for $I_{K,cAMP}$ inhibition and 1 for F_{in} activation. This suggests a highly cooperative mechanism in the case of the K^+ current inhibition, and a more direct mechanism for F_{in} activation. The sensitivity to ACh inhibition was independent of the agonist used for activating $I_{K,cAMP}$; thus ADE ($0{\cdot}2$ μM), dopamine ($0{\cdot}5$ μM) and noradrenaline (10 μM) gave similar results, and dose–response curves obtained at a holding potential of −60 mV showed no differences (not shown).

Blocking effects of cholinergic antagonists

As a first approach to discriminate the different cholinergic responses at a membrane receptor level, the effects of various antagonists were analysed. Neither of the follicle currents was affected by tubocurarine (1–10 μM) and neither was mimicked by nicotine (0·1–1 mM) (6 follicles, 3 frogs), and as noted before both were blocked by atropine; thus the responses were confirmed to be of the muscarinic type. The results of previous pharmacological studies in *Xenopus* oocytes suggested the presence of muscarinic receptors of the M_1 and M_3 subtypes (Van Wezenbeek *et al.* 1988; Davidson *et al.* 1991), whereas the amino acid sequence of XlmR shows that it is more comparable to the human m4 subtype (Herrera *et al.* 1994), although competitive inhibition binding curves show that XlmR exhibits similarities to the m2 subtype (Herrera *et al.* 1997). In view of this, we studied the antagonistic effects of PZP, MTT, 4-DAMP and TPC (Fig. 2), which have, respectively, a high affinity for M_1, M_2, M_3 and M_4 receptors in other preparations (e.g. Caulfield & Birdsall, 1998). For these experiments, the follicular responses were elicited by ACh doses around their respective EC_{50}, i.e. 7–10 nM ACh for $I_{K,cAMP}$ inhibition and 0·8–1 μM ACh for F_{in} activation. To study the effects on F_{in}, a given concentration of an antagonist was applied alone for 100–120 s, before ACh and the antagonist (same concentration) were coapplied (Fig. 2*A*). To examine the effects of antagonists on the ACh-induced

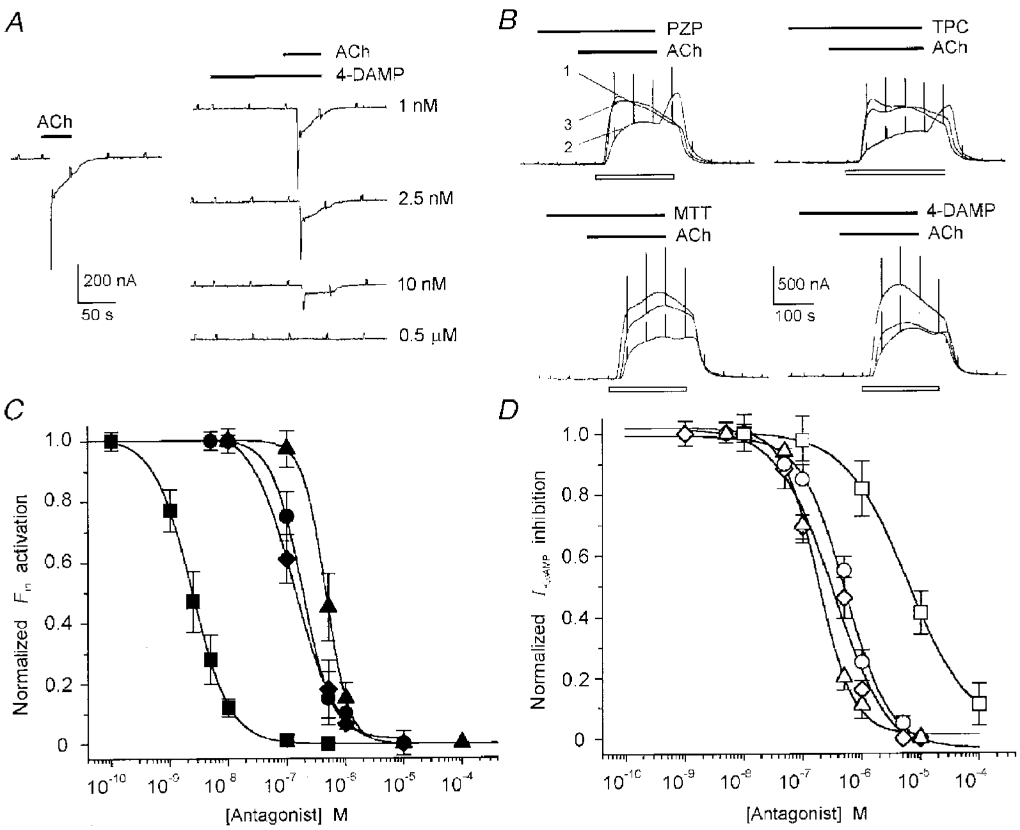

Figure 2. Dose–response relationships of muscarinic antagonists on follicular responses to ACh

