INHIBITION OF PHENYLEPHRINE-STIMULATED GLUCONEOGENESIS BY CHLORPROMAZINE IS MEDIATED BY α-ADRENERGIC RECEPTORS

Leann TILLEY, R. J. SUMMERS*, T. G. REDGRAVE and B. E. KEMP+

*Department of Physiology, University of Melbourne, Parkville, VIC 3052, *Clinical Pharmacology Unit, Department of Medicine, University of Melbourne, Austin Hospital, Heidelberg, VIC 3084* and *+Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, VIC 3052, Australia*

Received 3 March 1981

1. Introduction

It is now recognized that glucagon and epinephrine regulate gluconeogenesis by different mechanisms. Glucagon stimulation of gluconeogenesis appears to be mediated via cyclic AMP and the cAMP-dependent protein kinase system [1], whereas epinephrine is believed to stimulate hepatic gluconeogenesis via a cAMP-independent α-adrenergic receptor system [1].

Calcium has been implicated as an important regulator in the α-adrenergic control of glycogenolysis [2–4], however its regulatory role in hepatic gluconeogenesis is less well defined [1]. Intracellular mobilization of Ca^{2+} appears to follow α-adrenergic stimulation [5,6]. Moreover hepatic protein phosphorylation and inactivation of pyruvate kinase by angiotensin II and vasopressin are also Ca^{2+}-dependent [7]. The concept emerging is that protein phosphorylation is probably the primary regulator of the key enzymes in the control of gluconeogenesis and that either cAMP or Ca^{2+} may modulate it.

Since some Ca^{2+}-dependent protein kinases such as phosphorylase *b* kinase [8] and the myosin light chain kinase owe their Ca^{2+}-dependence to calmodulin [9] it was of interest to explore whether calmodulin was important in the α-adrenergic control of gluconeogenesis. Phenothiazine drugs are known to block the actions of calmodulin [10] trifluoperazine has been used as a probe to study the possible involvement of calmodulin in the control of insulin secretion from isolated pancreatic islets [11]. The purpose of this study was to determine whether α-adrenergic stimulation of gluconeogenesis could be inhibited by chlorpromazine and if so whether this was consistent with a role for calmodulin in the regulation of hepatic gluconeogenesis.

2. Materials and methods

2.1. *Experiments with hepatocytes*

Isolated hepatocytes were prepared from 24 h starved rats (250 g) by the procedure in [12] as modified [13]. The isolated hepatocytes (1×10^6 cells/ml) were incubated in plastic scintillation vials (20 ml) in 1.5 ml total vol. Krebs–Henseleit bicarbonate buffer containing 1% albumin and 10 mM lactate with shaking for 1 h at 37°C and gassed with O_2–CO_2 (95:5). Cell viability ranged from 80–95% as judged by trypan blue and nigrosin dye exclusion. Neither chlorpromazine nor phenylephrine had any measurable effect on cell viability during the course of cell incubation. Glucose output was assessed in neutralized perchloric acid precipitated samples by the glucose–oxidase–peroxidase method [14].

2.2. *Radioligand binding studies*

Liver plasma membranes for drug binding studies were prepared up to step 12 of the procedure in [15]. Plasma membrane protein concentration [16] and 5′-nucleotidase [17] were measured. [^{3}H]Prazosin was purified before use by thin-layer chromatography on silica gel plates (Anal. Techn. Inc.) using ethyl acetate:diethylamine (19:1) mobile phase. Plasma membranes were washed with 50 mM Tris–HCl buffer (pH 7.6) (49 000 × *g* for 15 min at 4°C) and resuspended in the same buffer at ~250 μg protein/ml. Binding assays containing 50 μg protein, [^{3}H]prazosin

