EXPLORATION OF THE ROLE OF SODIUM IN THE α-ADRENERGIC REGULATION OF HEPATIC GLYCOGENOLYSIS

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Received 6 October 1980

1. Introduction

A large body of evidence is accumulating which suggests the existence of an intracellular second messenger for the α-adrenergic system in liver [1–6]. Current findings suggest that, following catecholamine binding to hepatic α-adrenergic receptors located in the plasma membrane [3,7] a factor or signal is generated which produces an efflux of calcium from mitochondria [3,5,6]. The released calcium is then thought to activate phosphorylase *b* kinase allosterically. The nature of this putative messenger is unknown.

Several physiologically occurring factors are known to influence the release of calcium from mitochondria in vitro (e.g., Na^+, phosphoenolpyruvate, prostaglandins, oxidation of nicotinamide nucleotides) [8,9]. It was originally thought that the rat liver mitochondrial calcium-transport system is not affected by Na^+ [8]. However, it has recently been demonstrated that sodium does produce a release of calcium from rat liver mitochondria [10,11] as it does with heart and brain mitochondria [8,12]. It has also been suggested that sodium may act as the intracellular second messenger for the α-adrenergic system in rat liver [10,11] and that Na^+ is important for other hepatic α-adrenergic effects [13–15].

Here we show that Na^+ is able to alter the calcium buffering point of rat liver mitochondria in vitro. However, sodium depletion experiments together with studies utilizing the monovalent cationophore monensin [16] in isolated hepatocytes demonstrate that sodium is an unlikely candidate for the putative α-adrenergic second messenger.

2. Experimental

'Heavy mitochondria' (i.e., those sedimented between 4800–21 700 × *g* . min) were isolated from fed Sprague Dawley rats as in [2]. Mitochondrial Ca^{2+}-buffering was measured in a medium (6 ml final vol.) of 120 mM KCl, 10 mM Hepes/KOH, 5 mM K-succinate, 0.25 mM KP_i, 5 μM EGTA, 6 mg mitochondrial protein and 26 nmol $CaCl_2$/mg protein (pH 7.4 at 30°C). To monitor the $[Ca^{2+}]$ a F212Ca electrode (Radiometer) connected to a pH meter (pH meter 26, Radiometer) and a Servocord recorder (Datamark) was used.

The electrode was calibrated daily using EGTA–Ca^{2+} solutions added to the incubation medium (in the absence of mitochondria) as in [17]. In some experiments, the incubation medium contained 230 mM sucrose and 5 mM KCl in place of the 120 mM KCl. Similar results were obtained using both media.

Isolated liver cells were prepared as in [1]. When cells were washed and incubated in low sodium medium, the composition of the medium was as follows: choline chloride 130 mM, calcium chloride 2.4 mM, potassium dihydrogen phosphate 1.1 mM, magnesium sulfate 1.1 mM, potassium bicarbonate 10 mM. Sodium was 0.7 mM in this medium as measured by atomic absorption spectroscopy. However, it was found that 1.5% (w/v) gelatin was required to maintain cell viability >95%, but this raised the $[Na^+]$ to 1.7 mM. Essentially the same results were obtained with and without gelatin, but gelatin was routinely used in these experiments.

Phosphorylase *a* levels were determined as in [1]. Na^+ and K^+ content of cells was determined by atomic absorption spectroscopy. The cell separation

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procedure was similar to that used for calcium determinations [1], except that samples of cells were centrifuged through an ice cold solution of 150 mM choline chloride 10% (w/v) sucrose (to prevent mixing of sample with washing solution) and 10^{-5} M digitoxin [18]. The washing solution was buffered with 5 mM Hepes (pH 7.4). Cell pellets were suspended in distilled water then diluted with an equal volume of 0.6 M $HClO_4$ to precipitate protein.

3. Results and discussion

3.1. *Effect of Na^+ on the 'set point' for mitochondrial Ca^{2+} buffering*

As shown in [19,20], rat liver mitochondria were able to buffer Ca^{2+} at ~0.8 μM free Ca^{2+} (fig.1). The addition of Na^+ (20 mM) to the incubation resulted in an almost immediate (<10 s) increase in free [Ca^{2+}] of ~0.2 μM (fig.1). When further Ca^{2+} was added, the mitochondria were able to buffer the added Ca^{2+} at the altered 'set point' (fig.1). The effect of Na^+ on the 'set point' for mitochondrial Ca^{2+} buffering was dose-dependent, with a half-maximal effect obtained at ~7.5 mM Na^+ and a full effect obtained at 25 mM Na^+ (fig.2).