A, effect of 4-DAMP on F_{in} generated by 800 nM ACh. The traces on the right show 4-DAMP blockade of F_{in} elicited by ACh; the trace on the left is the control current. Wash periods were of 10 min. Data from a single follicle. *B*, blocking effect of 1 μM PZP, TPC, MTT or 4-DAMP on 8 nM ACh inhibition of $I_{K,cAMP}$ generated by 2 μM ADE (open bar below each trace). Superimposed traces in each group correspond to control current (ADE alone; 1), $I_{K,cAMP}$ inhibition in the presence of ACh and ADE (2), and the same as 2 plus the antagonist which was superfused for 100 s before ACh application (3). Wash intervals were of 15 min. Data from different follicles (2 frogs). *C* and *D*, dose–antagonist relationships for the effect of 4-DAMP (squares), MTT (circles), PZP (triangles) and TPC (diamonds) on F_{in} activation (*C*) and $I_{K,cAMP}$ inhibition (*D*) elicited by ACh at the EC_{50} concentration. Curves were fitted using the same equation as for Fig. 1*D*, and each point represents the mean of 4–5 follicles from 2 frogs. $I_{K,cAMP}$ was generated by 2 μM ADE or 2 μg ml^{-1} FSH.

inhibition of $I_{K,cAMP}$, a mixture of ACh plus antagonist and ADE or FSH was applied (Fig. 2*B*). F_{in} was more potently blocked by 4-DAMP than the ACh-induced inhibition of $I_{K,cAMP}$; all other compounds blocked the responses over a similar submicromolar range. The half-maximal inhibitory concentrations (IC_{50}) for F_{in} of PZP, MTT, 4-DAMP and TPC (Fig. 2*C*) (slope factor (n_H) in parentheses) were, respectively, 457 ± 3 nM (2), 183 ± 15 nM (1·7), $2·4 \pm 0·07$ nM (1·3) and 134 ± 9 nM (1·1). The IC_{50} values for block of the inhibition of $I_{K,cAMP}$ were 183 ± 20 nM (1·4), 530 ± 11 nM (1), 5900 ± 200 nM (0·8) and 341 ± 15 nM (1) (see Fig. 2*C* and *D*). The relative potencies for block of F_{in} activation or $I_{K,cAMP}$ inhibition by PZP, MTT and TPC were fairly similar; however, 4-DAMP was about 2000 times more potent in blocking F_{in}, thus supporting the notion that cholinergic actions upon F_{in} activation and $I_{K,cAMP}$ inhibition involve distinct muscarinic receptors. The potency sequences were 4-DAMP $\gg$ TPC $\approx$ MTT > PZP for the effect on F_{in}, and PZP > TPC $\approx$ MTT $\gg$ 4-DAMP for the effect on $I_{K,cAMP}$ inhibition. The higher sensitivity to 4-DAMP compared with that to PZP and MTT suggests that a population of M_3 receptors is involved in the activation of F_{in}.

In follicles from the same frogs used to study the antagonism of ACh effects, F_{in} activation or $I_{K,cAMP}$ inhibition produced by ATP (10 μM) through activation of purinergic receptors was not affected by any of the four cholinergic antagonists (100 μM) tested (13 follicles, 4 frogs; data not shown). Thus, these antagonists appear to act specifically on the follicular muscarinic receptors, and not on ion channels or some other molecular component of the membrane pathways involved.

