Volume 241, number 1,2, 177-180 FEB 06586 December 1988

Calcium mobilization by angiotensin II and neurotransmitter receptors expressed in *Xenopus laevis* oocytes

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Received 5 October 1988

Specific receptors for angiotensin II (AII) were expressed in albino *Xenopus laevis* oocytes co-injected with poly(A)$^+$ mRNA isolated from rat adrenal cortex and the calcium-specific photoprotein, aequorin. In such oocytes, AII elicited rapid, dose-dependent rises in cytosolic free calcium with light emission responses up to 100-fold above basal levels. Ligand-induced light emission was also observed in oocytes injected with rat brain mRNA and stimulated with acetylcholine and glutamate. These findings demonstrate that mammalian AII receptors expressed in *Xenopus* oocytes are functionally linked to intracellular Ca^{2+} mobilization, and indicate the potential value of aequorin-injected oocytes for rapid and specific screening of mRNAs transcribed from expression libraries containing cloned receptor cDNAs.

Ca^{2+} mobilization; Oocyte; Aequorin; Angiotensin II receptor; (*Xenopus laevis*)

1. INTRODUCTION

Angiotensin II (AII) exerts calcium-dependent regulatory actions in numerous target tissues including the adrenal, liver, pituitary, smooth muscle, heart, brain, and kidney [1]. Many of AII's diverse effects are related to cardiovascular regulation and fluid homeostasis. Studies on the molecular mechanisms of action of AII and other peptide hormones would be facilitated by the use of *Xenopus laevis* oocytes as a translation vehicle [2] for expressing the homologous receptors from exogenous mRNAs.

We recently demonstrated that *Xenopus* oocytes injected with bovine adrenal mRNA express functional AII receptors which upon binding AII trigger the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C to yield inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol [3]. Since $InsP_3$ generation is regarded as the primary mechanism by which Ca^{2+}-dependent hormones and neurotransmitters initiate calcium mobilization [4], we investigated the ability of exogenous AII receptors to elevate cytosolic calcium ($[Ca^{2+}]_i$) upon AII stimulation using the Ca^{2+}-specific photoprotein, aequorin [5].

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2. EXPERIMENTAL

RNA was extracted from rat adrenal cortex and rat brain and poly(A)$^+$-selected prior to injection into albino *X. laevis* oocytes by previously described methods [3]. Total mRNA (2 $\mu g/\mu l$) was resuspended with aequorin (2 $\mu g/\mu l$) in Ca^{2+}-free water (prepared by passing doubly deionized water over a Chelex 100 chelating resin) at a 1:1 ratio and injected into the oocyte cytoplasm (50 nl/oocyte). Injected oocytes were incubated at 18 °C in modified Barth's medium [82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM Hepes, pH 8, 1 mM sodium phosphate; pH 7.8] supplemented with aprotinin (100 kallikrein units/ml) for three days. Ca^{2+}-free modified Barth's medium was prepared by substituting $CaCl_2$ with $MgCl_2$ and supplementing the buffer with 200 μM EGTA. The number of photons emitted per 6 s intervals from aequorin-injected oocytes was measured in a liquid scintillation counter (Beckman model LS250) with the coincidence gate switched off.

3. RESULTS AND DISCUSSION

Examples of AII-evoked increases in light emission in oocytes co-injected with rat adrenal cortex

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00145793/88/$3.50

mRNA and aequorin are illustrated by the three experiments shown in fig.1A. The magnitude of the AII-induced $[Ca^{2+}]_i$ response was variable between individual oocytes, probably reflecting differences in the degree of oocyte integrity and/or the levels of functional receptor expression. However, out of 156 oocytes examined, more than 70% responded to AII with rapid increases in $[Ca^{2+}]_i$ within 2 min, followed by a second phase with a gradual return to basal levels over the subsequent 10–15 min. The initial lag time before reaching maximum light emission was related to the magnitude of the response in that the largest increases (82 ± 9.7-fold were reached at 0.6 min while smaller responses (2.3 ± 0.5-fold) occurred significantly later (1.4 min). These results are consistent with the characteristics of AII-induced Ca^{2+} mobilization in adrenal zona glomerulosa cells [6].

Fig.1B illustrates the effect of extracellular Ca^{2+} depletion on Ca^{2+} mobilization and light emission induced by AII. While the initial peak response was not significantly affected by the absence of extracellular Ca^{2+}, the return to basal levels was accelerated due to loss of the slower phase of the response. These data suggest that the role of extracellular Ca^{2+} in AII action is related to calcium influx subsequent to the initial peak of mobilization from intracellular stores, in accordance with AII's effects on Ca^{2+} mobilization in bovine adrenal glomerulosa cells [7].

