Mobilization of intracellular calcium by glucagon and cyclic AMP analogues in isolated rat hepatocytes

Jean-Pierre Mauger and Michel Claret

Unité de Recherches de Physiologie et Pharmacologie Cellulaire, INSERM U-274, Université Paris-Sud (Bât. 443), F. 91405 Orsay Cedex, France

Received 2 November 1985

Separate or combined addition of cyclic AMP-dependent and Ca^{2+}-linked hormones to isolated rat hepatocytes suspended in a low Ca^{2+} medium reduced the total cellular Ca. When the hormones were administered together, their effects were not additive. This suggests that both types of hormones mobilize Ca^{2+} from a common intracellular pool. In the presence of 1.8 mM extracellular Ca^{2+}, the Ca^{2+} influx counterbalanced or even exceeded the hormone-induced Ca^{2+} loss, depending on the ability of the hormones to stimulate the Ca^{2+} influx.

(Rat hepatocyte) Calcium content Glucagon cyclic AMP Ca^{2+}-dependent hormone

1. INTRODUCTION

In rat liver cells, several hormones like vasopressin and angiotensin or the α_1-adrenergic agonists exert their effects by raising the cytosolic Ca^{2+} concentration [1–4]. These agents induce the release of Ca^{2+} from intracellular stores [3–5] and enhance the entry of extracellular Ca^{2+} [4–6]. We recently demonstrated that addition to liver cell preparations of glucagon or cyclic AMP analogues together with a Ca^{2+}-dependent hormone stimulates the Ca^{2+} influx synergistically [7]. Several reports indicate that glucagon also induces either a loss of Ca or a Ca^{2+} efflux from rat intact liver or isolated hepatocytes, suggesting that it mobilizes Ca^{2+} from intracellular stores [8–12]. In agreement with these observations, glucagon increased the cytosplic Ca^{2+} in rat hepatocytes as measured by the quin2 method [1] and this effect was also transiently observed in cells suspended in a low Ca^{2+} medium (Berthon, B. and Mauger, J.-P., unpublished). Here, we have measured the effects of glucagon and cyclic AMP analogues on the total cellular Ca content in rat hepatocytes suspended in low Ca^{2+} medium. The results indicate that by increasing the cellular cyclic AMP content, glucagon mobilizes Ca^{2+} from a pool sensitive to the Ca^{2+}-dependent hormones.

Abbreviations: Bt_2cAMP, N^6,O^2-dibutyryladenosine 3′,5′-cyclic monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, *myo*-inositol triphosphate

2. MATERIALS AND METHODS

Hepatocytes were isolated from female Wistar rats weighing 200–250 g as described [7] and were resuspended at 5–7 × 10^6 cells/ml in Eagle's medium supplemented with 1.5% gelatin (Difco) and 1 mg/ml bacitracin.

The total hepatocyte Ca content was measured essentially as described by Blackmore and Exton [13] using an atomic absorption spectrophotometer (Perkin-Elmer model 2380). The results are expressed in nmol Ca/mg protein.

The suppliers of the reagents have already been listed [6,7].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

3. RESULTS

Addition of EGTA to the medium reduced the total cellular Ca content of the hepatocytes (fig.1A). This decrease was partly due to the release of Ca^{2+} from extracellular binding sites, and partly to the active extrusion of cytosolic Ca^{2+} by the Ca^{2+} pump; in low Ca^{2+} medium, such extrusion is not counterbalanced by the Ca^{2+} influx [6]. The addition of 10 nM vasopressin accelerated the loss of Ca^{2+} from the cells as expected, since this hormone induces the release of Ca^{2+} from an intracellular pool [2–5]. This mobilization of Ca^{2+} increases the $[Ca^{2+}]_i$ which in turn activates the plasma membrane Ca^{2+} pump [4] and the mobilized Ca^{2+} is partly extruded from the cells. However, some of this Ca^{2+} will be taken up into a nonionic intramitochondrial Ca pool and will not be available for the Ca^{2+} pump (see [14]). Consequently the Ca loss from the hepatocytes in the absence of Ca^{2+} influx is essentially indicative of the amount of Ca^{2+} released from the internal stores by the Ca^{2+}-dependent hormones but does not measure it exactly.