+ To whom correspondence should be addressed

(33 Ci/mmol; final conc. 0.07–0.7 nM) and 50 mM Tris–HCl buffer (pH 7.6) in 2 ml final vol. were incubated for 30 min at 25°C and then washed on Whatman GF/B glass fibre filters [18]. Non-specific binding was determined by addition of 10 μM indoramin (a selective α_1 adrenoceptor agonist at each [^{3}H]prazosin concentration). Specific binding was defined as the difference between total binding and non-specific binding, and represented from 80–95% of total binding depending on ligand concentration. Values for the dissociation constant (K_d) and maximal number of binding sites (B_{max}) for [^{3}H]prazosin binding were determined using computer-assisted iterative curve-fitting procedures [19]. In drug displacement studies sigmoid curves were fitted to the points using an iterative procedure utilising the logistic function:

$$\% \text{ Radioligand displaced} = 100\ [D]^P/([D]^P + (IC_{50})^P)$$

where $[D]$ is the concentration of displacing drug and P is the slope factor. From the value of the IC_{50} the inhibition constant (K_i) was obtained [20].

2.3. *Chemicals*

Chlorpromazine, collagenase (type IV), phenylephrine and Trizma were obtained from Sigma Chemical Co. (St Louis). Angiotensin II amide Arg-vasopressin and indoramin were obtained from Ciba, Ferring AB (Sweden) and Wyeth Labs, respectively. [^{3}H]Prazosin (33 Ci/mmol) was kindly donated by Pfizer UK Ltd.

3. Results

Chlorpromazine inhibited phenylephrine (50 μM)-stimulated gluconeogenesis over 0.1–10 μM with half-maximal inhibition at ~5 μM. Neither the basal rate of gluconeogenesis (25 ± 2 μg glucose . 10^6 cells^{-1} . h^{-1}) (fig.1A) nor the glucagon stimulated gluconeogenesis was affected by chlorpromazine (fig.1B) over this range. Angiotensin II (0.1 μM) or vasopressin (46 nM) produced 20% and 30% stimulation of gluconeogenesis, respectively (not shown) but again chlorpromazine was without effect.

Radioligand binding studies were used to investigate the possibility that chlorpromazine was inhibiting phenylephrine-stimulated gluconeogenesis by acting primarily as an α-adrenoceptor antagonist rather than by inhibiting calmodulin. Prazosin is known to be a

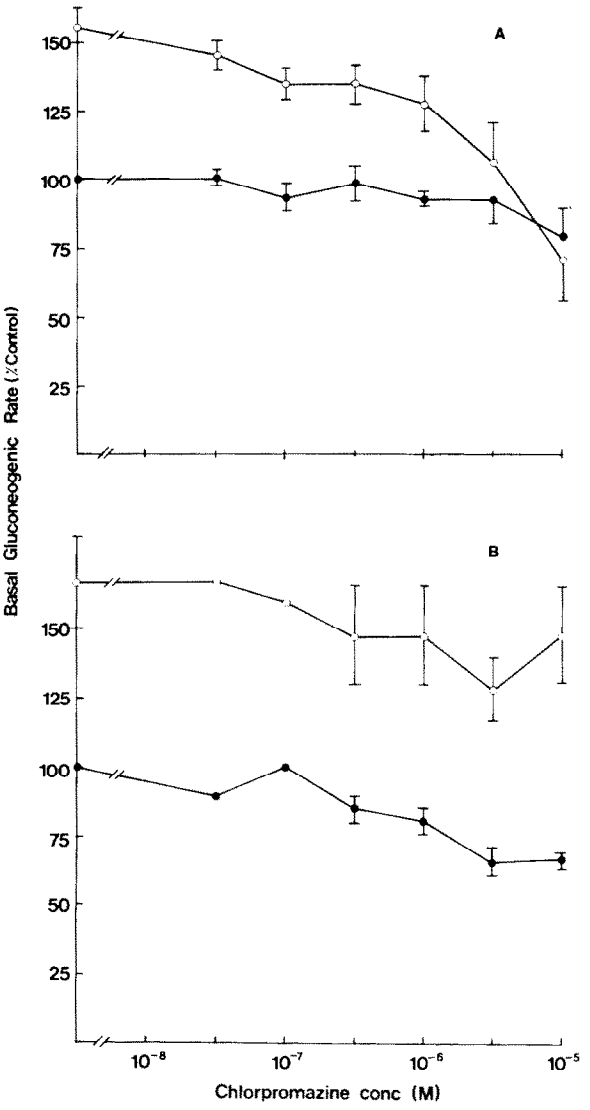

Fig.1. The effect of chlorpromazine on hepatic gluconeogenesis stimulated by phenylephrine and glucagon: (A) phenylephrine (50 μM); and (B) glucagon (2.7 μM) (•). The data are expressed as the % of the basal gluconeogenic rate measured in the absence of hormone and chlorpromazine. Values given are the mean ± SE for 5 (A) and 3 (B) different hepatocyte preparations.