Effects of cholinergic agonists on follicular ACh responses

The generation of follicular responses by activation of muscarinic receptors was then studied using different agonists. Carbachol, a non-specific agonist, elicited both ACh responses (Fig. 3*D*); dose–response curves gave EC_{50} values of $51·5 \pm 3$ and 329 ± 17 nM for $I_{K,cAMP}$ inhibition and F_{in} activation, respectively. Both responses elicited by carbachol (100 μM; 9 follicles, 3 frogs) were blocked by atropine (1 μM), 4-DAMP or PZP (both at 100 μM). In contrast, when the follicular responses were elicited by oxotremorine, an agonist with higher affinity for the M_2 receptor subtype, there was a clear specificity in the response elicited. Oxotremorine did not activate F_{in} responses as robustly as did ACh or carbachol in the same follicles (Fig. 3*A*). Indeed, the F_{in} responses elicited by oxotremorine (0·1–100 μM) were in all cases < 5% of those activated by the other agonists

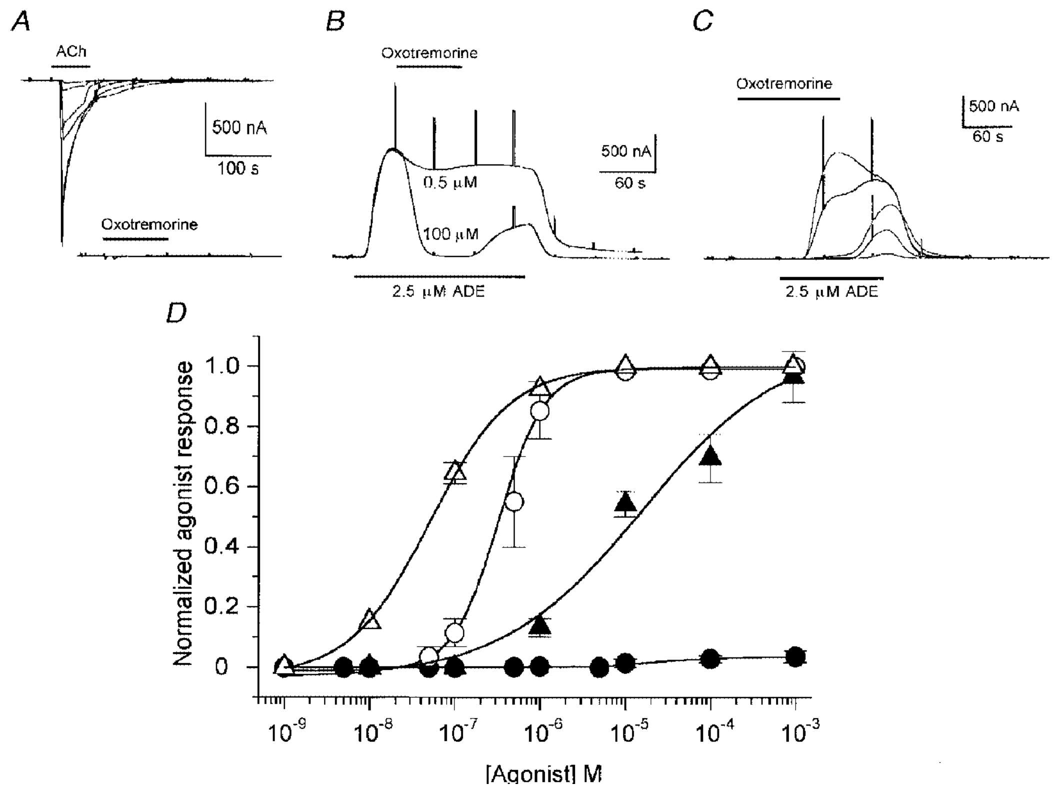

Figure 3. Dose–response relationships of muscarinic agonists on follicular responses

A, F_{in} generated by ACh and oxotremorine in a single follicle. The ACh concentrations were 0·05, 0·1, 0·5, 1, 10 and 100 μM, and the oxotremorine concentrations were 1, 10 and 100 μM. *B*, inhibition by oxotremorine of $I_{K,cAMP}$ (elicited by ADE). Oxotremorine (0·5 or 100 μM) was applied at the peak of the K^+ current. *C*, preincubation with oxotremorine also blocked $I_{K,cAMP}$. The oxotremorine concentrations were 0·1, 1, 10, 100 and 1000 μM. Note that at intermediate concentrations (1–100 μM) $I_{K,cAMP}$ inhibition was reversed rapidly on washout. *D*, carbachol (open symbols) and oxotremorine (filled symbols) dose–response relationships for F_{in} activation (circles) and $I_{K,cAMP}$ inhibition (triangles). Mean of 9–13 follicles from 5 frogs.