The maximum peak height of the light response was dependent on the dose of AII (fig.2), with threshold responses (2× basal) at 10^{-7} M and maximum responses (50× basal) at 10^{-5} M AII. In adrenal cells labeled with quin-2, minimum Ca^{2+} responses to AII were elicited by 10^{-10} M AII, and maximum responses by 10^{-7} M AII [8]. This difference in the sensitivity to AII between the adrenal receptors expressed in the two cell types may reflect differences in species, receptor-effector coupling, membrane environments, and post-translational processing of the receptor. The present AII dose-response data are consistent with those obtained by electrophysiological analysis of AII receptor expression in mRNA-injected oocytes [9,10].

The receptor-specific AII antagonist, [Sar^1, Ala^8]AII, blocked the calcium-mobilizing effect of 500 nM AII and did not itself elicit a Ca_i^{2+} response. However, after extensive washing the

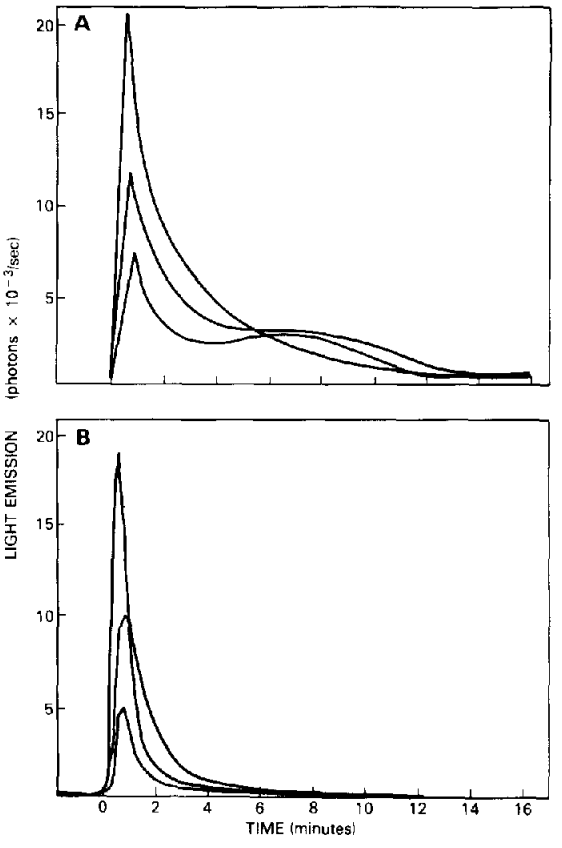

Fig.1. Time course of AII-evoked changes in light emission. Three representative examples are shown of data from oocytes stimulated with 1 μM AII in the presence (A) and absence (B) of extracellular calcium after co-injection with rat adrenal mRNA and aequorin 3 days previously.

same oocytes responded to AII with 10 ± 2-fold ($n = 5$) increases in maximum peak light emission (fig.3).

Control oocytes injected with aequorin alone did not respond to 1 μM of AII, although 10 μM AII occasionally elicited 2–4-fold increases in the maximum peak light emission in control oocytes examined on 1 day but not 3 days after aequorin injection. This indication of the presence of sparse endogenous AII receptors in the oocyte is consistent with reports of AII receptors in the mammalian ovary [11], where their possible function is indicated by the recent report that intraperitoneal injection of an AII antagonist inhibited ovulation in rats [12].

To compare AII's effects with those of

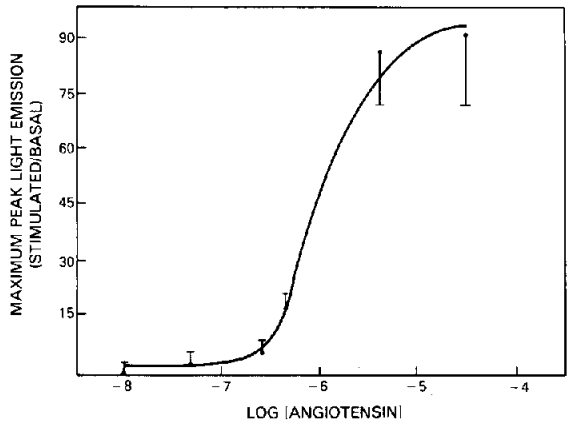

Fig.2. Dose-dependent stimulation of peak light intensity by increasing concentrations of AII. Each point represents the mean ± SE of data from 5–10 oocytes.

