Ca²⁺-mediated activation of phosphofructokinase in perfused rat heart

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Perfusion of the isolated rat heart with Ca²⁺ concentrations exceeding 3 mM activated phosphofructokinase and phosphorylase, and decreased the concentration of cyclic AMP. Half-maximal activation of phosphofructokinase occurred at 5 mM-CaCl₂; significant activation of phosphorylase did not occur until the concentration of CaCl₂ exceeded 12 mM. The time course for the activation of phosphofructokinase at 12 mM-CaCl₂ indicated that maximal activation occurred within 2 min; when the perfusion-medium Ca²⁺ concentration was re-adjusted to 3 mM, the phosphofructokinase activity returned to pre-activation values within 30 s. The addition of Ca²⁺ to extracts of heart did not activate phosphofructokinase. The activation of phosphofructokinase by sub-maximal doses of adrenaline and Ca²⁺ were not additive. The activation of phosphofructokinase by 1 μ M-adrenaline + 10 μ M-propranolol and by 1 μ M-isoprenaline was inhibited by high concentrations of K⁺ (22–56 mM). The activation of phosphofructokinase by 1 μ M-adrenaline + 10 μ M-propranolol, 12 mM-CaCl₂ and by 1 μ M-isoprenaline was blocked by the slow Ca²⁺-channel blocker nifedipine. These findings suggest that both the β - and α -adrenergic mechanisms for the activation of rat heart phosphofructokinase involve an increase in the myoplasmic Ca²⁺ concentration. This increase may result from an inhibition of Ca²⁺ efflux or a stimulation of Ca²⁺ influx.

In previous publications we have reported that phosphofructokinase is activated in perfused rat heart by catecholamine treatment (Clark & Patten, 1981a,b; Clark *et al.*, 1982a; Patten *et al.*, 1982). Although a β -adrenoceptor mechanism for the activation of phosphofructokinase was noted (Patten *et al.*, 1982), the predominant mechanism initiated by adrenaline was mediated by α -receptors (Patten *et al.*, 1982). In addition, the adrenaline-mediated activation of phosphofructokinase occurred independently of changes in cyclic AMP (Clark & Patten, 1981a) and phosphorylation of phosphofructokinase (Patten *et al.*, 1981), but was found to be dependent on Ca²⁺ (Patten *et al.*, 1981). In the present work Ca²⁺-mediated activation of phosphofructokinase is reported. The possible relevance of this activation in relation to the adrenergic control of rat heart phosphofructokinase is considered.

Experimental

Perfusions

Non-recirculating Langendorff perfusions of rat hearts with glucose-free medium were conducted

as described previously (Clark & Patten, 1981a). Hearts were pre-perfused for 8–10 min with medium containing 3 mM-CaCl₂ and 0.5 mM-EDTA. After this time, media containing different concentrations of CaCl₂ were introduced, as described in the Figures. Unless indicated otherwise, hearts were exposed to the different agonists etc. for 5 min.

Assays

Details for extract preparation (Clark & Patten, 1981a,b) and for the determination of phosphofructokinase and phosphorylase activity ratios were as given previously (Clark & Patten, 1981a; Clark *et al.*, 1982a). The activity ratio of phosphofructokinase is defined as the activity at 1 mM-ATP and 10 μ M-hexose 6-phosphate/activity at 0.1 mM-ATP and 10 μ M-hexose 6-phosphate (Clark & Patten, 1981a). Cyclic AMP was determined by the method of Gilman (1970). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. The methods for preparing membranes and for conducting binding studies were as described by Williams & Lefkowitz (1978). All determinations were made in duplicate.

Animals

Male hooded Wistar rats of 180–250 g body wt. were used and were maintained *ad libitum* on the Division's 'colony' high-carbohydrate diet. Details for this diet are given elsewhere (Clark *et al.*, 1982b).

