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Angiotensin receptors from rat liver, brain and pituitary gland

Expression of two subtypes in Xenopus oocytes

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INTRODUCTION

Angiotensin II (A II) and/or angiotensin III (A III) exert a wide variety of actions on multiple targets in the kidney, the cardiovascular and endocrine (pituitary gland, adrenals) systems, the genital tract and the liver. Also, several areas in the central or peripheral nervous system (see [1] for a review) have been identified as putative targets for locally produced A II or A II metabolites [I–4]; the existence of several subtypes of receptors was then suggested. Two angiotensin-receptor subtypes, named A and B, [5–9] have recently been characterized. They can be distinguished on the basis of differences in affinity for recently designed non-peptide or peptide antagonists and differences in sensitivity to dithiothreitol. Yet, in the brain, the problem of identifying and characterizing angiotensin-receptor subtypes has not been completely resolved. Bennett & Snyder [10,11] have shown that A II had a lower affinity for the brain receptors than A III. According to Harding & Felix [12], A III is more potent than A II in eliciting an electrophysiological response in the rat paraventricular nucleus [13,14]. Moreover, 18I-A III binds specifically, and with high affinity, to rat brain membranes [15]. The relationship between the putative brain A III receptors and the A-receptor subtype identified at the periphery has not yet been explored. The major difficulties encountered for the characterization of brain angiotensin receptors on the basis of direct binding measurements are linked to receptor heterogeneity, low density and uncontrolled degradation of the labelled and unlabelled ligands used.

A number of recent studies demonstrated that receptors for several peptide hormones can be expressed in oocytes from Xenopus laevis after injection of mRNA from receptor-containing tissues [16–20]. The present work was intended to evaluate the usefulness of the Xenopus oocyte expression system to investigate and compare the pharmacological properties of brain angiotensin receptors and peripheral A II receptors from liver and adeno-

hypophysis. A II receptors were detected and quantified by measuring hormone-induced Ca2+ release from 45Ca2+-loaded oocytes. This method was used in the present study for the following reasons: (1) each individual determination is derived from a group of ten oocytes; the influence of cell-to-cell variability in the number and properties of angiotensin receptors is therefore minimized; (2) for the determination of dose–response relationships, each group of ten oocytes is utilized only once; thus, interference by agonist-induced desensitization and (or) time-dependent changes in responsiveness is eliminated.

We show that A II receptors expressed from mRNA extracted from liver and adenohypophysis exhibited pharmacological properties similar to those found on liver and adenohypophysial membranes. Results obtained with brain mRNA revealed that receptors which very poorly discriminate between A II and A III contributed predominantly to the overall oocyte response.

MATERIALS AND METHODS

Materials

Animal used were Wistar rats (180–200 g body wt.) purchased from IFFA-Credo (Lyon, France). Xenopus laevis were obtained from a local (Montpellier, France) breeding centre. A I, A II and A III, and acetylcholine, were purchased from Sigma (St. Louis, MO, U.S.A.). The angiotensin antagonist [Sar1,Ala8]A II (Saralasin) was purchased from Bachem (Bubendorf, Switzerland). The iodinated peptide [Sar1,18I-Tyr4,Phe7]A II (18I-[Sar,Phe]-A II) was prepared by the method of Fraker & Speck [23].

Isolation and purification of polyadenylated [poly(A)+] RNA

Poly(A)+ RNA was isolated from rat liver, brain and pituitary gland. Immediately after isolation, the organs were rinsed twice with phosphate-buffered saline (PBS), pH 7.4, at 0°C. The temperature was maintained at 0°C during the whole process. Total RNA was obtained by the method of Chirgwin et al. [24].

Abbreviations used: A I, A II and A III, angiotensin I, II and III; 18I-[Sar,Phe]A II, [Sar1,18I-Tyr,Phe7]angiotensin; poly(A)+, polyadenylated; PBS, phosphate-buffered saline, pH 7.4; MBSH, modified Barth’s saline solution (composition given in text).
as modified by Chomczynski & Sacchi [25]. Poly(A)^+ RNA was isolated by oligo(dT)-cellulose chromatography and quantified spectrophotometrically [26]. Poly(A)^+ RNA was dissolved at a concentration of 0.5–1.0 mg/ml in autoclaved twice-distilled water and stored at −80 °C until use.