Addition of 10 nM glucagon also accelerated the Ca^{2+} loss from the hepatocytes, suggesting that this hormone also mobilizes intracellular Ca^{2+} (fig.1A). However, the effect of glucagon was slower than that of vasopressin, and 5 min after its addition, Ca^{2+} loss was smaller than that caused by vasopressin (0.9 vs 1.4 nmol/mg). Fig.1B shows the dose-response curve for the effect of glucagon on the cell Ca content. The EC_{50} of the response is about 0.2 nM and is in the range of the physiological concentrations of glucagon found in the plasma. The diterpene forskolin, which activates adenylate cyclase and augments the cellular cyclic AMP content, and the permeant cyclic AMP analogue Bt_2cAMP also accelerated the Ca^{2+} loss from cells incubated in low Ca^{2+} medium in the same way as glucagon (table 1). This strongly suggests that glucagon mobilizes intracellular Ca^{2+} by raising the cellular cyclic AMP content.

Fig.2A shows that when maximal doses of glucagon and vasopressin were simultaneously added to the cells, the Ca^{2+} loss was only slightly larger than that observed in the presence of glucagon alone and was the same as that observed in the presence of vasopressin alone. Therefore the effects of glucagon and vasopressin were not ad-

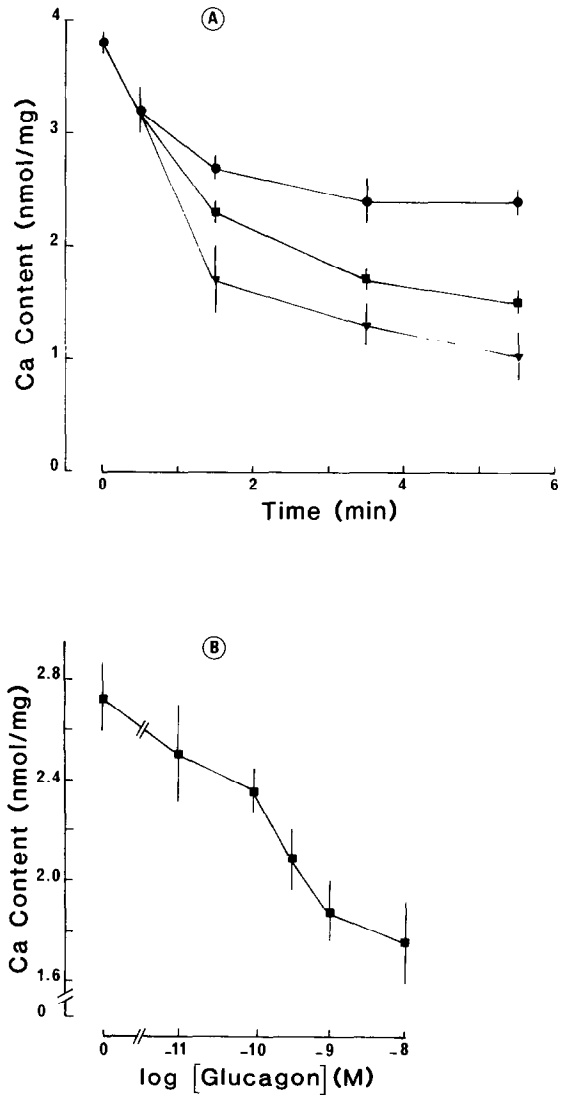

Fig.1. Effects of glucagon and vasopressin on the Ca content of hepatocytes suspended in a low Ca^{2+} medium. (A) Hepatocytes were preincubated in Eagle's medium containing 1.8 mM Ca^{2+}. 3 mM EGTA was added at time 0. After 30 s, vehicle (●) or 10 nM glucagon (■) or 10 nM vasopressin (▼) was added. At the indicated times, 1-ml samples of the cell suspension were removed for determination of cell Ca. Values are means ± SE of 9 or 12 determinations in 3 or 4 experiments. (B) Hepatocytes were incubated as in A with the indicated concentration of glucagon. Cell Ca was determined 5 min after the addition of glucagon. Values are means ± SE of at least 6 determinations in at least 3 experiments.