(same concentration range). For example, in eight follicles from one frog the F_{in} response elicited by ACh (10 μM) was 1387 ± 340 nA, while the F_{in} response generated by oxotremorine (100 μM) was 36 ± 8 nA. All follicles ($n = 28$) from different frogs ($n = 6$) behaved similarly. However, oxotremorine completely inhibited the $I_{K,cAMP}$ elicited by FSH or ADE (Fig. 3*B–D*). The EC_{50} for oxotremorine-elicited inhibition was $15{\cdot}8 \pm 1{\cdot}4$ μM (13 follicles, 5 frogs).

The inhibition of $I_{K,cAMP}$ induced by 10–100 μM oxotremorine (14 follicles, 6 frogs) was blocked by cholinergic antagonists such as atropine (1 μM), 4-DAMP, PZP and TPC (all at 100 μM), thus indicating that oxotremorine was acting on muscarinic receptors (Fig. 4*A*). Oscillatory Ca^{2+}-dependent Cl^- currents generated by ACh in follicles ($n = 8$) from some donors (3 frogs) were not mimicked by applications of oxotremorine (0·01–100 μM; Fig. 4*B*). In addition, oxotremorine itself had a potent inhibitory effect on F_{in} generated by ACh (10 μM). This effect had an IC_{50} of $2{\cdot}3 \pm 0{\cdot}7$ μM, and was reversible on washout with NR solution for 7–10 min (Fig. 5*A* and *B*).

Cholinergic membrane mechanisms and follicular cell–oocyte communication

Further evidence for the idea that different types of muscarinic receptor are involved in the generation of F_{in} and in the inhibition of $I_{K,cAMP}$ by ACh was derived from a comparison of the rates of development of both responses. These rates were estimated as the time necessary for the currents to reach 50% of their maximum. In the case of F_{in} evoked by ACh, the rate of activation decreased linearly with the logarithm of the increase in agonist concentration, while the rate of $I_{K,cAMP}$ inhibition decreased sigmoidally (Fig. 4*C*).

To study these responses further, and to obtain information about the possible mechanisms of $I_{K,cAMP}$ inhibition, we designed the following experiment in order to see whether muscarinic stimulation of follicular receptors decreased the coupling between the follicular cells and the oocyte, as has been suggested previously (e.g. Greenfield *et al.* 1990), and proved for some other cellular systems coupled through gap-junction channels (see Neyton & Trautmann, 1986).

If the ACh inhibition of $I_{K,cAMP}$ is due, at least in part, to a muscarinic uncoupling of follicular cells from the oocyte, one would assume that any other current, such as F_{in} elicited by ATP, which also originates in the follicular cells, should suffer a similar inhibition. To examine this question, follicles were stimulated with ATP (10 μM) or ACh (50 μM) alone, or with either agonist co-applied with oxotremorine, after a brief (approximately 60 s) preincubation with oxo-

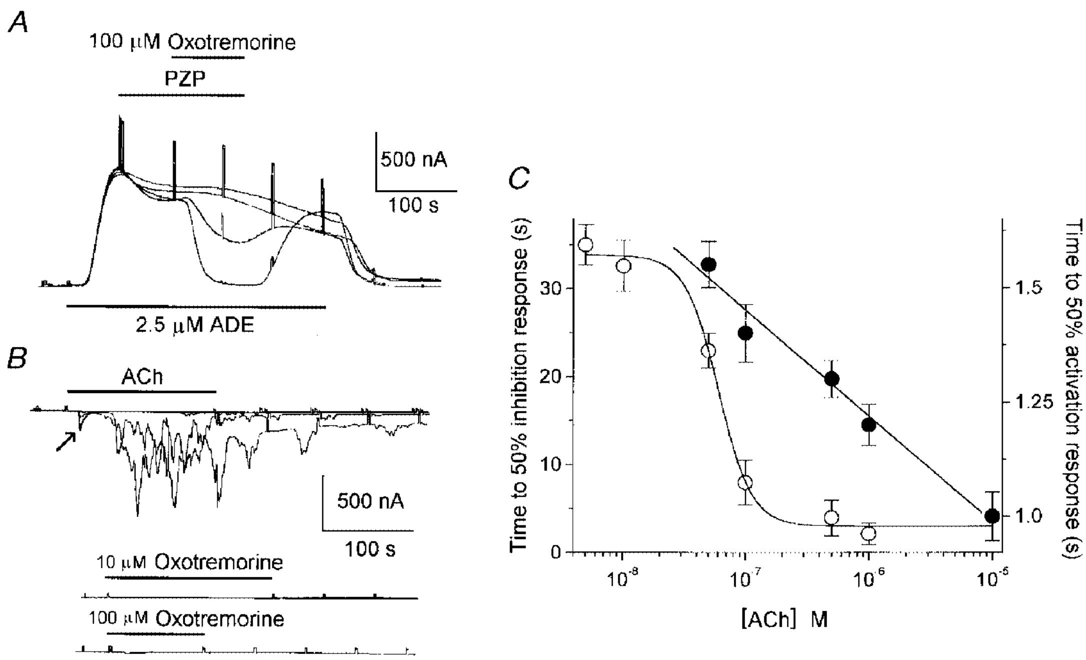

Figure 4. Oxotremorine effect on follicular responses and activation rates of muscarinic responses