potent competitive antagonist which acts specifically at α_1 adrenoceptors [21]. Here, prazosin was a potent inhibitor of phenylephrine-stimulated gluconeogenesis with complete inhibition of the process being obtained at 10 nM prazosin (fig.2). These results are consistent with the finding that prazosin is a potent

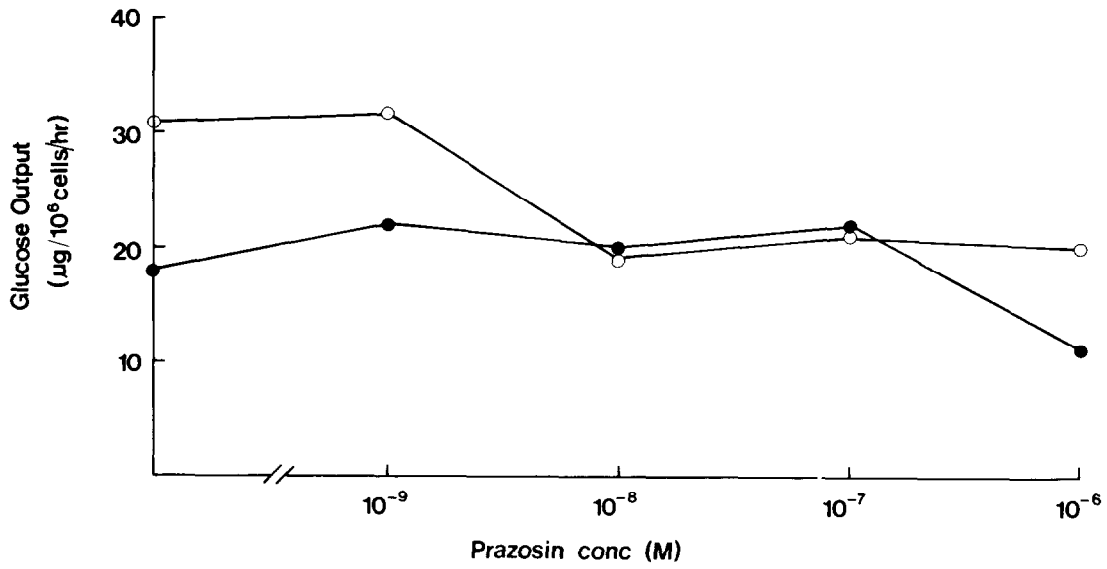

Fig.2. The effect of prazosin on phenylephrine-stimulated gluconeogenesis. Hepatocytes were incubated with (○) and without (●) phenylephrine (50 μM). Results shown for 1 expt.

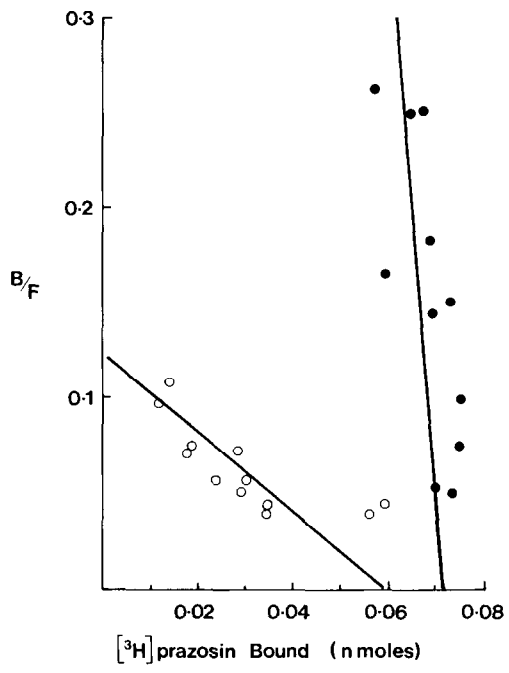

Fig.3. Scatchard plot of specific [^{3}H]prazosin binding to plasma membranes from rat liver. Each point is the mean value of duplicate determination of 2 expt. The lines are those of best fit determined by the iterative procedure [19]: (●) control [^{3}H]prazosin binding, K_d = 17.2 fM, B_{max} = 1.44 pmol/mg protein; (○) binding in the presence of 4 nM chloropromazine, K_d = 239 fM, B_{max} = 1.16 pmol/mg protein.