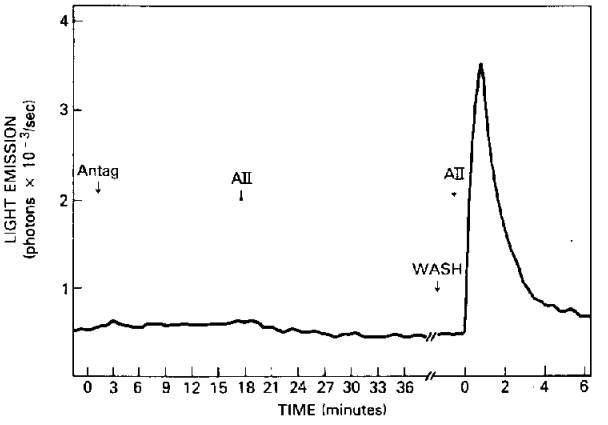

Fig.3. Antagonist blockade of AII-induced changes in light emission. The time course of light emission is representative of responses in 5 co-injected oocytes treated individually with [Sar^1,Ala^8]AII (5 μM) followed by 500 nM AII, then washed for 1 h and exposed to another pulse of 500 nM AII.

Ca^{2+}-mobilizing neurotransmitters, we examined the $[Ca^{2+}]_i$ response to acetylcholine (100 μM) and glutamate (100 μM) in oocytes injected with rat brain mRNA (table 1). Acetylcholine (ACh) and glutamate (Glu) also elicited major increases in light emission (5–15× basal), consistent with recent evidence that binding of these ligands to their respective receptors in the oocyte leads to the formation of $InsP_3$ [13,14]. Table 1 illustrates the effect of extracellular Ca^{2+} depletion on Ca^{2+} mobilization and light emission induced by ACh and Glu. The maximum peak light response was not significantly affected by the absence of extracellular Ca^{2+}, indicating that mobilization of intracellular pools was primarily responsible for the ligand-induced response. Taken together, these findings clearly illustrate the ability of aequorin-injected oocytes to assess the activation of exogenous receptors by Ca^{2+}-mobilizing hormones and neurotransmitters.

By directly measuring light responses resulting from changes in intracellular Ca^{2+} levels, we have shown that exogenous AII receptors expressed in the oocyte are coupled to Ca^{2+} mobilization with properties similar to those of the native receptor. Recently, oocytes injected with mRNA from rat brain, rat liver and bovine adrenal have been shown to respond to AII by changes in Ca^{2+} efflux [15] and in membrane potential and current [9,10]. These electrophysiological responses were attributed to inward chloride currents, but the latter were not shown to be independent of extracellular Ca^{2+}. The demonstration of Ca^{2+}-independent chloride currents in the oocyte [16] makes the direct evaluation of $[Ca^{2+}]_i$ changes imperative to a full understanding of the mechanisms governing the responses to activation of exogenous AII receptors.

Direct analysis of $[Ca^{2+}]_i$ changes in oocytes not only permits analysis at the single cell level of receptor coupling to Ca^{2+} mobilization, but also provides a rapid and specific assay for cloning Ca^{2+}-mobilizing hormones and neurotransmitters.

Table 1

Ligand-stimulated peak light emission from oocytes injected with rat brain mRNA and stimulated with 100 μM acetylcholine (ACh) or 100 μM glutamate (Glu) in the presence or absence of extracellular Ca^{2+} $(Ca^{2+})_e$

	Light emission (photons $\times 10^{-3}$/s)			
	$+(Ca^{2+})_e$		$-(Ca^{2+})_e$	
Ligand	Basal	Peak	Basal	Peak
ACh	1.2 ± 0.1	16.6 ± 4.1	1.6 ± 0.1	15.0 ± 4.3
Glu	1.2 ± 0.2	18.4 ± 4.2	1.1 ± 0.2	13.1 ± 2.9

The magnitudes of the light responses to the neurotransmitters did not change significantly in the absence of $(Ca^{2+})_e$. Each value represents the mean ± SE of data from 15–20 oocytes. No responses to the transmitters were observed under these conditions in oocytes injected with aequorin alone

This approach offers several advantages over assay techniques which are currently being used to clone Ca^{2+}-mobilizing receptors [15,17,18]. The present method is rapid and convenient; no radioisotopes are required and no electrophysiological expertise or equipment is necessary.

The fluorescent indicator fura-2 was recently employed to show that serotonin $5HT_{1c}$ receptors expressed in brain mRNA-injected oocytes mobilize Ca^{2+} in response to serotonin [19]. For this purpose, oocytes previously injected with mRNA had to be re-injected on day 3 with fura-2, 15 min before fluorescence intensity was measured. The photoprotein aequorin, because of its long half-life, offers the distinct advantage over other calcium indicators that it can be co-injected with mRNA and remains available for several days. The use of a single injection of aequorin with the mRNA is more convenient and avoids the traumatic nature of double injection. The aequorin-light emission reaction is highly responsive to changes in Ca^{2+} levels because the amplitude of the aequorin signal is proportional to the cube of the Ca^{2+} concentration [5]. Aequorin is thus more sensitive to localized increases in Ca^{2+} than linear indicators such as quin-2 and fura-2 which monitor whole cell Ca^{2+} [20]. Finally, not only can 1 oocyte be screened in less than 5 min, but several oocytes from the same group can be pooled and screened at once.