**Injection into Xenopus oocytes**

Oocytes (Stage V/VI) from *Xenopus laevis* were manually selected and stored in modified Barth’s saline solution (MBSH) containing Heps buffer (10 mM, pH 7.4), penicillin (10 μg/ml), streptomycin (2 μg/ml) and ovomucoid trypsin inhibitor (0.1 mg/ml). The selected oocytes were injected in the animal pole (part of the egg’s surface with the least concentration of yolk) with 50 nl of aq. mRNA or the same volume of ultrapure water. Microinjections were performed using a 10 μl Drummond micropipette fixed on a MM-3 Narishige (Kyoto, Japan) micromanipulator.

**Measurement of calcium outflux from injected oocytes**

Ca^2+ outflux from injected oocytes was measured as previously described [21,22]. Briefly, immediately after injection, oocytes were incubated for 48 h at 18 °C in MBSH buffer containing 50 μCi of ^45^Ca^2+/. This long incubation period was selected in order to eliminate angiotensin-induced Ca^2+ release by non-injected oocytes. Indeed, it has been shown [27,28], and confirmed in the present study (results not shown), that A II and A III can induce a Ca^2+ response by freshly water-injected oocytes. This A II-mediated mobilization of Ca^2+ takes place via an action on follicle cells that is transmitted to the oocyte through gap junctions [27]. This indirect oocyte response is markedly attenuated after incubating isolated oocytes for 24–48 h. ^45^Ca^2+/-loaded oocytes were selected on the basis of their morphological integrity, as evaluated from examination under a stereoscopic (M-3) Wild–Leitz microscope. They were washed four times with 2 ml of MBSH buffer at room temperature and distributed in groups of ten in Nunc multi-well dishes (16 mm in diameter). Each well contained 0.5 ml of MBSH buffer at 18 °C. Ca^2+ released during a 5–10 min period after washing was determined. Groups of oocytes exhibiting very high spontaneous release (about 1/5) were discarded. For the remaining groups, the medium was renewed every 5 min and its ^45^Ca^2+ content determined. ^45^Ca^2+ efflux was first measured under basal conditions during a period of 30 min. The agonist to be tested was then applied for 5 min, and the Ca^2+ efflux during six successive 5 min periods. In all experiments, oocytes injected with 50 nl of water were used as a control. The response to these oocytes to a stimulation of muscarinic cholinergic receptors, which are naturally expressed by oocytes [29], was tested.

**Binding assays on purified membranes**

Rat liver plasma membranes were prepared from male Wistar rats as described by Neville [30] up to step 11. Membranes were stored in liquid N₂ until use. After thawing, the membrane suspension (5–10 μg of protein/ml) was diluted in a solution (binding buffer) composed of 50 mM-Tris/HCl, pH 7.4, 10 mM-MgCl₂ and BSA (1 mg/ml). The membranes were centrifuged at 27000 g for 10 min at 4 °C and resuspended in the binding buffer. Binding experiments were conducted as described previously by Bonafous et al. [31]. Membranes (10 μg/assay) were incubated for 30 min at 30 °C in a total volume of 100 μl of binding buffer containing various amounts of ^125^I-[Sar,Phe]A II. Non-specific binding was determined in the presence of unlabelled [Sar,Phe]A II (1 μM). The binding constants for the unlabelled peptides were determined in competition experiments. Binding was measured in the presence of a constant amount (0.07–0.1 nM) of ^125^I-[Sar,Phe]A II and increasing amounts of the competing peptides.