inhibitor of epinephrine stimulated phosphorylase activity in isolated hepatocytes [22]. [^{3}H]Prazosin of high specific activity was used to label α_1 adrenoceptors in purified preparations of rat liver plasma membranes to study whether chlorpromazine could displace it from the α receptor. [^{3}H]Prazosin binding to rat liver plasma membranes was rapid, saturable and reversible. Equilibrium studies revealed that the binding site has a K_d of 17.2 fM and a B_{max} of 1.44 pmol/mg protein (fig.3). In the presence of a low cencentration of chlorpromazine (4 nM) the K_d for [^{3}H]prazosin binding was increased ~14-fold to 239 fM with only a small reduction in B_{max} to 1.16 pmol/mg protein. These results are consistent with chlorpromazine acting as a potent competitive antagonist of [^{3}H]prazosin binding. Increasing concentrations of either chlorpromazine or trifluoperazine produced concentration-dependent inhibition of [^{3}H]prazosin binding (fig.4) with K_i-values of 0.77 and 4.16 nM, respectively. Hill plots of the displacement curves had gradients near unity indicating a lack of cooperativity in binding.

Inhibition of [^{3}H]prazosin binding by either chlorpromazine or trifluoperazine was independent of Ca^{2+}. The K_i-values in the presence of either Ca^{2+} (100 μM) or EGTA (1 mM) were 0.75 and 1.05 nM for chlorpromazine and 4.17 and 4.03 nM for trifluoperazine, respectively.