3.2. *Ability of monensin and the α-agonist phenylephrine to activate phosphorylase and modulate intracellular sodium in hepatocytes*

The results in fig.3A,B show the effects of various concentrations of monensin on the intracellular level

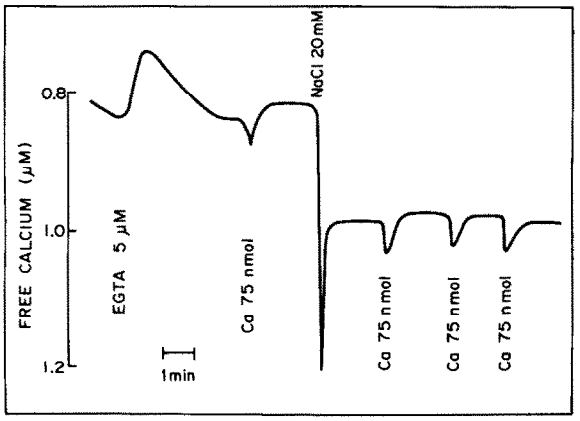

Fig.1. Effect of Na^+ on the 'set point' for mitochondrial Ca^{2+} buffering. Mitochondrial Ca^{2+} buffering was measured as in section 2.

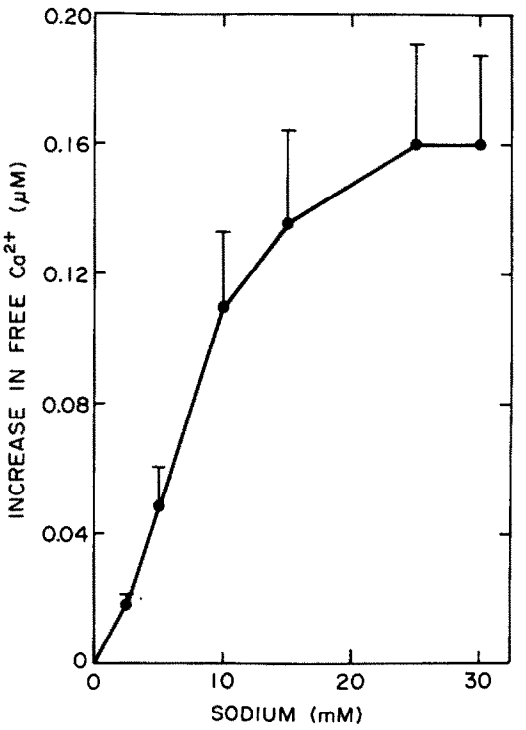

Fig.2. Concentration dependence of the effect of Na^+ on mitochondrial Ca^{2+} buffering. Experimental conditions were the same as in fig.1 except that the [Na^+] added to the incubation was varied as shown. The data are the mean ± SEM of 3 expt. A blank incubation (i.e., with mitochondria absent) was performed, and the values were subtracted from those obtained in the presence of mitochondria, to correct for the effect of Na^+ on the electrode.

of sodium and the activity of phosphorylase in isolated hepatocytes. Monensin (5×10^{-5} M) produced a 4-fold increase in intracellular sodium. This increase was accompanied by a very small increase in phosphorylase *a* levels (18.8–21.2 units/g wet wt). The high basal phosphorylase *a* activity (18.8 *vs* 13.2, 3C) was due to the dimethyl sulfoxide solvent used to dissolve the ionophore. This phenomenon had been reported [1].