A, PZP blockade of oxotemorine-elicited (100 μM) inhibition of $I_{K,cAMP}$. Decreasing concentrations of PZP were applied (100, 10, 1 and 0·1 μM), with 15 min wash intervals. Similar results were obtained in 3 other follicles from 2 frogs. *B*, examples of follicles in which ACh generated both F_{in} (arrow) and oscillatory Ca^{2+}-dependent Cl^- currents (top traces), but in which oxotremorine failed to generate any response (middle and bottom traces). The 4 superimposed traces (top) show currents elicited by ACh (0·1, 1, 10 and 100 μM) applied to the same follicle. Similar results were obtained in follicles from 2 other frogs. *C*, ACh concentration *vs.* rate of F_{in} activation (●) or inhibition of $I_{K,cAMP}$ (○). Rates are expressed as the time necessary to reach 50% of the maximal response for each concentration. For F_{in} activation the rate decreased linearly while for $I_{K,cAMP}$ inhibition it increased following a sigmoidal relationship (3 follicles, 2 frogs).

tremorine alone. The concentrations of oxotremorine used ranged from 0·01 to 1000 μM but special attention was given to concentrations (10–100 μM) where oxotremorine completely inhibited $I_{K,cAMP}$ but weakly activated F_{in}. Under such conditions it would be expected that oxotremorine would not directly cross-affect F_{in} responses very strongly, in the way that ACh affects ATP-activated F_{in} (see Arellano *et al.* 1998). The results of these experiments are illustrated in Fig. 5. Oxotremorine, at doses which produced complete inhibition of $I_{K,cAMP}$, reduced by only 8 ± 5% the F_{in} elicited by ATP. This suggests that muscarinic stimulation did not induce an important closing effect of the follicular cell–oocyte gap-junction channels, and that ACh inhibited $I_{K,cAMP}$ more directly by acting on the K^+ channels. Moreover, as we have noted above, concentrations (100 μM) of oxotremorine that almost completely blocked the ACh-elicited F_{in} inhibited only a small fraction of the F_{in} elicited by ATP (Fig. 5*A* and *C*), indicating that the inhibitory effect of oxotremorine on F_{in} is more probably a direct effect on the muscarinic receptor.

Molecular detection of XlmR in isolated follicular cells and oocytes

The differences found for the follicular muscarinic receptors stimulated during F_{in} activation and $I_{K,cAMP}$ inhibition already gave some clues as to the possible receptor subtypes involved. For example, in the case of $I_{K,cAMP}$ inhibition the receptor (1) had a high sensitivity to PZP and MTT, and (2) was fully activated by oxotremorine, a preferential agonist of M_2 receptors. The muscarinic receptor (XlmR) encoded by the gene cloned from a *Xenopus* oocyte cDNA library (Herrera *et al.* 1994) resembles the human m4 receptor, although a clear functional or pharmacological distinction cannot be made between M_2 or M_4 subtypes (Herrera *et al.* 1997). Therefore, as a first step to see whether XlmR plays a role during $I_{K,cAMP}$ inhibition, we examined whether XlmR was present in follicular cells.

Using primers specific for XlmR, RT-PCR amplification of total RNA was performed for two different types of cellular preparations: defolliculated oocytes and isolated follicular cells. RT-PCR amplification resulted in a product of 430 bp in both preparations, as expected for the transcript of *XlmR* gene (Herrera *et al.* 1994), while no amplification was obtained in controls in which the reverse transcriptase was omitted (Fig. 6*A*). This result indicates that the receptor may be expressed not only in the membrane of the oocyte (see Herrera *et al.* 1994), but also in the membrane of the follicular cells. In order to confirm that the RT-PCR product represented the transcript of *XlmR* gene and was not due to non-specific amplification, the positive product was then identified by hybridization with an *XlmR* probe in Southern blots (Fig. 6*B*). Again, this resulted in a signal of the expected size and thus suggested the presence of XlmR in the follicular cells. All PCR amplifications from 4 frogs gave the same result. Electrophysiological experiments revealed that follicles ($n = 6$) from the same frogs exhibited ACh-elicited inhibition of $I_{K,cAMP}$, while defolliculated oocytes ($n = 7$) did not generate any response to ACh.