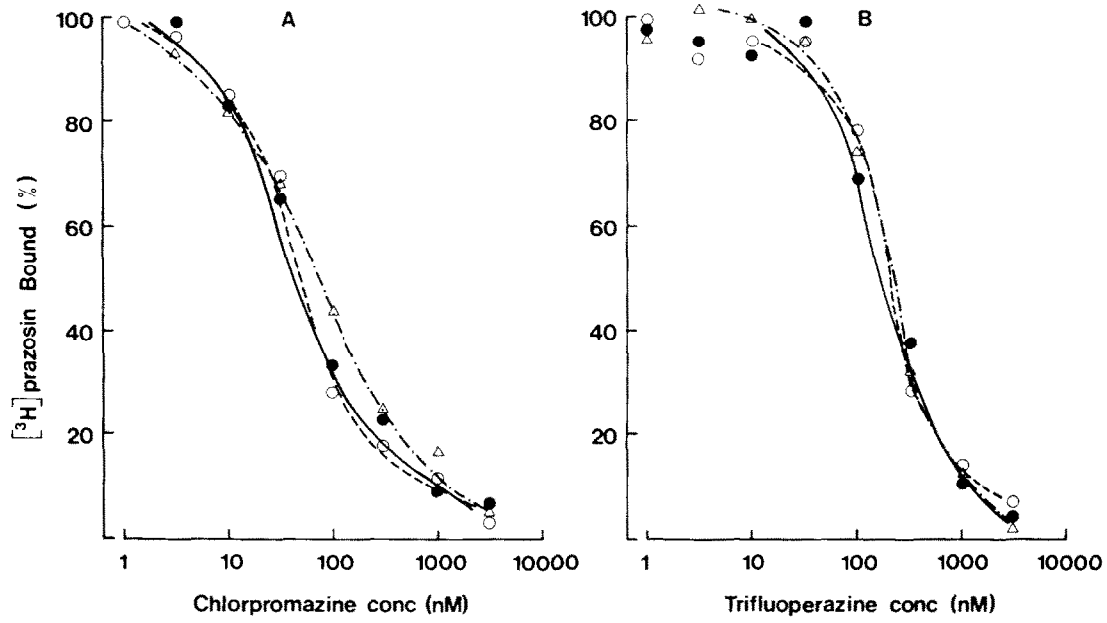

Fig.4. Displacement of [^{3}H]prazosin binding from rat liver plasma membranes by phenothiazine drugs: (A) Effect of chlorpromazine alone (•) and in the presence of 100 μM Ca^{2+} (○) or 1 mM EGTA (△). Curves are those of best fit determined by the iterative procedure [19]. (B) Effect of trifluoperazine alone (•) and in the presence of 100 μM Ca^{2+} (○) or 1 mM EGTA (△).

4. Discussion

These results show that α-adrenergic-stimulated gluconeogenesis from lactate in isolated hepatocytes can be blocked by chlorpromazine with half-maximal inhibition occurring at ~5 μM. Similar concentrations of chlorpromazine are required to inhibit phenylephrine-stimulated gluconeogenesis, calmodulin-dependent phosphodiesterase [10] and phosphorylase *b* kinase [23]. In contrast, glucagon-stimulated gluconeogenesis was unaffected by chlorpromazine over 10 nM–10 μM. The inhibition of phenylephrine-stimulated gluconeogenesis occurs at concentrations of chlorpromazine below those required to observed non-saturating uptake of the drug into isolated hepatocytes [24].

Although these results would appear to be consistent with the concept that calmodulin may be involved, chlorpromazine failed to inhibit either angiotensin II- or vasopressin-stimulated gluconeogenesis. Both of these hormones require Ca^{2+} to inactivate hepatic pyruvate kinase [7]. The results of these radioligand binding studies suggest that chlorpromazine inhibition of phenylephrine-stimulated gluconeogenesis results entirely from its α-antagonist activity at the cell membrane rather than from effects on calmodulin. The binding of the highly selective α_1 adrenoceptor antagonist [^{3}H]prazosin to the liver plasma membrane fraction was potently inhibited by chlorpromazine. Scatchard plots of [^{3}H]prazosin binding conducted in the presence of a low concentration of chlorpromazine showed increases in K_d with no significant change in B_{max}, indicating competitive inhibition. Displacement of a saturating concentration of [^{3}H]prazosin by increasing concentrations of chlorpromazine and trifluoperazine revealed that both drugs had high potency at hepatic α_1 adrenoceptors. Radiolabelled chlorpromazine of sufficiently high specific activity was not available to confirm that phenylephrine could block its binding.

A further possibility is that calmodulin could be a component of the α_1 adrenoceptor but this seems unlikely as:

(i) Chlorpromazine was >5-times more potent than trifluoperazine in displacing bound [^{3}H]prazosin which is the reverse order of potency to that observed against calmodulin-dependent phosphodiesterase [10].

(ii) Displacement of bound [^{3}H]prazosin by both chlorpromazine and trifluoperazine was indepen-

dent of Ca^{2+} in contrast to the Ca^{2+}-dependent binding of phenothiazines to calmodulin [10].

These results indicate that chlorpromazine and trifluoperazine inhibit α-adrenergic-stimulated gluconeogenesis by acting as α-antagonists at the plasma membrane level. These observations together with the report of a calmodulin-independent protein kinase [25] inhibited by chlorpromazine highlight the difficulties in attempting to use phenothiazine drugs as probes for calmodulin function in cellular systems. Furthermore, when calmodulin is bound as the δ-subunit of muscle phosphorylase *b* kinase it is relatively insensitive to phenothiazine drugs [23].

Acknowledgements

This work was supported by grants from the National Health and Medical Research Council and National Heart Foundation of Australia. The authors wish to acknowledge the excellent technical assistance of Judi Trask.

Note added in proof

Blackmore et al. [26] have recently reported that inhibition of phenylephrine stimulated activation of hepatic phosphorylase *a* by chlorpromazine and trifluoroperazine was due to α-antagonist activity. While we agree with the conclusions of this report regarding α-adrenergic control of hepatic gluconeogenesis we feel that their use of [^{3}H]epinephrine as a ligand to identify α-adrenoreceptors in liver plasma membranes was inappropriate for two reasons:

(i) [^{3}H]Epinephrine labels predominantly α_2 receptors in the high affinity (α_{2H}) state which constitute <20% of the total hepatic α adrenoreceptors [27];

(ii) Evidence from this study would indicate that the adrenoreceptor controlling gluconeogenesis is of the α_1-type.