Pituitary glands were isolated from Wistar rats, washed at 0 °C in 1 ml of a solution composed of 10 mM-phosphate buffer, pH 7.2, 1 mM-EGTA and 10 % (w/w) sucrose. Plasma membranes were prepared as previously described [32], with small modifications. Briefly, pituitary glands were homogenized in a 1 ml-capacity glass/Teflon homogenizer in washing buffer (0.5 ml for three glands). The crude homogenate was filtered through a nylon filter (200 μm pore size) and centrifuged at 10000 g for 15 min. The pellet was resuspended in 0.75 ml of buffer, then filtered and centrifuged as indicated above. The pellet was then resuspended in 20 ml-phosphate buffer, pH 7.5, containing BSA (1 mg/ml), bacitracin (0.5 mg/ml) and 10 mM-MgCl₂. Binding assays were performed on freshly prepared membranes. Conditions were as indicated for binding assays on liver membranes, except for the membrane protein content, which was 50 μg/assay.

**RESULTS**

Fig. 1 shows the results of a typical experiment performed with a group of ten oocytes injected with 50 ng of rat liver mRNA and two control groups of ten water-injected oocytes. Basal Ca^2+ outflux measured during six successive 5 min periods was stable and comparable for mRNA- and water-injected oocytes. In some experiments a time-dependent decrease in basal Ca^2+ outflux was observed, but in most cases a stable value was reached within 30 min. Addition of A II (100 nM) to the incubation medium of liver mRNA-injected oocytes induced a marked, but transient,
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Fig. 2. Effect of increasing the amount of injected mRNA on the magnitude of A II-induced increase in $^{45}$Ca$^{2+}$ outflux

Groups of ten oocytes were injected with the indicated amounts of liver mRNA (0–25.2 ng), $^{45}$Ca$^{2+}$-loaded, and tested for their responsiveness to a maximal concentration of A II. The experimental design was similar to that shown in Fig. 1. The magnitude of the response to A II stimulation was measured as the increase over basal value of $^{45}$Ca$^{2+}$ outflux after the 5 min period exposure to A II. The Figure shows the results of one experiment representative of the three experiments performed.

increase in Ca$^{2+}$ outflux. Subsequent stimulation by acetylcholine also induced an increase in Ca$^{2+}$ outflux. Water-injected oocytes were insensitive to the addition of A II (100 nM), but responded to acetylcholine (1 mM). The magnitude and duration of acetylcholine-induced transient increase in Ca$^{2+}$ outflux were comparable with those of the A II-induced response of liver mRNA-injected oocytes. The residual $^{45}$Ca$^{2+}$ oocyte content determined at the end of the experiment represented about 10 times the cumulative release which occurred during the entire experiment. It therefore appears that the size of the intracellular $^{45}$Ca$^{2+}$ pool was not limiting for the observed A II- or acetylcholine-induced $^{45}$Ca$^{2+}$ release. The magnitude of the responses to A II increased with the amount of injected liver mRNA (Fig. 2) over a fairly narrow range (between 0 and 5 ng). Increasing the amount of injected mRNA from 5 to 25 ng (experiment shown) and up to 50 ng (not shown) did not lead to a further increase in the magnitude or duration of the response to a maximal (100 nM) dose of A II. All further experiments were performed with oocytes injected with 25–50 ng of mRNA, i.e. in a situation where one can reasonably assume that the magnitude of the response to maximal doses of A II or related molecules would reflect the intrinsic properties (full or partial agonistic properties) of these molecules rather than differences in the level of receptor expression. Note that water-injected (indicated as 0 ng of mRNA injected in Fig. 2) did not respond to A II (100 nM).

Fig. 3 illustrates the results of six representative experiments in which dose-dependencies for A II- and A III-induced increase in $^{45}$Ca$^{2+}$ release by oocytes injected with mRNA from liver, adrenohypophysis and brain were determined. Groups of ten oocytes were stimulated by A II at concentrations ranging from 1.0 to 1000 nM. Two criteria could be used to quantify the magnitude of A II-induced responses: either the increase over basal level of $^{45}$Ca$^{2+}$ release during the 5 min period during which A II was applied (see the Materials and methods section) or the cumulative increase of $^{45}$Ca$^{2+}$ release during the 30 min period after A II application. Whatever the criterion used, dose–response relationships were similar (results not shown). For the sake of simplicity, increase over basal level of $^{45}$Ca$^{2+}$ release during the 5 min period of drug application was used as the response index. Curves on the graph were constructed from parameters deduced from Hill plots of the experimental data;
Table 1. $K_d$ values for binding of A II and related peptides to rat liver and adenohypophyseal membranes

