Sustained oscillations in extracellular calcium concentrations upon hormonal stimulation of perfused rat liver

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Sustained oscillations in extracellular free Ca^{2+} were shown to occur on addition of vasopressin, phenylephrine or angiotensin II in isolated rat liver perfused with low ($10\ \mu\text{M}$)- Ca^{2+} medium. The amplitude and frequency of oscillation depend on hormone concentration. In contrast, Ca^{2+} releases on addition of ATP, t-butyl hydroperoxide or arachidonate do not exhibit oscillatory behaviour. The vasopressin-induced oscillations were suppressed by glucagon and dibutyryl 3',5'-cyclic AMP, but not by dibutyryl 3',5'-cyclic GMP. These observations in the extracellular space complement observations by Woods, Cuthbertson & Cobbold [(1986) *Nature* (London) 319, 600–602] on oscillations in intracellular free Ca^{2+} in single liver cells.

INTRODUCTION

Stimulation by vasoactive hormones has long been known to lead to an increase in intracellular and extracellular free Ca^{2+} concentration in liver. Using low extracellular $[\text{Ca}^{2+}]$ of 5 or $10\ \mu\text{M}$, we observed that, after the initial peak of Ca^{2+} ejection on addition of, e.g., phenylephrine, there were distinct shoulders in the trace recorded from a Ca^{2+} -sensitive electrode (Sies *et al.*, 1981). Similar observations can also be made from recordings published by others, e.g. Althaus-Salzmann *et al.* (1980). These initial observations of an apparent oscillatory behaviour of extracellular Ca^{2+} found an interesting intracellular counterpart in the elegant work by Woods *et al.* (1986), showing repetitive transient increases in cytosolic free Ca^{2+} in hormone-stimulated hepatocytes, as detected by aequorin photoemission. It is thus clear that the recent advances in knowledge on the intracellular signalling sequence involving inositol trisphosphate and other products of inositol phospholipid breakdown will be further expanded by analysis on a time-resolved basis.

Against this background, we report here an examination of the conditions for the occurrence of free- Ca^{2+} oscillatory transients in the extracellular space of isolated perfused rat liver. For some types of study, this system may be well suited, as synchronized responses apparently are demonstrable in the whole organ.

MATERIALS AND METHODS

Haemoglobin-free rat liver perfusion

Livers of male Wistar rats (200–250 g body wt.), fed on stock diet (Altromin, Lage, Germany), were perfused as described previously (Sies, 1978) without recirculation (open system) at 37°C . Additions were made by micro-pumps directly before the portal vein. Perfusate flow was constant at 4 ml/min per g wet wt.

Assays

O_2 concentration, pH and Ca^{2+} and K^+ in the effluent perfusate were monitored continuously with Clark-type, pH-glass and Ca^{2+} - or K^+ -sensitive electrodes respectively (Sies *et al.*, 1981).

For measurement of inositol phosphates, the animals

were pretreated 15–20 h before the experiment by intraperitoneal injection with $100\ \mu\text{Ci}$ of *myo*-[2- ^3H]inositol in 0.9% NaCl (see Rhodes *et al.*, 1983). Samples (1–2 g) were freeze-clamped and further processed, eluted from columns ($0.5\ \text{cm} \times 2.0\ \text{cm}$) of Dowex 1X (formate form) as described by Downes & Michell (1981) and Berridge *et al.* (1983), and data were expressed as d.p.m./150 mg of protein (approx. 1 g wet wt.).

Materials

[Lysine]vasopressin (grade I-S, oxytocin-free), angiotensin II, glucagon and phenylephrine were obtained from Sigma (Munich, Germany). *myo*-[2- ^3H]inositol ($16.3\ \text{Ci/mmol}$) was from Amersham Buchler (Braunschweig, Germany). Biochemicals were from Boehringer (Mannheim, Germany) and other chemicals from Merck (Darmstadt, Germany).

RESULTS AND DISCUSSION

Oscillations in extracellular Ca^{2+} obtained with vasopressin, phenylephrine and angiotensin II

Numerous laboratories have previously shown that the addition of vasopressin, phenylephrine and angiotensin II is followed by a release of Ca^{2+} into the extracellular space. In the isolated rat liver perfused with Krebs–Henseleit medium, this is demonstrated particularly well if the Ca^{2+} concentration is decreased from the usual $1.25\ \text{mM}$ to $10\ \mu\text{M}$, measured with a Ca^{2+} -sensitive electrode. As mentioned above, after the initial peak in the recording of Ca^{2+} concentration in the effluent perfusate there are secondary peaks (sometimes also called shoulders). We found that, after a first period of hormone infusion and a subsequent interval of a few minutes, a second addition of hormone reveals the oscillations in Ca^{2+} concentration more clearly. Therefore, in the experiments reported below, the 'standard' condition is the $10\ \mu\text{M}$ - Ca^{2+} medium with a first addition of vasopressin ($13\ \text{nM}$) for 8 min followed by an interval of 12 min before the second addition. In Fig. 1(a) (left-hand trace), for example, vasopressin ($0.5\ \text{nM}$) was infused at such a time point in a perfusion experiment; likewise, effects of phenylephrine ($1.5\ \mu\text{M}$) or angio-