These results suggest that XlmR may be involved in the cholinergic responses from follicular cells, particularly in the mechanisms that produce $I_{K,cAMP}$ inhibition.

DISCUSSION

Our study shows that in *Xenopus* follicles there is a molecular heterogeneity in the muscarinic receptors involved in generating the current responses elicited by

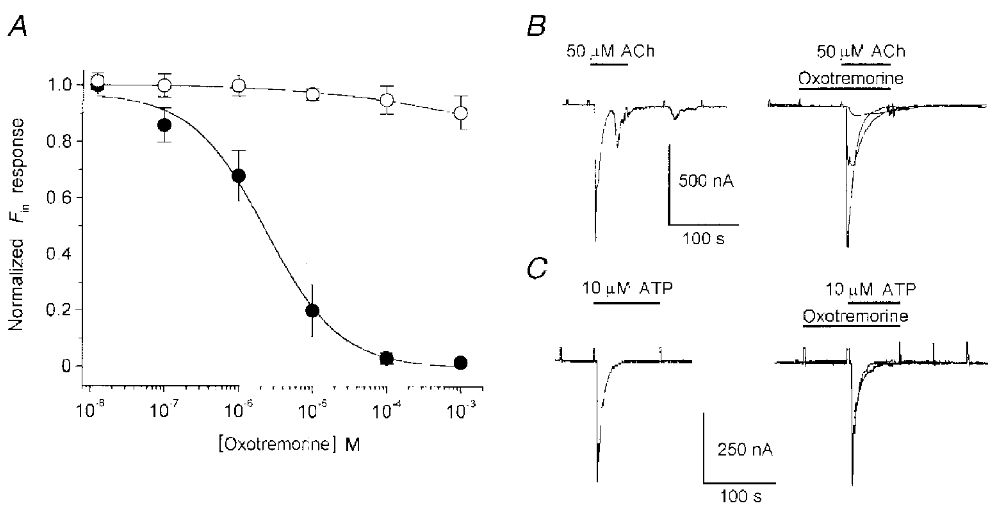

Figure 5. Oxotremorine effect on F_{in} elicited by ACh or ATP

A, dose–response relationships for oxotremorine inhibition of F_{in} elicited by 50 μM ACh (●) or 10 μM ATP (○). Inhibition curves were adjusted as in Fig. 1*D*. The IC_{50} for F_{in} elicited by ACh was $2·3 \pm 0·7$ μM. Each point represents the mean of 8 follicles from 3 frogs. *B* and *C*, sample traces of inhibition produced by oxotremorine on F_{in}. The trace on the left in each panel is the control current elicited by the agonist alone, and the superimposed traces on the right show the effects of oxotremorine: for F_{in} activated by ACh the oxotremorine concentrations were 10 nM, 1 and 10 μM, and for F_{in} activated by ATP the concentrations were 10 and 100 μM. All traces were obtained from a single follicle.

ACh. Pharmacological and kinetic evaluations of these responses revealed that two different muscarinic receptor subtypes appear to be coupled to different sets of membrane proteins producing completely distinct responses. Both muscarinic receptors seem to be in the membrane of the follicular cells. Their principal differences are: (1) the receptor that produces inhibition of the channel underlying $I_{K,cAMP}$ displayed a 100-fold higher sensitivity to ACh than the receptor involved in F_{in} activation. (2) The antagonist 4-DAMP was about 2000-fold more potent on the receptor mediating F_{in} activation. (3) Oxotremorine partially mimicked the actions of ACh and inhibited $I_{K,cAMP}$. In sharp contrast, this cholinergic agonist potently inhibited F_{in} generated by ACh. (4) The rate of development of $I_{K,cAMP}$ inhibition was non-linearly dependent on the dose of ACh, while that for F_{in} generation was linear.