The results with monensin are in contrast to those obtained with the α-agonist phenylephrine (fig.3C,D). Phenylephrine at a max dose (10^{-5} M) produced a large activation of phosphorylase (~2-fold). However, no increase in intracellular sodium was seen. In fact, a small decrease in intracellular sodium was sometimes observed, but this was not a consistent result (fig.4). It can also be observed that basal values for intracellular sodium varied slightly from one cell

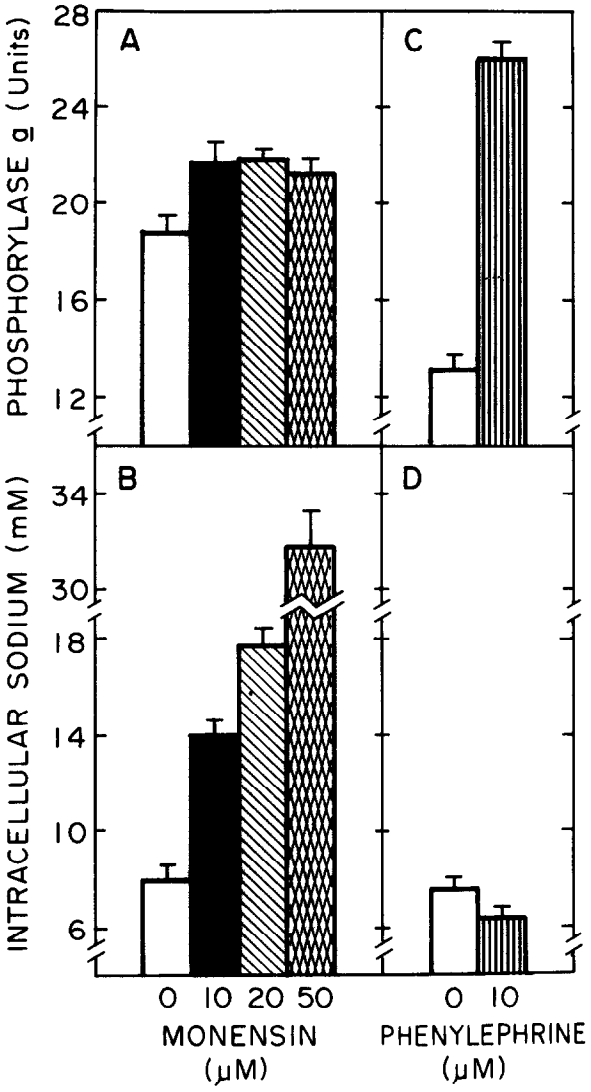

Fig.3. Effect of several concentrations of monensin and phenylephrine on Na^+ content and phosphorylase activation in isolated liver cells. Samples of 0.5 ml were removed for phosphorylase and sodium determinations after 3 min incubation. The control for the monensin incubations was 1% (v/v) dimethyl sulfoxide, while the control for phenylephrine was physiological saline. Each values is the mean of duplicate assays performed on triplicate incubations.

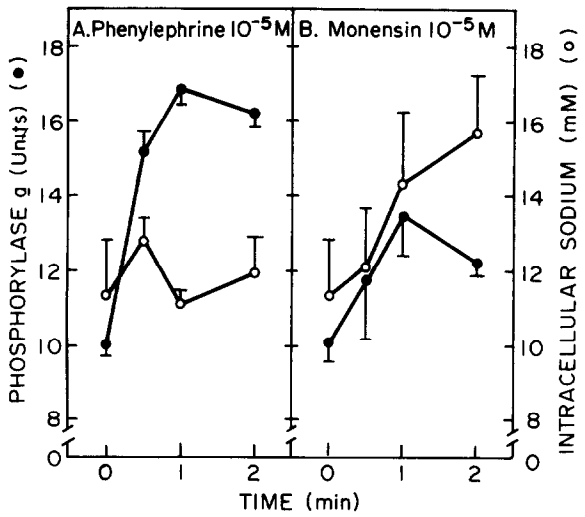

Fig.4. Time course of the effects of phenylephrine and monensin on phosphorylase *a* and intracellular sodium levels. For other details see legend to fig.3.

preparation to another, ranging from 11.5–8.8 mM. It should also be noted that these values are lower than those in [21,22].

The results in fig.4A show that during 2 min exposure to phenylephrine no consistent change in sodium level was observed, whereas phosphorylase was maximally activated between 30–60 s. On the other hand monensin produced a significant increase in sodium content and phosphorylase activation after 1 min exposure.

Monensin produced a small efflux of calcium (9% decrease) from the hepatocytes. This was less than that observed with 10^{-5} M phenylephrine (22% decrease).