Fig. 1. Oscillatory time course of Ca^{2+} release into the extracellular space upon stimulation with vasopressin in perfused rat liver

Perfusion medium contained 10 μM - Ca^{2+} (instead of the usual 1.25 mM). Vasopressin was given for 8 min, followed by a 12 min hormone-free interval (not shown), then vasopressin (0.5 nM) was infused. Later, also 13 nM vasopressin was infused as shown. In (b), the time periods of Ca^{2+} release of third-to-fourth peak in several similar experiments are plotted as cycles/min.

tensin II (14 nM) were recorded in different perfusion experiments shown in Fig. 2. It is noteworthy that there are oscillations in the extracellular Ca^{2+} . The amplitude and frequency of oscillation for vasopressin were dependent on the concentration of the hormone. As shown in Fig. 1(a) (right-hand trace), a saturating concentration of vasopressin (13 nM) elicited oscillations similar to those with phenylephrine and angiotensin II at saturating concentration. The frequency of Ca^{2+} oscillation increased from about 0.2 cycle/min at 0.5 nM to about 0.7 cycle/min at 13 nM-vasopressin (Fig. 1b), measured for the third-to-fourth oscillation data points in Fig. 1(a). The extracellular K^{+} concentration did not

Fig. 2. Oscillations in extracellular Ca^{2+} with phenylephrine or angiotensin II

Conditions were as in Fig. 1. Instead of vasopressin (0.5 nM), phenylephrine or angiotensin II was infused as the second hormone addition.

Fig. 3. Time course of Ca^{2+} release and portal pressure upon ATP addition

Conditions were as in Fig. 1.

exhibit oscillatory transients related to the Ca^{2+} oscillations.

Non-oscillatory Ca^{2+} release with ATP, t-butyl hydroperoxide and arachidonate

The property of oscillatory behaviour is unique to the hormone-stimulated Ca^{2+} release. Other means of

Fig. 4. Dependence of Ca²⁺ oscillations on influent Ca²⁺ concentration

Perfusion medium contained 10 μM , 50 μM - or 100 μM -Ca²⁺ as indicated. Vasopressin (15 nM) was given as second addition of hormone in each of the three different perfusions.

Fig. 5. Suppression of vasopressin-induced oscillations by cyclic AMP (55 μM)

In this trace, the first addition of vasopressin is also shown.

introducing such a release, again at the time points in the standard experiment, were explored. The addition of ATP, for example, generated a substantial increase in Ca²⁺ release, comparable in size with that observed with the hormones at saturating concentration. However, there was no oscillation (Fig. 3). Likewise, the release of Ca²⁺ induced by the addition of t-butyl hydroperoxide (1 mM) or arachidonate (11 μM) did not occur in the form of oscillations, as shown by Sies *et al.* (1984) (results not shown).

Dependence on extracellular Ca²⁺

The oscillations are still detectable when the Ca²⁺ concentration in the medium is raised to 50 μM , but were

not seen at 100 μM - (Fig. 4) or at 1.25 mM-Ca²⁺. This could be due to a decrease in signal/noise as the Ca²⁺ is increased, or to a net suppression of the oscillations in the presence of sufficiently high extracellular Ca²⁺ concentration. The comparison of the traces for 50 μM - and 100 μM -Ca²⁺ shows similar signal/noise, so that a biological cause for the lack of oscillations appears possible. It is important to note that, in the experiments with isolated hepatocytes, Woods *et al.* (1986) used physiological (i.e. high) extracellular Ca²⁺ and were able to record intracellular oscillations.

Suppression of vasopressin-induced oscillation by cyclic AMP and glucagon

Dibutyl 3',5'-cyclic AMP (Fig. 5), but not dibutyl 3',5'-cyclic GMP, at 55 μM concentration is able to suppress the vasopressin-induced oscillations. A distinct suppression is already noticeable with 1 μM -dibutyl 3',5'-cyclic AMP. Similarly, glucagon (1 nM) suppresses the oscillations, suggesting that cyclic AMP may be crucial in controlling oscillations.