Table 1

Effect of Bt_2cAMP and forskolin on the Ca content of hepatocytes suspended in a low Ca^{2+} medium

Addition	Ca content (nmol/mg)
None	2.6 ± 0.1
Vasopressin (10^{-8} M)	1.4 ± 0.1
Bt_2cAMP (10^{-4} M)	1.5 ± 0.1
Vasopressin (10^{-8} M) + Bt_2cAMP (10^{-4} M)	1.4 ± 0.1
DMSO (0.5%)	2.2 ± 0.1
Forskolin (5×10^{-5} M)	1.5 ± 0.1

Hepatocytes were incubated as described in fig.2A. Values are means ± SE of 6 determinations in 3 experiments

ditive, as also observed for the effects of Bt_2cAMP and vasopressin (table 1). This lack of additivity of the effects induced by cyclic AMP and the Ca^{2+}-linked hormones implies that they mobilize Ca^{2+} from a common intracellular store. Stimulation of the β-adrenoceptors by 1 μM isoproterenol or 1 μM adrenaline in the presence of 10 μM phentolamine also induced a Ca^{2+} loss from the hepatocytes (fig.2A), as does stimulation of α-adrenoceptors by the addition of 1 μM adrenaline in the presence of 10 μM propranolol. When both the α- and β-adrenoceptors were stimulated by the addition of 1 μM adrenaline without antagonists, the Ca^{2+} loss was similar to that observed when only the α-adrenoceptors were stimulated, indicating that the α- and β-adrenergic responses were not additive.

As fig.2B shows, the separate addition of glucagon, vasopressin, β- or α-adrenergic agonists to cells suspended in a medium containing 1.8 mM Ca^{2+} either had no effect on the total cell Ca content, or slightly reduced it. However, simultaneous application of glucagon and vasopressin raised the cell Ca content as observed by Morgan et al. [15]. These observations indicate that in the presence of a normal extracellular Ca^{2+} concentration, the Ca^{2+} influx partly counterbalances or even exceeds the Ca^{2+} loss resulting from the mobilization of Ca^{2+} from the intracellular pool. We previously observed that, when added singly the cyclic AMP-dependent and Ca^{2+}-linked hormones stimulated the Ca^{2+} influx in isolated rat hepatocytes, and

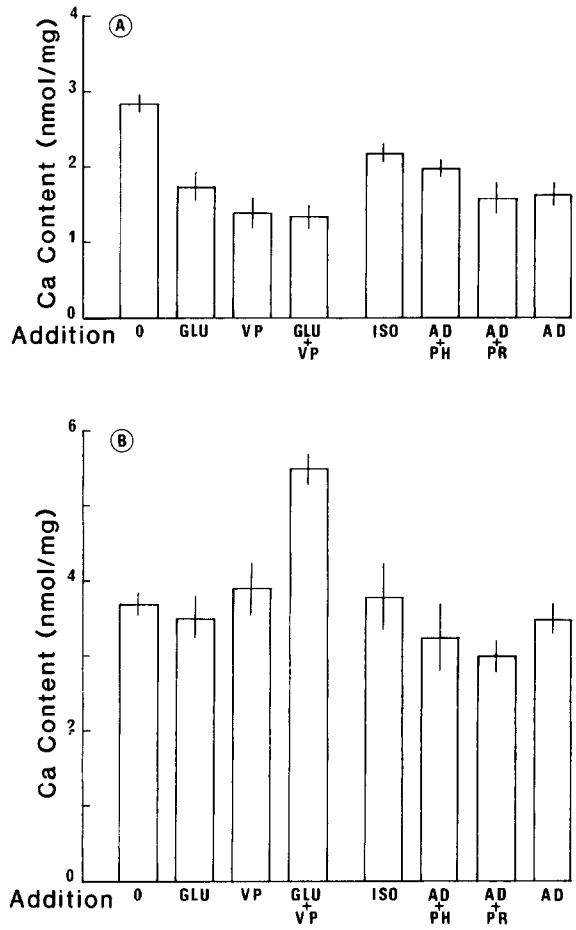

Fig.2. Effects of glucagon, vasopressin, α- and β-adrenergic agonists on the Ca content of hepatocytes incubated in low or normal Ca^{2+} medium. (A) Hepatocytes were incubated as described in fig.1. Hormones were added 30 s after EGTA and samples were removed for cell Ca determination 5 min after the addition of the hormones. Glucagon (GLU) and vasopressin (VP) were added at 10 nM; isoproterenol (ISO) and adrenaline (AD) were added at 1 μM. When used, phentolamine (PH) and propranolol (PR) were added at 10 μM, 5 min before the addition of adrenaline. (B) As in A except that EGTA was not added. Values are means ± SE of at least 6 determinations in at least 3 experiments.