$K_d$ values for the indicated unlabelled peptides were deduced from competition experiments using $^{125}$I-Sar,Phel-A II (0.15–0.25 nM) as the labelled ligand. Experimental data were fitted by means of the following linear relationship:

$$\log\left(\frac{B_o}{B}\right) = \log\left(\frac{[L^*]K_d^*}{[L^*]K_d^*+1}\right) + \log\left(\frac{K_d}{K_d}ight)$$

in which $B_o$ and $B$ are the amounts of labelled ligand bound in the absence or presence of the competing ligand (I) respectively; $[L^*]$ is the concentration of labelled ligand, and $K_d^*$ is the corresponding dissociation constant. $K_d^*$ values (ranging from 0.25 to 0.4 nM) were deduced from parallel determinations of dose-dependent $^{125}$I-[Sar²]-A II binding. Values in the Table are means ± S.D. of three independent determinations.

<table>
<thead>
<tr>
<th>Competing ligand</th>
<th>Liver membranes</th>
<th>Adenohypophyseal membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A I</td>
<td>380 ± 115</td>
<td>290 ± 75</td>
</tr>
<tr>
<td>A II</td>
<td>2.4 ± 0.8</td>
<td>0.76 ± 0.2</td>
</tr>
<tr>
<td>A III</td>
<td>7.0 ± 1.3</td>
<td>12.3 ± 7.5</td>
</tr>
<tr>
<td>Saralasin</td>
<td>1.0 ± 0.5</td>
<td>3.2 ± 0.6</td>
</tr>
</tbody>
</table>

Table 2. Effects of A I and Saralasin on $^{45}$Ca$^{2+}$ outflux from liver-, adenohypophyseal- or brain-mRNA-injected oocytes

A series of experiments similar to those shown in Figs. 1 and 3 were performed using oocytes injected with liver, adenohypophyseal or brain mRNA. The responses (increase over basal value of $^{45}$Ca$^{2+}$ outflux during the 5 min drug application period; see the Materials and methods section) to increasing concentrations of A I or Saralasin were determined. In all series the effects of the indicated concentrations of A I or Saralasin were determined on homogeneous batches of oocytes. Each series included the determination of the response to a maximal dose of A II (100 nM). Responses to A I or Saralasin are expressed as a percentage of the A II-induced response.

<table>
<thead>
<tr>
<th>Agent tested</th>
<th>Conc. (µM)</th>
<th>Receptor origin…</th>
<th>Liver</th>
<th>Adenohypophysis</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A I</td>
<td>0.01</td>
<td>3.0</td>
<td>3.8</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>3.0</td>
<td>4.9</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>6.8</td>
<td>6.5</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>12.4</td>
<td>9.1</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td>Saralasin</td>
<td>0.01</td>
<td>6.0</td>
<td>8.4</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>9.6</td>
<td>6.8</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>64.0</td>
<td>54.2</td>
<td>38.7</td>
<td></td>
</tr>
</tbody>
</table>

reproducible manner. Receptors expressed from liver and adenohypophyseal mRNA discriminated between A II and A III. $K_{act}$ values for A III were similar in both cases and about 5 times higher than the corresponding values determined for A II. In contrast, receptors expressed from brain mRNA failed to discriminate between A II and A III, although the $K_{act}$ value for A III (50 nm) was similar to the corresponding values determined for receptors expressed from liver (44 nm) and adenohypophyseal (58 nm) mRNA. $K_v$ values for the binding of A II and A III to liver and adenohypophyseal membranes are given in Table 1. Comparison of these values with the corresponding $K_{act}$ values given in Fig. 3 (legend) indicate that there is a good correspondence between the relative potencies of A II and A III in eliciting increased $^{45}$Ca$^{2+}$ release by liver or adenohypophyseal mRNA-injected oocytes and their relative affinities for binding to liver and adenohypophyseal membranes. However, in all cases, the $K_{act}$ values were 3–6 times higher than the corresponding $K_d$ values.