The use of the specific α-antagonist ([^{3}H]prazosin) as a ligand to identify α_1-receptors in liver plasma membranes is clearly more relevant.

References

[1] Exton, J. H. (1979) J. Cyclic Nucl. Res. 5, 277–287.
[2] Van de Werve, G., Hue, L. and Hers, (1977) Biochem. J. 162, 135–142.
[3] Keepens, S., Vandenheede, J. R. and De Wulf, H. (1977) Biochim. Biophys. Acta 496, 448–457.
[4] Assimacopoulos-Jaennet, F. D., Blackmore, P. F. and Exton, J. H. (1977) J. Biol. Chem. 252, 2662–2669.
[5] Blackmore, P. F., Brumbley, F. T., Marks, J. L. and Exton, J. H. (1976) J. Biol. Chem. 254, 7147–7156.
[6] Chen, J. L. J., Babcock, D. F. and Lardy, H. A. (1978) Proc. Natl. Acad. Sci. USA 75, 2234–2238.
[7] Garrison, J. C., Borland, M. K., Florio, V. A. and Twible, D. A. (1979) J. Biol. Chem. 254, 7147–7156.
[8] Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C. and Nairn, A. C. (1978) FEBS Lett. 92, 287–298.
[9] Cheung, W. Y. (1980) Science 207, 19–27.
[10] Weiss, B. and Levin, R. M. (1978) Adv. Cyclic Nucl. Res. 9, 285–303.
[11] Sugden, M. C., Christie, M. R. and Ashcroft, S. J. H. (1979) FEBS Lett. 105, 95–100.
[12] Berry, M. N. and Friend, D. S. (1969) J. Cell. Biol. 43, 506–520.
[13] Edwards, K., Nagashima, M., Dryburgh, H., Wykes, A. and Schreiber, G. (1979) FEBS Lett. 100, 269–272.
[14] Bergmeyer, H. O. and Bernt, E. (1974) Methods in Enzymatic Analysis (Bermeyer, H. E. ed) 3rd edn, pp. 1202–1215, Academic Press, New York.
[15] Neville, D. M. (1968) Biochim. Biophys. Acta 154, 540 552.
[16] Lowry, H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[17] Fischer, D. B. and Muller, G. C. (1971) Biochim. Biophys. Acta 248, 434–448.
[18] Summers, R. J., Jarrott, B. and Louis, W. J. (1980) Neurosci. Lett. 20, 347–350.
[19] Priore, R. L. and Rosenthal, H. E. (1976) Anal. Biochem. 70, 231–240.
[20] Cheng, Y. and Prusoff, W. J. (1973) Biochem. Pharmacol. 22, 3099–3108.
[21] Cambridge, D., Davey, M. J. and Massingham, R. (1977) Brit. J. Pharmacol. 59, 514–515P.
[22] Aggerbeck, M., Guellaen, G. and Hanoune, J. (1980) Biochem. Pharmacol. 29, 1653–1662.
[23] Shenolikar, S., Cohen, T. W., Cohen, P., Nairn, A. C. and Perry, S. W. (1979) Eur. J. Biochem. 100, 329–337.
[24] Salhab, A. S. and Dujoune, C. A. (1979) Proc. Soc. Exp. Biol. Med. 161, 270–274.
[25] Mori, T., Takai, Y., Minakuchi, R., Yu, B. and Nishizuka (1980) J. Biol. Chem. 255, 8378–8380.
[26] Blackmore, P. F., El-Refai, M. F., Dehaye, J. P., Strickland, W. G., Hughes, B. P. and Exton, J. H. (1981) FEBS Lett. 123, 245–248.
[27] Hoffman, B. B., Mullikin-Kilpatrick, D. and Lefkowitz, R. J. (1980) J. Biol. Chem. 255, 4645–4652.