that when added together, their effect was synergistic [6,7]. In fig.3, the difference between the total Ca content observed in the presence of extracellular Ca^{2+} (fig.2B) and in its absence (fig.2A) has been plotted as a function of the Ca^{2+} influx,

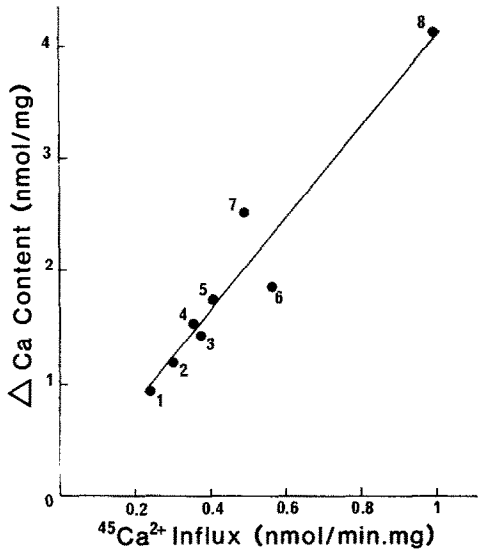

Fig.3. Correlation between the cell Ca content and the Ca^{2+} influx. The differences in the Ca content observed in low and high Ca^{2+} medium were determined from the values shown in fig.2A and B. The values for $^{45}Ca^{2+}$ influx were taken from Mauger et al. [7] and Poggioli, J., Mauger, J.-P. and Claret, M. (submitted). The figures indicate the different hormones tested as follows: 1, none; 2, 1 μM adrenaline + 10 μM phentolamine; 3, 1 μM adrenaline + 10 μM propranolol; 4, 1 μM isoproterenol; 5, 10 nM glucagon; 6, 1 μM adrenaline; 7, 10 nM vasopressin; 8, 10 nM glucagon + 10 nM vasopressin.

determined with the same hormones from the initial $^{45}Ca^{2+}$ uptake rate. The 2 effects were closely correlated, indicating that the Ca^{2+} influx plays an important role in the regulation of the cellular Ca content by the hormones in rat hepatocytes.

4. DISCUSSION

This report indicates that by increasing cellular cyclic AMP content, glucagon accelerates the loss of Ca^{2+} from rat hepatocytes incubated in a low Ca^{2+} medium. This could be due to primary activation of the plasma membrane Ca^{2+} pump. However, it has been shown that glucagon or cyclic AMP analogues do not increase the ATP-dependent Ca^{2+} transport by plasma membrane vesicles [16,17]. The other possible explanation of our results is that glucagon and the cyclic AMP analogues mobilize Ca^{2+} from the same intracellular pool as the one affected by the addition of vasopressin to the cells. In this connection it is now known that the Ca^{2+}-linked hormones such as vasopressin initiate the degradation of membranous PIP_2 leading to an enhancement of cell IP_3 content [18–21]. IP_3 in turn releases Ca^{2+} from the endoplasmic reticulum when it is added to permeabilized hepatocytes [22,23] or isolated liver microsomes [24]. Consequently the present results suggest that cyclic AMP also releases Ca^{2+} from the endoplasmic reticulum in rat hepatocytes.

There are 3 possible mechanisms by which cyclic AMP induces this release. The first hypothesis might be that cyclic AMP increases the IP_3 content of the cells. However, glucagon which increases cellular cyclic AMP content does not induce measurable degradation of PIP_2 or measurable IP_3 production in rat hepatocytes [18,25]. Nevertheless we cannot exclude the possibility that cyclic AMP produced an amount of IP_3 too small to be detected but sufficient to induce Ca^{2+} release from the endoplasmic reticulum. A second hypothesis could be that cyclic AMP inhibits the pumping of Ca^{2+} into the endoplasmic reticulum. But here again several reports rather indicated that pretreatment of the liver or isolated hepatocytes with glucagon or cyclic AMP analogues enhanced the Ca^{2+} uptake by the microsomes or endoplasmic reticulum-rich fractions [26,27]. The third hypothesis is that cyclic AMP increases the efflux of Ca^{2+} from the endoplasmic reticulum in the same way as IP_3. Such an effect of cyclic AMP has been described for example in the cardiac sarcoplasmic reticulum. In this system cyclic AMP appears to increase both active Ca^{2+} transport into the sarcoplasmic reticulum and the Ca^{2+} efflux from this intracellular membrane structure by stimulating a cyclic AMP-dependent protein kinase. Both these effects are concomitant with the phosphorylation of phospholamban (review [28]).

