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Native *Xenopus* oocytes express two types of muscarinic receptors

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We have recently described two types of muscarinic responses in native *Xenopus* oocytes of different donors (common and variant) that display qualitative and quantitative differences (Lupu-Meiri et al., 1990). Here we characterized the muscarinic receptors mediating these two types. The anti-muscarinic toxins from *Dentroaspis* significantly inhibited responses in oocytes of common donors, but had little effect on responses in oocytes of variant donors, possibly indicating expression of different receptor subtypes. Using specific muscarinic antagonists, we found that oocytes of common donors exhibit a pattern compatible with the M3 subtype of muscarinic receptors, while oocytes of variant donors appear to possess receptors of the M1 subtype. To more directly determine the subtypes of muscarinic receptors in oocytes of both populations of donors, we have microinjected antisense oligonucleotides into native oocytes. Antisense oligonucleotides to unique sequences in the N-terminal and the third cytoplasmic loop of M3 muscarinic receptors caused a significant inhibition of the response of common oocytes, but had virtually no effect on responses in oocytes of variant donors. Conversely, oligonucleotides complementary to the unique sequences of the m1 muscarinic receptors inhibited the response in variant oocytes, but not in oocytes of common donors. We conclude that native *Xenopus* oocytes of different donors phenotypically express either M3-like (majority) or M1-like (minority) muscarinic receptor subtypes. The differences in receptor subtype expression may explain the different characteristics of responses in the two populations.

Muscarinic receptor subtype; *Xenopus* oocyte; Antisense oligonucleotide

1. INTRODUCTION

Muscarinic membrane electrical responses in *Xenopus laevis* oocytes have been first described by Kusano et al. [1] and characterized in detail by Dascal and Landau [2]. We have recently reported that oocytes of approximately 40% of all donors exhibit muscarinic responses. Of those, the majority (36%) conform to the characteristics described by Dascal and Landau [2], i.e. a rapid transient depolarizing current (D1) followed by a slow, prolonged depolarizing current (D2) and superimposed depolarizing current fluctuations (F). These responses have been denoted as 'common'. Oocytes of approximately 4% of donors also exhibit a three-component depolarizing response. This response, though superficially similar to the 'common' response described above, is characterized by a much larger amplitude of the transient component, significantly larger ^{45}Ca efflux, prolonged latency, lower sensitivity to collagenase treatment, profound desensitization, inhibition due to activation of protein kinase C and different magnitude of the response on the two hemispheres of the oocyte [3,4]. This response has been denoted as 'variant'.

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The qualitative and quantitative differences between the responses observed in 'common' and in 'variant' oocytes could be related to many factors, e.g. slightly different coupling of the signal transduction pathway. However, it is also possible that oocytes of the two types of donors express different subtypes of muscarinic receptors. Van Wezenbeck et al. [5] have partially characterized the responses in oocytes of common donors as mediated by M3'receptor subtype. We, on the other hand, have observed that responses to ACh in oocytes injected with rat brain RNA (and coding predominantly for M1-receptor subtype) resemble responses observed in oocytes of variant donors. Here, we characterize the sub-types of muscarinic receptors expressed in both 'common' and 'variant' oocytes and conclude that while 'common' oocytes express mainly the M3-like, 'variant' oocytes express predominantly the M1-like muscarinic receptor subtype. These findings may be important for the understanding of the apparent redundance of different muscarinic receptor subtypes.

2. MATERIALS AND METHODS

2.1. *Experimental animals*

Adult *Xenopus* females, purchased from South African Xenopus Facility, were maintained and fed as described [3]. The animals were cold-anaesthesized and ovary fragments were dissected into ND96 medium. Stage 5 or 6 oocytes were separated and maintained at 20°C

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in NDE96. When the same donors were used repeatedly, dissections were spaced 2–3 weeks apart to allow a full recovery of the animals from the surgery. Where indicated, follicular cells were removed by collagenase as described [6].

2.2. *Electrophysiology*

The electrophysiological methods were described in detail elsewhere [3,7]. Briefly, all experiments were performed under two-electrode voltage clamp using Dagan 8500 intracellular clamp. Oocytes were routinely voltage-clamped at −100 mV to minimize potassium current. Agonists were added rapidly and directly to the bath in >1 ml of ND96 in order to avoid the dead time of the perfusion system and the gradual build-up of the drug's concentration.