Our results suggest that F_{in} activation is mediated by an M_3-like receptor subtype. This conclusion is based on the pharmacological results which show that 4-DAMP was by far the most effective antagonist of F_{in}. A similar conclusion was reached for the muscarinic receptor involved in generating the oscillatory Ca^{2+}-dependent Cl^- currents arising in the oocyte (Van Wezenbeek *et al.* 1988). Despite this similarity, these two responses to ACh differ. For example, they are located in different cellular compartments (i.e. oocyte *vs.* follicular cells), and F_{in} (and the *slow* inward current named S_{in}; Arellano & Miledi, 1993) are not eliminated when the oocytes are loaded with Ca^{2+} chelators (e.g. EGTA, BAPTA). This strongly suggests that an intracellular Ca^{2+} increase is not a central element in the activation cascade of F_{in}.

The effect of oxotremorine on F_{in} is particularly interesting. As far as we know, this is the first report of an inhibitory action of this drug on the function of muscarinic receptors, but further experiments will be necessary to determine the mechanism of action of oxotremorine on this receptor (e.g. antagonism *vs.* partial agonism), and to determine whether this effect is specific for follicular muscarinic receptors.

On the other hand, the muscarinic receptor involved in the inhibition of $I_{K,cAMP}$ seems to be an M_2-like receptor, because it was fully stimulated by oxotremorine and it was more sensitive to PZP, TPC and MTT, than to 4-DAMP. The molecular characteristics of XlmR, and the functional membrane mechanisms it controls when expressed in transfected cell lines, closely resemble those of both the M_4 and M_2 subtypes. By using RT-PCR we showed that follicular cells, as well as the oocyte, possess *XlmR* transcripts. Based on the functional and pharmacological characteristics of the receptor involved in $I_{K,cAMP}$ inhibition, and the possible expression of an M_2 or M_4-like receptor encoded by the *XlmR* gene in follicular cells, we suggest that this receptor subtype is responsible for $I_{K,cAMP}$ inhibition, although, in competitive inhibition binding experiments (Herrera *et al.* 1997), XlmR transiently expressed in COS-7 (African green monkey kidney) cells also exhibited a higher affinity for 4-DAMP than for PZP and MTT.

The XlmR couples efficiently to the system that produces inhibition of adenylyl cyclase and activation of mitogen-activated protein (MAP) kinase in COS-7 and human embryonic kidney 293 cells (Herrera *et al.* 1997), but its mode of function in the oocyte or in the follicular cells is still unknown. Nevertheless, from our results and those of other studies it is clear that the inhibition of $I_{K,cAMP}$ does not involve a reduction of cAMP synthesis, because $I_{K,cAMP}$ activated by direct intracellular injection of cAMP or cGMP is also blocked by ACh (Dascal *et al.* 1985; Miledi & Woodward, 1989; Greenfield *et al.* 1990). Another important fact is that the electrical communication between the oocyte and the follicular cells is not greatly affected by muscarinic stimulation, because oxotremorine-sensitive receptors did not produce a strong effect on F_{in} generated by ATP, and these responses are dependent on electrical coupling between

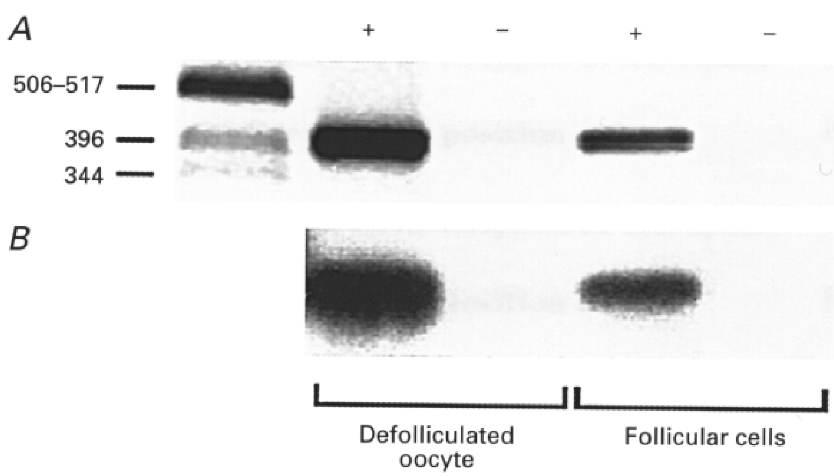

Figure 6. RT-PCR amplification of *XlmR* transcripts in follicular cells

A, ethidium bromide-stained gel of RT-PCR-amplified products. The left-hand lane contains DNA markers (in bp). +, cDNA from defolliculated oocytes or isolated follicular cells. −, negative controls, RNA without reverse transcriptase. *B*, Southern blot of the same gel as in *A*, hybridized with *XlmR* probe.

those cells (Arellano *et al.* 1998). This lack of effect on electrical coupling supports the idea that the eventual XlmR expression in the oocyte membrane does not play a fundamental role in $I_{K,cAMP}$ inhibition. All this strongly suggests that the ACh inhibitory effect on $I_{K,cAMP}$ is a more direct effect on the K^+ channels, a conclusion similar to that reached previously using different experimental procedures (e.g. Miledi & Woodward, 1989; Honoré & Lazdunski, 1993).