3.3. *Sodium depletion of hepatocytes*

In an effort to rule out the involvement of sodium influx during α-adrenergic action, cells were washed and incubated in low sodium medium (1.7 mM Na^+ as opposed to 154 mM Na^+). When these cells were exposed to various concentrations of phenylephrine, a shift in dose–response curve for phosphorylase activation was observed (fig.5B). (The half-maximally effective concentration in normal cells was 0.3 μM, and in sodium depleted was >1 μM.) However, glucagon action was inhibited to a much greater extent (fig.5A), with only partial activation being observed at a maximally effective dose of 10^{-9} M. These results indicate that if anything, glucagon action is more affected by extracellular sodium than is α-action.

3.4. *Effects of digitoxin*

Further experiments designed to exclude a role for Na^+ influx in α-adrenergic actions involved the use

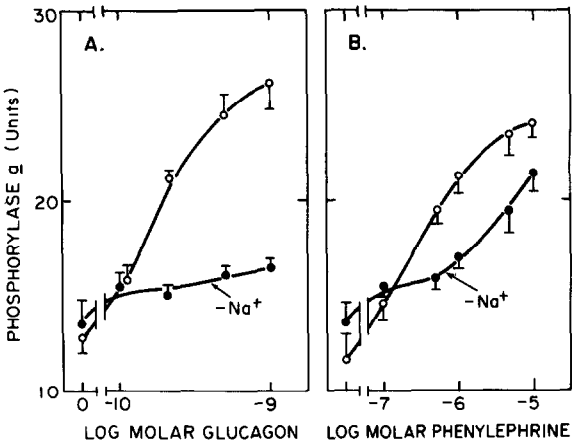

Fig.5. Dose–response for phosphorylase activation by glucagon and phenylephrine in normal and sodium-depleted hepatocytes. Samples were removed at 3 min for phosphorylase *a* determinations. See legend of fig.3 for other details.

of digitoxin an inhibitor of the $Na^+ + K^+$-ATPase [18]. Cells were incubated with 0.1 mM digitoxin and the intracellular [Na^+] measured together with the [K^+]. The results in fig.6A show that 5×10^{-6} M monensin produced a 38% increase in intracellular Na^+ in agreement with fig.3B. A relatively low concentration of digitoxin (10^{-4} M) produced the same effect, and both agents together elicited a large increase. Corresponding, changes were observed for intracellular potassium (fig.6B). The effect observed with monensin on potassium was probably due to the lack of specificity of the ionophore for monavalent cations [16]. Despite the changes in intracellular Na^+ induced by digitoxin in the absence and presence of

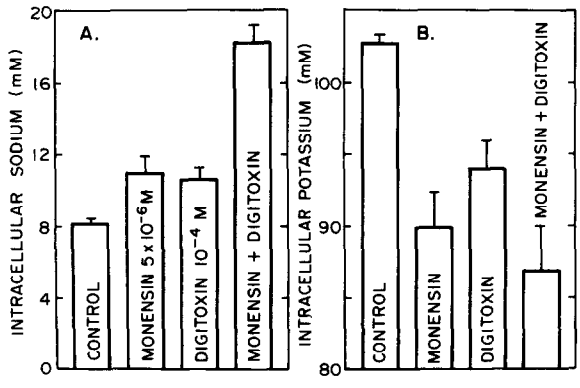

Fig.6. Effects of digitoxin and monensin on intracellular Na^+ and K^+ levels. See legend of fig.3 for other details.

monensin, it caused no significant alterations in phosphorylase *a* (not shown). The inhibitor was also without effect on the action of a submaximally effective concentration of phenylephrine on glucose release (not shown).

These results show that rat liver mitochondria calcium transport activity is modulated by Na^+. However, it would appear from the intracellular Na^+ measurements (9.8 ± 1.0 mM, $n = 6$) that this sodium-induced calcium efflux pathway would be almost saturated under normal basal conditions (see fig.2). Confirmation of this is shown in fig.3,4,6 where monensin is able to produce a large increase in intracellular sodium especially in the presence of digitoxin, but elicits only minimal activation of phosphorylase. On the other hand, a large activation of phosphorylase is observed with phenylephrine, with no significant change in intracellular [Na^+].