2.3. *Assay of* Dendroaspis *toxin activity*

The antimuscarinic toxin fraction was a generous gift from Alomone Laboratories (Jerusalem) and was prepared as described by Adem et al. [8]. The final concentration of the toxin was 0.1 mg protein/ml in ND96. Oocytes were pre-incubated in the toxin-containing solution for more than 45 min and then assayed for responses to 10 μM ACh. Oocytes of 'common' and 'variant' donors were assayed at the same experiment intermittently.

2.4. *Assay of muscarinic antagonists*

Five muscarinic antagonists were used. Atropine, pirenzepine, AF-DX 116, 5-diphenylacetoxy-*N*-methyl-piperidine methobromide (4-DAMP) and himbacine. Follicle-enclosed oocytes of both types of donors were pre-incubated with different concentrations of the various antagonists for 15–20 min and then exposed simultaneously to 10 μM ACh and the given concentration of the antagonist. Considering the slow dissociation of the antagonists in follicle-enclosed oocytes (Davidson and Oron, unpublished), these measurements were equivalent to determining the fraction of receptors unoccupied by the antagonist. The results were always normalized to standard responses to 10 μM ACh in the same experiment.

2.5. *Intracellular injections of oligonucleotides*

The oligonucleotides were synthesized on a 380B Applied Biosystems DNA synthesizer and purified on a 20% acrylamide/8 M urea preparative sequencing gel for each muscarinic ACh receptor mRNA sequence [9]. They were complementary to the nucleotide sequence corresponding either to the amino terminus or to the third cytoplasmic loop. Oligomers were complementary to bases 189–225 (anti m1/N), 1169–1210 (anti m1/CL) of the m1 receptor and to bases 47–97 (anti m3/N), 1184–1231 (anti m3/CL) of the m3 receptor. The specificity of the different oligonucleotides has been previously shown by Northern analysis in rat brain, where a hybridizing band could be detected with both the appropriate size and brain region distribution and by in situ hybridization histochemistry [10,11].

The oligonucleotides were dissolved in distilled water and 0.5–10.0 ng (38–675 fmol)/oocyte were injected into single oocytes (in 30–50 nl/oocyte) using a VWR digital microdispenser (10 μl maximal delivery) with the capillary pulled to a 5–20 μm tip. Control oocytes were sham injected with the same volume of distilled water. Oocytes were assayed for responses 2.5–5.0 h following their injection.

2.6. *Analysis of results*

All experiments were repeated several times in oocytes from different frogs. The number of oocytes assayed for each condition is denoted by *n* and the number of donors by *N*. Experiments were performed by assaying 4–10 oocytes within each experiment and mean ± S.E.M. values were determined. Statistical significance was determined by Student's *t*-test.

2.7. *Solutions and chemicals*

The composition of ND96 was (in mM): NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, Na-Hepes 5, pH 7.5. NDE96 included additionally Na-pyruvate (2.5 mM). ACh, atropine, pirenzepine and collagenase (type IA) were purchased from Sigma. AF-DX 116 was a gift of Dr. Karl Thomae, GmbH(Biberach, Germany), 4-DAMP was a gift of Dr. R.B. Barlow (University of Bristol), himbacine hydrochloride was a gift of Dr. W.C. Taylor (University of Sidney). All other chemicals were of analytical grade.

3. RESULTS

3.1. *The effect of anti-muscarinic toxins from* Dendroaspis

Muscarinic responses in oocytes of common and variant donors exhibit multiple differences [3]. Typical responses are shown in Fig. 1A,B. To obtain preliminary evidence that these two types of responses result