Materials

The following agonists and antagonists were used: L-adrenaline bitartrate, DL-propranolol hydrochloride, L-isoprenaline hydrochloride (all from Sigma Chemical Co., St. Louis, MO, U.S.A.), phenoxybenzamine hydrochloride (Rohm Pharma, Darmstadt, West Germany), nifedipine (Bayer, Leverkusen, West Germany), [^3H]prazosin hydrochloride (New England Nuclear, Boston, MA, U.S.A.), verapamil and methoxyverapamil (compound D-600) (both from Knoll-Schering, Sydney, N.S.W., Australia). Each agent was freshly prepared as a concentrated solution in either ethanol (nifedipine, verapamil and compound D-600) or 0.9% NaCl. Controls involved the addition of ethanol or saline where appropriate.

Results

Activation by Ca^{2+}

The effects of perfusate Ca^{2+} concentration on the activity ratios of phosphofructokinase and phosphorylase are shown in Fig. 1, together with the activity of phosphofructokinase at 1 mM-ATP and 10 μM -hexose 6-phosphate. The activation of phosphorylase and thus glycogenolysis in heart by high perfusate Ca^{2+} concentrations has been reported by other groups (Friesen *et al.*, 1967; Namm *et al.*, 1968). This phenomenon was also observed in the present studies (Fig. 1). In addition, a marked activation of phosphofructokinase occurred, and this latter activation appeared to be initiated by a lower concentration of Ca^{2+} (3–4 mM) than that causing an effect on phosphorylase (12 mM). Moreover, maximal activation of phosphofructokinase occurred at approx. 12 mM- CaCl_2 , but maximal activation of phosphorylase had not occurred even at 15 mM- CaCl_2 (results not shown). These findings suggest that the mechanism for the activation of phosphofructokinase is more sensitive to the concentration of extracellular Ca^{2+} than that for the activation of phosphorylase.

Fig. 2 shows the time courses for the activation and deactivation of phosphofructokinase and phosphorylase and for the changes in the amplitude and rate of contraction by perfusion medium containing high and low concentrations of CaCl_2 . After introduction of 12 mM- CaCl_2 , there was a rapid increase in the amplitude of contraction (Figs. 2c and 2d). This was complete within the

Fig. 1. Effect of perfusate Ca^{2+} concentration on the activity ratios of phosphofructokinase and phosphorylase

Hearts were pre-perfused for 8 min as described in the Experimental section. After this time the perfusion medium was changed to one containing 0.5 mM-EDTA and the concentrations of Ca^{2+} shown. The hearts were freeze-clamped after 4 min. Supernatants (from centrifugation at 8000g for 30 s) were prepared and assayed for phosphofructokinase (●) at 0.1 mM-ATP and 0.01 mM-hexose 6-phosphate and at 1 mM-ATP and 0.01 mM-hexose 6-phosphate. The ratio of the two activities is shown in (a). Phosphorylase activity ratio, $a/(a+b)$ (○), was determined on supernatants after passage through small columns of Sephadex G-25. The activity of phosphofructokinase at 1 mM-ATP and 0.01 mM-hexose 6-phosphate is shown in (b). Methods are given in the Experimental section. The numbers of hearts are shown in parentheses. Where appropriate, standard errors have been calculated and are represented as bars.

initial 15 s after exposure to 12 mM- CaCl_2 (Fig. 2d). A lag phase of at least 10 s preceded the first detectable change in phosphorylase activity ratio, and then this increased rapidly to reach completion over the next 30 s. Phosphofructokinase was the slowest to respond. The first detectable change in activity ratio occurred 45 s after exposure to 12 mM- CaCl_2 . The activity ratio then increased over the next 60–90 s to reach the maximum value 90–120 s after medium containing 12 mM- CaCl_2 had