The fact that protein kinase C (PKC) activators such as phorbol esters, like ACh, inhibit $I_{K,cAMP}$ suggests that PKC might participate in the cascade stimulated by the M_2-like muscarinic receptor of the follicular cells. However, this needs to be reconsidered, since it has been shown that phorbol esters effectively reduce the communication between *Xenopus* follicular cells and oocytes (Greenfield *et al.* 1990), as they do in follicles from other species (e.g. Cerdá *et al.* 1993). Thus, the phorbol ester-induced inhibition of $I_{K,cAMP}$ monitored from the follicle clearly involves a different mechanism (i.e. closing of gap-junction channels) from that activated by ACh.

It still remains to be determined whether different muscarinic receptor subtypes coexist in the membrane of the same follicular cells, or if the heterogeneity of receptors and their membrane responses is a consequence of differences among the 10000 or so follicular cells that surround the oocyte. Under the latter scheme, there could be at least two types of follicular cells, i.e. a group with M_3-like receptors capable of eliciting the F_{in} and S_{in} responses, and a second group of follicular cells endowed with channels underlying $I_{K,cAMP}$ and M_2-like receptors. It should be noted that whether the receptors are M_3 or M_2 (or M_4) requires further support from pharmacological experiments (i.e. detailed competitive inhibition binding studies), and more thorough information concerning the molecular nature of the receptors involved. In any case, the finding of two different receptors and membrane mechanisms in the whole follicle will help to determine their role in important physiological events such as maturation, as well as their participation in the regulation of other currents such as the osmolarity-dependent S_{in}. For example, it has been reported that oocyte maturation is modulated by stimulation of muscarinic receptors (Dascal *et al.* 1984) and by ionic currents carried by K^+ and Cl^- (Wibrand *et al.* 1992; Skoblina & Huhtaniemi, 1997). However, the cellular mechanisms involved are poorly understood. It has been suggested that there is an important effect of Cl^- currents on the progesterone production pathway, and that the opening of K^+ channels favours maturation by increasing the oocyte sensitivity to progesterone. In addition, increasing evidence has accumulated regarding the participation of different neurotransmitters (e.g. noradrenaline, dopamine, ACh) in mammalian ovarian physiology, not only during oocyte growth and maturation, where it has been shown that neurotransmitters modulate ovarian steroidogenesis (e.g. Adashi & Hsueh, 1981), but also in fertilization, where ACh has been proposed to be involved in the activation process stimulated by sperm–egg interaction (Eusebi *et al.* 1984). Moreover, studies in different species have shown that such neurotransmitters may have various sources in the ovarian follicle. For example: (1) autonomic catecholaminergic and cholinergic innervation (e.g. Sporrong *et al.* 1985), (2) ovarian intrinsic neuronal-like cells that in non-human primates have been shown to contain tyrosine hydroxylase (Dees *et al.* 1995), and (3) the synthesis of noradrenaline by the oocyte itself (e.g. Mayerhofer *et al.* 1998). Thus, information regarding the nature of the receptors present in the membranes of follicular cells and oocytes and their mechanisms of action are important for a comprehensive understanding of their role in reproductive function.

In conclusion, we have shown the presence of two different types of muscarinic receptor in the membrane of *Xenopus* follicular cells. These receptors are like the M_3 and M_2 (or M_4) subtypes, and they trigger distinct membrane pathways that lead to two types of ionic channel regulation. The characteristics of XlmR suggest that it resembles the receptor involved in $I_{K,cAMP}$ inhibition, and we show here that *XlmR* transcripts are present in follicular cells, strongly suggesting that XlmR mediates the inhibition. Further information regarding the molecular nature of the receptors, ion channels and membrane mechanisms stimulated is needed to understand their functional role. The results presented here are a step towards that goal, and in the meantime, will also facilitate the rational use of the follicle-enclosed *Xenopus* oocyte as a cellular model in biology.

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