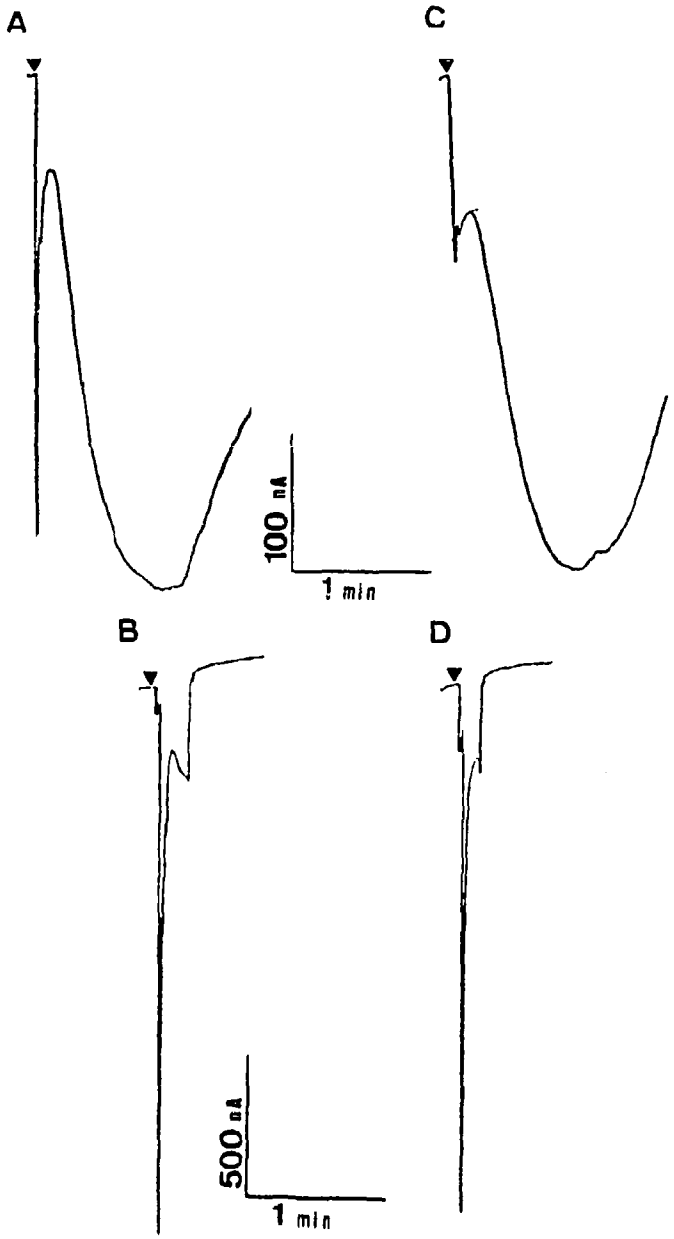

Fig. 1. Representative responses of common (A) and variant (B) oocytes to 10 μM ACh. The holding potential was −100 mV. Note the different calibration. Similar responses after 65–75 min incubation with 0.1 mg/ml Dendroaspis toxins are shown for common (C) and variant (D) oocytes. The slow component of the response was not recorded in variant oocytes.

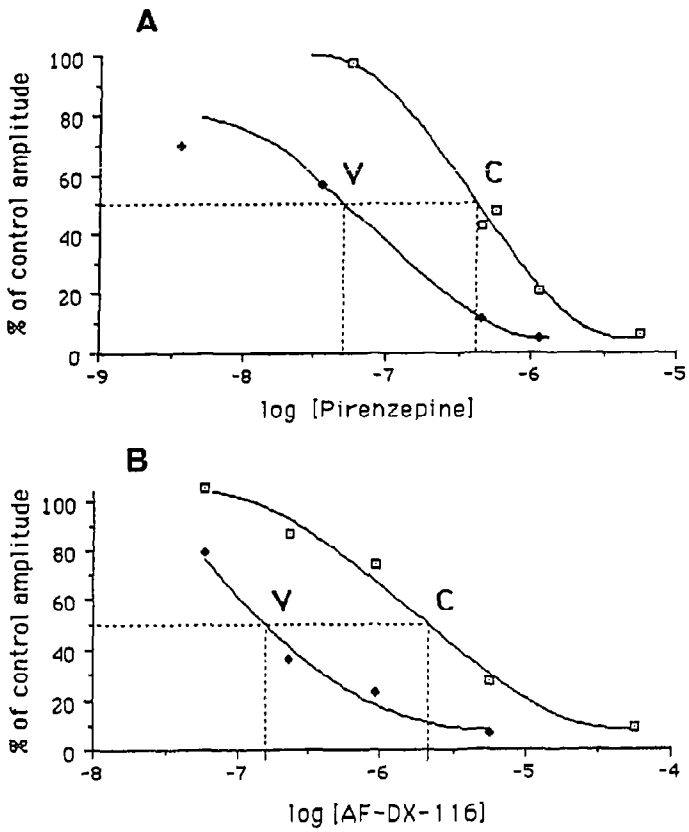

Fig. 2. Dose-response curves for the inhibition of the rapid component (D1) of the responses to 10 μM ACh by pirenzepine (A) or AF-DX-116 (B). Each point represents the mean of 18-30 determinations in individual oocytes from 3-5 different donors. In each experiment, values were normalized as percent of the control response to 10 μM ACh (100%). C, common, V, variant oocytes.

from the activation of two different receptor subtypes, we have used anti-muscarinic toxins from the green mamba snake (*Dendroaspis angusticeps*). It has been reported that these toxins compete for approximately 50% of specific muscarinic ligand binding in brain homogenates [8]. This implies that the toxins recognize some, but not all muscarinic receptor subtypes and may be used as tools for receptor discrimination.