In conclusion, these results show that AII receptors expressed from mammalian target cell mRNA in aequorin-containing *Xenopus* oocytes are functionally linked not only to phosphoinositide hydrolysis [3] but also to Ca^{2+} mobilization, similar to native AII receptors. The use of this self-contained expression-detection system will be particularly suitable for structure-function studies of AII receptor subtypes and ultimately for identification and cloning of their mRNAs. This system should also be generally applicable to the screening of mRNAs produced from expression libraries containing cloned cDNAs of plasma-membrane receptors for other Ca^{2+}-mobilizing hormones and neurotransmitters.

Acknowledgements: We thank Drs Shun-Ichiro Izumi, Pilar Carvallo, Sharon Zemel, Laszlo Hunyady and Greti Aguilera for helpful advice and discussion. A.J.M. was supported by a Fellowship grant provided by Sigma-Tau, Rome, Italy. Aequorin was obtained from Dr John R. Blinks, Dept of Pharmacology, Mayo Foundation, Rochester, MN 55905.

REFERENCES

[1] Catt, K.J., Carson, M.C., Hausdorff, W.P., Leach-Harper, C.M., Baukal, A.J., Guillemette, G., Balla, T. and Aguilera, G. (1987) J. Steroid Biochem. 27, 915–927.
[2] Gurdon, J.B. and Wakefield, L. (1986) in: Microinjection and Organelle Transplantation Techniques (Celis, J.E. et al. eds) pp. 269–299, Academic Press, London.
[3] McIntosh, R.P. and Catt, K.J. (1987) Proc. Natl. Acad. Sci. USA 84, 9045–9048.
[4] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
[5] Blinks, J.R., Wier, W.G., Hess, P. and Prendergast, F.G. (1982) Prog. Biophys. Mol. Biol. 40, 1–65.
[6] Quinn, S.J., Williams, G.H. and Tillotson, D.L. (1988) Proc. Natl. Acad. Sci. USA 85, 5754–5758.
[7] Capponi, A.M., Lew, P.D., Jornot, L. and Vallotton, M.B. (1984) J. Biol. Chem. 259, 8863–8869.
[8] Braley, L., Menachery, A., Brown, E. and Williams, G. (1984) Biochem. Biophys. Res. Commun. 123, 810–815.
[9] Cross, D., Cifuentes, F., Huidobro-Toro, J.P., Vio, C.P. and Inestrosa, N.C. (1987) Mol. Brain Res. 2, 268–270.
[10] Meyerhof, W., Morley, S., Schwarz, J. and Richter, D. (1988) Proc. Natl. Acad. Sci. USA 85, 714–717.
[11] Pucell, A.G., Bumpus, F.M. and Husain, A. (1987) J. Biol. Chem. 262, 7076–7080.
[12] Pellicer, A., Palumbo, A., Decherney, A.H. and Naftolin, F. (1988) Science 240, 1660–1661.
[13] Oron, Y., Dascal, N., Nadler, E. and Lupu, M. (1985) Nature 313, 141–143.
[14] Sugiyama, H., Ito, I. and Hirono, C. (1987) Nature 325, 531–533.
[15] Williams, J.A., McChesney, D.L., Calayag, M.C., Lingappa, V.R. and Logsdon, C.D. (1988) Proc. Natl. Acad. Sci. USA 85, 4939–4943.
[16] Parker, I., Gundersen, C.B. and Miledi, R. (1985) Neurosci. Res. 2, 491–496.
[17] Noma, Y., Sideras, P., Naito, T., Bergstedt-Linquist, S., Azuma, C., Severinson, E., Tanabe, T., Kinashi, T., Matsuda, F., Yaoita, Y. and Honjo, T. (1986) Nature 319, 640–646.
[18] Masu, Y., Nakayama, K., Tamaki, H., Harada, Y., Kuno, M. and Nakanishi, S. (1987) Nature 329, 836–838.
[19] Takahashi, T., Neher, E. and Sakmann, B. (1987) Proc. Natl. Acad. Sci. USA 84, 5063–5067.
[20] Cobbold, P.H. and Rink, T.J. (1987) Biochem. J. 248, 313–328.