Possible relation to inositol lipid metabolism

The mechanism of hormone-stimulated Ca²⁺ release includes the generation of inositol trisphosphate, which in turn releases the Ca²⁺ (Streb *et al.*, 1983). We therefore considered the possibility of intracellular fluctuations in inositol trisphosphate or other related second-messenger compounds. Initial measurements of inositol trisphosphate in freeze-clamped liver samples showed an increase 30 s after the first infusion of hormone, whereas there was no substantial difference in radioactivity attributable to inositol trisphosphate in samples taken at the top or at the bottom of an oscillation (third-to-fourth peak) during the second vasopressin addition (Fig. 6). More refined methods need to be developed to study better the possible involvement of inositol-related compounds during the oscillations of extracellular Ca²⁺.

Concluding remarks

We described here conditions in the intact organ in which oscillations of extracellular Ca²⁺ triggered by vasopressin, α -adrenergic agonist or angiotensin II are observable. The concentration-dependence and the frequency of the oscillations match well with the observations by Woods *et al.* (1986) on intracellular Ca²⁺ transients in isolated hepatocytes. However, there are also differences from the observations on single cells. For example, the oscillations were sustained also at high hormone concentrations in perfused liver, whereas this was not the case in the experiments by Woods *et al.* (1986). At this stage, it should be mentioned that the intracellular transients observed in single cells and the synchronized response detected in the extracellular space in perfused liver might also be unrelated phenomena.

The oscillations are receptor-specific, since no such phenomenon occurs when Ca²⁺ releases of similar magnitude are initiated, e.g., by the addition of extracellular ATP, which acts through purinergic receptors, and may cause a different metabolic response.

The experimental model presented here may permit studies on the underlying mechanisms of the oscillation. The concentration of hormone required and the frequency pattern are similar to those observed in single cells. Thus

Fig. 6. Elution patterns of inositol phosphates for samples taken at different time points

Perfused liver was freeze-clamped at 30 s after the beginning of the first infusion of vasopressin (13 nM) (●), or at the top (▲) or the bottom (□) of the third-to-fourth peak during the second addition of vasopressin (13 nM). Samples were taken from different livers. This pattern is representative of six perfusions. Abbreviations: IP₁, inositol 1-phosphate; IP₂, inositol 1,4-bisphosphate, IP₃, inositol trisphosphate (1,3,4- or 1,4,5-).

the synchrony of the response in the intact organ makes it possible to obtain more samples amenable to biochemical analysis at a given time point of an oscillation. Biological and biochemical oscillators have long attracted interest, mainly from the standpoint of theory and from experimental model systems *in vitro* (see, e.g., Chance *et al.*, 1973; Berridge & Rapp, 1979; Boiteux *et al.*, 1980). Extracellular Ca²⁺ was found to be oscillating in suspensions of differentiating *Dictyostelium* on addition of attractant (Bumann *et al.*, 1984, 1986). Intracellular Ca²⁺ was observed to undergo sustained oscillations in mouse oocytes on addition of phorbol esters and spermatozoa (Cuthbertson & Cobbold, 1985). Thus the phenomenon of oscillatory behaviour, widespread in Nature, seems also to be used in the intricate mechanisms of Ca²⁺-dependent control in mammalian liver cells.

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REFERENCES

- Althaus-Salzmann, M., Carafoli, E. & Jakob, A. (1980) *Eur. J. Biochem.* **106**, 241–248
- Berridge, M. J. & Rapp, P. E. (1979) *J. Exp. Biol.* **81**, 217–279
- Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* **212**, 473–482
- Boiteux, A., Hess, B. & Sel'kov, E. E. (1980) *Curr. Top. Cell. Regul.* **17**, 171–203
- Bumann, J., Wurster, B. & Malchow, D. (1984) *J. Cell Biol.* **98**, 173–178
- Bumann, J., Wurster, B. & Malchow, D. (1986) *Differentiation* **31**, 85–91
- Chance, B., Hess, B., Ghosh, A. & Pye, E. (eds.) (1973) *Biological and Biochemical Oscillators*, Academic Press, New York
- Cuthbertson, K. S. R. & Cobbold, P. H. (1985) *Nature (London)* **316**, 541–542
- Downes, C. P. & Michell, R. H. (1981) *Biochem. J.* **198**, 133–140
- Rhodes, D., Prpic, V., Exton, J. H. & Blackmore, P. F. (1983) *J. Biol. Chem.* **258**, 2770–2773
- Sies, H. (1978) *Methods Enzymol.* **52**, 48–59
- Sies, H., Graf, P. & Estrela, J. M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3358–3362
- Sies, H., Graf, P. & Cadenas, E. (1984) in *Prostaglandins and Membrane Ion Transport* (Braquet, P., Garay, R. P., Frölich, J. C. & Nicosia, S., eds.), pp. 119–128, Raven Press, New York
- Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. (1983) *Nature (London)* **306**, 67–69
- Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. (1986) *Nature (London)* **319**, 600–602