Fig. 2. Time course for the effects of perfusate Ca²⁺ concentration on phosphofructokinase, phosphorylase and contraction. Hearts were perfused in a non-recirculating Langendorff manner with medium containing 0.5 mM-EDTA and 3.0 mM-CaCl₂ initially for 10 min and then the time indicated. At 2 min identical medium containing 12 mM-CaCl₂ was introduced. At 7 min the medium was changed back to the one containing 3 mM-CaCl₂ and at 10 min back again to 12 mM-CaCl₂. Hearts were freeze-clamped at the times indicated, and the activity ratios of phosphofructokinase (●) and phosphorylase (○) determined as described in the Experimental section. The rate (□) and amplitude (△) of beating was determined by using a Harvard Apparatus Rotary Motion Transducer; composite curves from four hearts are shown. Panels (d) and (e) show the early time points for the activation and de-activation time courses respectively. The actual times for the change in CaCl₂ concentration at the heart are shown. Each value for phosphofructokinase (PFK, ●) and phosphorylase (PHOS, ○) is the mean of duplicate determinations from a minimum of two hearts.

reached the heart. When the medium was changed back to one containing 3 mM-CaCl₂, the amplitude of contraction and the activity ratio of phosphofructokinase rapidly decreased. De-activation was complete within 30 s. The de-activation of phosphofructokinase corresponded to the decrease in amplitude of contraction (Fig. 2e).

The effect of high extracellular Ca²⁺ concentration on the myocardial concentration of cyclic AMP was also determined (results not shown). Within the 5 min exposure to medium containing 12 mM-CaCl₂, the concentration of cyclic AMP decreased from a control value of 0.30 ± 0.02 nmol/g wet wt. of heart (mean $\pm$ S.E.M. for five hearts) to

Table 1. *Effect of perfusate Ca^{2+} concentration and adrenaline on the activity ratio of phosphofructokinase*

Hearts were pre-perfused for 8 min as described in the Experimental section. After this time the perfusion medium was changed to one containing 0.5 mM-EDTA, saline or adrenaline and the concentrations of Ca^{2+} shown. The hearts were freeze-clamped after 4 min. Supernatants (from centrifugation at 8000g at 30s) were prepared and assayed for phosphofructokinase at 0.1 mM-ATP and 0.01 mM-hexose 6-phosphate and at 1 mM-ATP and 0.01 mM-hexose 6-phosphate. The ratio of the two activities is shown. Means $\pm$ S.E.M. were calculated and the numbers of hearts are given in parentheses.

Concn. of Ca^{2+} (mM)	Addition	Activity ratio of phosphofructokinase
1	Saline	0.24 ± 0.02 (11)
1	10 nM-Adrenaline	0.41 ± 0.01 (3)
2	Saline	0.24 ± 0.05 (3)
2	10 nM-Adrenaline	0.41 ± 0.03 (3)
3	Saline	0.25 ± 0.04 (11)
3	10 nM-Adrenaline	0.39 ± 0.07 (5)
3	0.1 μM -Adrenaline	0.47 ± 0.03 (6)
5	Saline	0.34 ± 0.05 (4)
5	10 nM-Adrenaline	0.40 ± 0.03 (3)
5	0.1 μM -Adrenaline	0.49 ± 0.01 (3)
7	Saline	0.44 ± 0.01 (4)
7	10 nM-Adrenaline	0.48 ± 0.01 (3)
7	0.1 μM -Adrenaline	0.50 ± 0.03 (3)

zero and did not increase back to control values when Ca^{2+} concentration was returned to 3 mM. The decrease in cyclic AMP elicited by high Ca^{2+} concentration was similar to that previously noted by Namm *et al.* (1968).

The mechanism whereby elevated extracellular Ca^{2+} concentration activates phosphorylase is believed to involve an initial activation of phosphorylase *b* kinase, a Ca^{2+} -dependent protein kinase (Ozawa & Ebashi, 1967; Krebs *et al.*, 1968; Brostrom *et al.*, 1971). If extracts of heart are assayed for phosphorylase kinase after perfusions with different concentrations of Ca^{2+} , there is no permanent change; this is presumed to occur because the Ca^{2+} effect is diluted out when the extract is prepared. However, if Ca^{2+} is added directly to the purified muscle, enzyme activation occurs. To test whether Ca^{2+} directly activates extract phosphofructokinase, various concentrations of Ca^{2+} were added to extracts (1:10) of control hearts. It was found (results not shown) that Ca^{2+} (0–1.5 mM) had no effect on the activity ratio of phosphofructokinase. These observations were consistent with our previous data (Patten *et al.*, 1982), suggesting that direct interaction between Ca^{2+} and phosphofructokinase was unlikely.