To sum up, our results indicate that in rat hepatocytes, glucagon and the cyclic AMP analogues induce the release of Ca^{2+} from the IP_3 sensitive pool, presumably the endoplasmic reticulum. The mechanism by which cyclic AMP increases the Ca^{2+} efflux from this compartment remains to be elucidated.

ACKNOWLEDGEMENTS

We thank R. Leuillet and J. Tansini for their technical assistance.

REFERENCES

[1] Charest, R., Blackmore, P.F., Berthon, B. and Exton, J.H. (1983) J. Biol. Chem. 258, 8769–8773.
[2] Berthon, B., Binet, A., Mauger, J.-P. and Claret, M. (1984) FEBS Lett. 167, 19–24.
[3] Exton, J.H. (1985) Am. J. Physiol. 248, E633–E647.
[4] Williamson, J.R., Cooper, R.H., Joseph, S.K. and Thomas, A.P. (1985) Am. J. Physiol. 248, C203–C216.
[5] Reinhart, P.H., Taylor, W.M. and Bygrave, F.L. (1984) Biochem. J. 223, 1–13.
[6] Mauger, J.-P., Poggioli, J., Guesdon, F. and Claret, M. (1984) Biochem. J. 221, 121–127.
[7] Mauger, J.-P., Poggioli, J. and Claret, M. (1985) J. Biol. Chem. 260, 11635–11642.
[8] Friedmann, N. and Park, C.R. (1968) Proc. Natl. Acad. Sci. USA 61, 504–508.
[9] Chen, J.L., Babcock, D.F. and Lardy, H.A. (1978) Proc. Natl. Acad. Sci. USA 75, 2234–2238.
[10] Blackmore, P.F., Assimacopoulos-Jeannet, F., Chan, T.M. and Exton, J.H. (1979) J. Biol. Chem. 254, 2828–2834.
[11] Kimura, S., Kugai, N., Tada, R., Kojima, I., Abe, K. and Ogata, E. (1982) Horm. Metab. Res. 14, 133–138.
[12] Whiting, J.A. and Barritt, G.J. (1982) Biochem. J. 210, 73–77.
[13] Blackmore, P.F. and Exton, J.H. (1985) Methods Enzymol. 109, 550–558.
[14] Rasmussen, H. and Barrett, P.Q. (1984) Physiol. Rev. 64, 938–984.
[15] Morgan, N.G., Blackmore, P.F. and Exton, J.H. (1983) J. Biol. Chem. 258, 5110–5116.
[16] Prpic, V., Green, K.C., Blackmore, P.F. and Exton, J.H. (1984) J. Biol. Chem. 259, 1382–1385.
[17] Lotersztajn, S., Epand, R.M., Mallat, A. and Pecker, F. (1984) J. Biol. Chem. 259, 8195–8201.
[18] Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H. and Kirk, C.J. (1983) Biochem. J. 212, 733–747.
[19] Thomas, A.P., Marks, J.S., Coll, K.E. and Williamson, J.R. (1983) J. Biol. Chem. 258, 5716–5725.
[20] Thomas, A.P., Alexander, J. and Williamson, J.R. (1984) J. Biol. Chem. 259, 5574–5584.
[21] Seyfred, M.A. and Wells, W.W. (1984) J. Biol. Chem. 259, 7666–7672.
[22] Burgess, G.M., Godfrey, P.P., McKinney, J.S., Berridge, M.J., Irvine, R.F. and Putney, J.W. (1984) Nature 309, 63–66.
[23] Joseph, S.K., Thomas, A.P., Williams, R.J., Irvine, R.F. and Williamson, J.R. (1984) J. Biol. Chem. 259, 3077–3081.
[24] Dowson, A.P. and Irvine, R.F. (1984) Biochem. Biophys. Res. Commun. 120, 858–864.
[25] Charest, R., Prpic, V., Exton, J.H. and Blackmore, P.F. (1985) Biochem. J. 227, 77–90.
[26] Andia-Waltenbaugh, A.M. and Friedmann, N. (1978) Biochem. Biophys. Res. Commun. 82, 603–608.
[27] Taylor, W.M., Bygrave, F.L., Blackmore, P.F. and Exton, J.H. (1979) FEBS Lett. 104, 31–34.
[28] Katz, A.M. (1982) Handbook Exp. Pharmacol. 58, part II, 347–364.