Indeed, incubation of oocytes of common donors with 0.1 mg/ml of the toxins solution resulted in significant (48 ± 6%) inhibition of the D1 component of the response ($n = 53$, $N = 4$). The same batches of toxins had little effect on the rapid component of the response in oocytes of variant frogs (10 ± 3% inhibition, $n = 32$, $N = 3$). Representative responses are shown in Fig. 1C,D. These results implied that oocytes of common and variant donors express different receptor subtypes.

3.2. *The effect of muscarinic antagonists*

The order of potency of muscarinic antagonists was similar in inhibiting the muscarinic responses in oocytes of both common and variant donors. In both cases, the order of potency was: atropine > DAMP > pirenzepine > AF-DX 116 (see Fig. 2 for representative curves of pirenzepine and AF-DX-116 inhibition). The M2-selective antagonist, himbacine, exhibited very low potency against responses in oocytes of both 'common' and 'variant' donors. The IC_{50} values for the various antagonists in common and variant oocytes were very close to those reported in radioligand binding studies in salivary gland (M3) and brain (M1), respectively [12] (see Table I). Hence, judging by the order of potency and similarities to receptor binding data in representative tissues, common oocytes appear to express mainly M3-like, while variant oocytes exhibit M1-like muscarinic receptor subtypes.

3.3. *The effect of complentary oligonucleotide probes*

Although the results obtained with the muscarinic antagonists are suggestive, they do not make a definite proof. This is due to the poor selectivity of the antagonists, the rapid and transient nature of the response (which precludes the use of competitive pharmacological analysis (e.g. Schild plots)) and the relatively low signal-to-noise ratio in radioligand binding experiments [3]. More importantly, the pharmacological tools are very poor in discriminating between the recently cloned m4 abtypes [13].

For all the reasons described above, we have decided

Table 1

$-\log(IC_{50})$ values of muscarinic antagonists

Antagonist	Common		Salivary gland*	Variant		Brain*
	D1	D2		D1	D2	
Atropine	8.9	8.9	(8.9)	9.0	8.8	(9.2)
4-DAMP	8.0	7.2	(8.5)	8.5	6.8	(8.7)
Pirenzepine	6.4	6.5	(6.4)	7.3	6.5	(7.4)
AF-DX-116	5.4	5.2	(5.3)	6.7	4.8	(6.1)
Himbacine	4.0	4.7	-	5.5	>5.0	-

Dose-response curves were generated to the various antagonists as described in Fig. 2 for the rapid component of the responses (D1). Data for the slow component of the responses (D2) were obtained in an identical manner. *The values in parentheses represent $-\log K_i$ for [^{3}H]-N-methylscopolamine binding taken from Doods et al. [12].

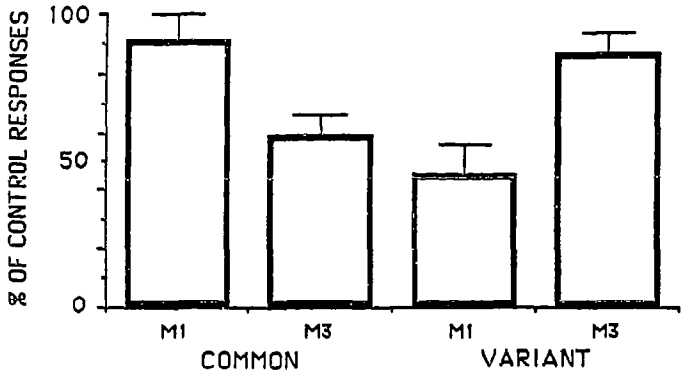

Fig. 3. The effect of anti-m1 or anti-m3 oligonucleotides. The bars represent the means ± SE of 9 individual experiments in common and variant oocytes, injected with 0.5 to 10.0 ng of the anti-m1 or anti-m3 oligonucleotides. Each value represents assay results of 25–68 oocytes from 4–6 different donors.

to conclusively identify the oocyte receptor subtypes by synthesizing antisense oligonucleotides complementary to unique sequences at the N-terminal and the third cytoplasmic loop regions of the m1 and m3 receptors. Injection of a large excess of a suitable oligonucleotide should result in the formation of DNA-mRNA hybrids in vivo. These double-stranded hybrids are rapidly degraded by RNase H, resulting in a loss of specific mRNA activity [14,15]. Provided that the turnover of membrane receptors is rapid, injection of a suitable probe would result in a rapid decrease in its activity.