The effects of A I and saralasin on mRNA-injected oocytes were also tested, as illustrated in Table 2. A I used at concentrations up to 30 times the apparent dissociation constants for binding to liver or adenohypophyseal membranes had only moderate effects on $^{45}$Ca$^{2+}$ outflux. Responses to 10 µM A I represented 9–16% of the maximal A II-induced responses measured during the course of the same experiments. It therefore appears that A I acting on receptors expressed from liver adenohypophyseal or brain membranes mRNA behaved like a partial agonist. Saralasin used at concentrations up to 0.1 µM, i.e. about 30–100 times the dissociation constants, indicated in Table 1 failed to increase $^{45}$Ca$^{2+}$ outflux. However, at 1 µM Saralasin elicited marked responses.

**DISCUSSION**

We show in the present study that the *Xenopus* oocyte expressing angiotensin receptors from tissues of different origin does represent a valuable system for evaluating the pharmacological properties of these receptors. First, dose-response relationships to A II and structurally related molecules could be established in a reproducible and fairly precise manner. The response index which was used, namely the increase in Ca$^{2+}$ outflux from groups of injected oocytes, offers the following advantages: (i) it provides a high signal/noise ratio; (ii) it eliminates interferences due to receptor desensitization (oocytes are stimulated only once; and (iii) it limits the influence of individual fluctuations (determinations on groups of oocytes). Secondly, the pharmacological properties of A II receptors from tissue of a given origin appear to be preserved when these receptors are expressed in oocytes from RNA of the same origin. In the case of liver A II receptors, which constitute an apparently homogeneous population of receptors of the B-subtype, we demonstrate a fairly good parallelism between the relative potencies of A I, A II and A III in eliciting an increase in $^{45}$Ca$^{2+}$ outflux in liver mRNA-injected oocytes and the relative affinities of these peptides for the receptors present on rat liver membranes. However $K_{act}$ values determined on mRNA-injected oocytes were slightly higher than the corresponding $K_v$ values determined on liver or adenohypophyseal membranes. This might reflect differences in the heterotropic interactions between the receptors and other components of the signal-transduction machinery as they operate in the native membranes and the oocyte membrane. The difference between $K_{act}$ and $K_v$ could also reflect the expression of more than one subtype of receptor in the oocyte, but in ratios different from those in the situation *in vitro*.

The main advantages of exploring the pharmacological properties of A II receptors from tissues of different origin when expressed in *Xenopus* oocytes are to eliminate or attenuate tissue-specific interferences, such as the influence of the membrane environment, differences in post-receptor signal amplification and uncontrolled metabolic conversion of the agent being tested.

An obvious limitation to the use of *Xenopus* oocytes as a pharmacological model might arise in all situations where the populations of receptors and mRNA encoding for these receptors are heterogeneous. From this point of view, the results derived from brain-mRNA-injected oocytes deserve special comment. Our results clearly suggest that, among mRNAs encoding for brain angiotensin receptors, the more abundant (or the more efficiently translated) encodes for a receptor subtype which is different from that of hepatic and adenohypophyseal receptors. The relative low potency of A II (equivalent to that of A III) contrasts with the efficient A II/A III discrimination observed
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with receptors expressed from liver or adenophophyseal mRNA. Incidentally, it is worth noting that our results indirectly support the conclusion derived from several previous studies, namely that in brain A III is at least as potent as A II (see the Introduction). Further studies using selective antagonists of the A and B receptor subtypes will be necessary to identify the non-A II/A III-discriminative subtype(s) of angiotensin receptor which is (are) expressed in brain-mRNA-injected oocytes. Whereas only the A-subtype was detected in bovine cerebellum [7], Chang and colleagues [33] recently showed the presence of the A- and B-subtypes in the brain (thalamus, hypothalamus, and midbrain). It is not clear yet whether the 'A III receptor' [15] present in brain could be identified as the predominant receptor expressed in brain-mRNA-injected oocytes and if this receptor represents the A-subtype. It must be mentioned, however, that Chang & Lotti [34] have shown some remarkable similarities between the properties of A III-binding sites present in rat adrenals [35] and those of angiotensin receptors of the A-subtype.

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