The increase in intracellular Na^+ induced by monensin would be expected to occur with an exchange of H^+ for Na^+ [16]. Consequently, the small activation of phosphorylase observed in hepatocytes incubated in the presence of monensin is not likely to be due to a decrease in cytoplasmic pH and subsequent release of mitochondrial Ca^{2+}, as shown in vitro [23].

From these studies it would appear unlikely that changes in the intracellular [Na^+] are responsible for the release of mitochondrial calcium during α-adrenergic stimulation of glycogenolysis. Changes in the intracellular [Na^+] can, however, regulate that level of phosphorylase *a* in hepatocytes, as demonstrated by these data with monensin and the pioneering studies of Cahill et al. [24]. The nature of the putative intracellular messenger of the α-adrenergic system is presently being sought in this laboratory.

Acknowledgement

This work was supported in part by research grant AM 18660 from the National Institutes of Health, US Public Health Service.

References

[1] Blackmore, P. F., Brumley, F. T., Marks, J. L. and Exton, J. H. (1978) J. Biol. Chem. 253, 4851–4858.
[2] Blackmore, P. F., Dehaye, J.-P. and Exton, J. H. (1979) J. Biol. Chem. 254, 6945–6950.

[3] Dehaye, J.-P., Blackmore, P. F., Venter, J. C. and Exton, J. H. (1980) J. Biol. Chem. 255, 3905–3910.
[4] Chen, J.-L. J., Babcock, D. F. and Lardy, H. A. (1978) Proc. Natl. Acad. Sci. USA 75, 2234–2238.
[5] Babcock, D. F., Chen, J.-L. J., Yip, B. P. and Lardy, H. A. (1979) J. Biol. Chem. 254, 8117–8120.
[6] Murphy, E., Coll, K., Rich, T. L. and Williamson, J. R. (1980) J. Biol. Chem. 255, 6606–6608.
[7] El-Refai, M. F., Blackmore, P. F. and Exton, J. H. (1979) J. Biol. Chem. 254, 4375–4386.
[8] Crompton, M., Moser, R., Ludi, H. and Carafoli, E. (1978) Eur. J. Biochem. 82, 25–31.
[9] Lehninger, A. L., Vercesi, A. and Bababunmi, E. A. (1978) Proc. Natl. Acad. Sci. USA 75, 1699–1694.
[10] Haworth, R. A., Hunter, D. R. and Berkhoff, H. A. (1980) FEBS Lett. 110, 216–218.
[11] Nedergaard, J. and Cannon, B. (1980) Acta Chem. Scand. B34, 145–151.
[12] Nicholls, D. G. and Scott, I. D. (1980) Biochem. J. 186, 833–839.
[13] Haylett, D. G. and Jenkinson, D. H. (1972) J. Physiol. (Lond.) 225, 721–750.
[14] Haylett, D. G. and Jenkinson, D. H. (1972) J. Physiol. (Lond.) 225, 751–772.
[15] Lambotte, L. and Kestens, P. J. (1971) in: Research Methods in Reproductive Endocrinology, 4th Karolinska Symp., Perfusion Techniques (Diezfalusky, E. ed) pp. 217–236.
[16] Pressman, B. C. (1976) Annu. Rev. Biochem. 45, 501–530.
[17] Bartfai, T. (1979) Adv. Cyclic Nucl. Res. 10, 219–242.
[18] Beigelman, P. M. and Thomas, L. J. jr (1972) J. Membr. Biol. 8, 181–188.
[19] Nicholls, D. G. (1978) Biochem. J. 176, 463–474.
[20] Brand, M. D. and DeSelincourt, C. (1980) Biochem. Biophys. Res. Commun. 92, 1377–1382.
[21] Krebs, H. A., Cornell, N. W., Lund, P. and Hems, R. (1974) in: Regulation of Hepatic Metabolism (Lundquist, F. and Tygstrup, N. eds) pp. 726–750, Munksgaard, Copenhagen.
[22] Baur, H., Kasperek, S. and Pfaff, E. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 827–838.
[23] Akerman, K. E. O. (1978) Arch. Biochem. Biophys. 189, 256–262.
[24] Cahill, G. F., Ashmore, J., Zottu, S. and Hastings, A. B. (1956) J. Biol. Chem. 218, 237–250.