Indeed, injection of antisense oligonucleotides for the m3 receptor, significantly ($P<0.01$) inhibited the response to ACh in oocytes of common donors (by $41 \pm 7\%$), but had little effect on the response in oocytes of variant donors. Conversely, oligonucleotides for the m1 receptor inhibited by $55 \pm 11\%$ ($P<0.01$) the response in oocytes of variant donors, but had negligible effect on the response in oocytes of common donors. These results are shown in Fig. 3.

4. DISCUSSION

Xenopus oocytes exhibit two types of intrinsic muscarinic responses [3]. Both types appear to utilize the G-protein-PIP_2-IP_3-calcium cascade and both consist of a multicomponent increase in chloride conductance. The many quantitative and qualitative differences in their characteristics cannot be ascribed to the large discrepancy in amplitude alone, since the magnitudes of the responses in oocytes of the two populations of donors sometimes overlap considerably. Likewise, the number of muscarinic receptors appears to be similar in oocytes of both types of donors [3]. We have investigated the possibility that native oocytes may express two subtypes of muscarinic receptors that couple to the same signal transduction pathway.

The pharmacological evidence strongly suggests that indeed common and variant donors express different types of muscarinic receptors. To briefly summarize the evidence, the two populations appear to be affected differently by the muscarinic toxins of the *Dendroaspis* venom and the EC_{50} values to a series of muscarinic antagonists imply the predominance of M3 subtype in common and of M1 subtype in variant oocytes, respectively.

Because of the relative shortcomings of classical pharmacological tools in this system, we have validated these findings by selectively inhibiting the synthesis of M1 and M3 receptors in vivo, using specific antisense oligonucleotides directed against non-homologous regions of cloned m1 and m3 receptors. These experiments conclusively demonstrated that the muscarinic responses in common oocytes are mainly mediated by the stimulation of M3-like receptors, while those of the variant oocytes are due to M1-like receptors. We cannot exclude the possibility that oocytes express receptors that are slightly different from but highly homologous to the cloned m1 and m3 subtypes, at least in the regions probed by the oligonucleotide sequences. The success of identifying a gene product in an intact cell by injection of antisense oligonucleotides opens a new pharmacologic tool for distinguishing among polymorphous species that may be expressed in large cells and that exhibits a relatively rapid turnover.

Several groups have demonstrated that both m1 and m3 receptor subtypes couple to the PIP_2-IP_3-calcium cascade [16–18]. This evidence is particularly convincing when both subtypes are transiently overexpressed in CHO-K1 cells [19]. We should ask, therefore, why two receptors that couple to the same signal transduction pathway in the same cell exhibit multiple qualitative and quantitative differences in their responses' characteristics [3] and hemispheric distribution [4]. One possibility is interaction with different populations of G-proteins. This possibility is supported by the reports of Kubo et al. [20] and Bujo et al. [21]. Overexpression of m3 receptors in *Xenopus* oocytes resulted in membrane electrical responses that were identical to those obtained by overexpression of m1 receptors. Similarly, m2 and m4 receptors, usually involved in the inhibition of the adenylate cyclase and in G-protein-mediated activation of ionic channels, when overexpressed in model systems may couple to the PIP_2-IP_3-calcium cascade. These findings have been ascribed to the promiscuity of the G-proteins, especially at high receptor levels [13].

The differences between the responses to the activation of the two types of receptors in native oocytes may represent the true specificity of the various subtypes of muscarinic receptors and a search for the native G-proteins participating in these responses may yield important information. The differential coupling to various G-proteins may be also a function of the topography of their expression. We have demonstrated that native M1 receptors show marked preference for the animal hemisphere of the oocyte, while M3 recep-

tors appear to be distributed evenly over the surface of the cell [4]. The reason for these differences in receptor sorting may be related to the primary structure of the two peptides and future studies of their non-homologous regions should be potentially rewarding.

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