Addition of Ca^{2+} plus adrenaline

The proposition that adrenergic activation of phosphofructokinase involves increasing the movement of Ca^{2+} from outside to the interior of the cell was investigated by assessing the possible additivity of Ca^{2+} and adrenaline. Table 1 shows that the activation produced by 10 nM- (and 100 nM-)adrenaline was not additive to that pro-

Fig. 3. *Effect of nifedipine on the activation of phosphofructokinase and phosphorylase by α - and β -agonists* Hearts were pre-perfused for 8 min. After this time the perfusion medium was changed to one containing 0.5 mM-EDTA, 3.0 mM- CaCl_2 and the concentration of nifedipine shown. This perfusion was continued for 3 min, at which time 1 μM -isoprenaline or 1 μM -adrenaline + 10 μM -propranolol were added. The hearts were freeze-clamped 4 min after addition of the adrenergic agonists. Heart extracts were prepared and each was assayed for phosphofructokinase (a) and phosphorylase (b) activities. Experimental details were as given for Fig. 1. The symbols are: phosphofructokinase activity at 1 mM-ATP and 10 μM -hexose 6-phosphate after adrenaline + propranolol ($\square$) and after isoprenaline ($\blacksquare$); phosphofructokinase activity ratio after adrenaline + propranolol ($\circ$) and after isoprenaline ($\bullet$); phosphorylase activity ratio after adrenaline + propranolol ($\triangle$) and after isoprenaline ($\blacktriangle$). Data from a representative experiment of three are shown involving a minimum of two hearts at each concentration of nifedipine.

Fig. 4. Effect of nifedipine on binding of [³H]prazosin to heart membranes

Heart membranes were prepared and incubated with 0.86 nM [³H]prazosin and the concentrations of phentolamine (●), nifedipine (○), or propranolol (△) shown for 30 min at 25°C. Means of duplicate determination are shown. The inset shows specific binding of [³H]prazosin to the heart membranes as a function of prazosin concentration. K_D was estimated to be 0.2 nM.

duced by concentrations of Ca²⁺ up to 7 mM. Thus these findings imply that adrenaline and high perfusate concentrations of Ca²⁺ activate phosphofructokinase by a common mechanism, possibly involving movement of Ca²⁺ across the sarcolemma to the cytosol.

Involvement of Ca²⁺ channels

Ca²⁺ channels for the entry of Ca²⁺ into the cell in response to a membrane signal have been classified into two major types. Potential-dependent channels are activated by membrane depolarization, and receptor-operated channels are associated with, and activated by, specific receptors independent of membrane potential. One approach to assessing the role of potential-dependent channels and the relationship between these and the activation of phosphofructokinase has been to use Ca²⁺-channel blockers. These agents are believed to exert their actions primarily at the potential-dependent Ca²⁺ channels (Triggle, 1981), a conclusion based on the finding that they inhibit the slow inward Ca²⁺ current in heart in the same concentration range at which they produce a negative inotropic effect. In Fig. 3 the effect of varying the concentration of nifedipine on the activation of phosphofructokinase by 1 μM-adrenaline + 10 μM-propranolol and 1 μM-isoprenaline as

well as on the activation of phosphorylase by 1 μM-isoprenaline, is shown. Nifedipine inhibited the α- and β-adrenergic activation of phosphofructokinase, with a half-maximal effect at approx. 0.1 μM. There was no effect of nifedipine on the activation of phosphorylase by isoprenaline (Fig. 3b). Adrenaline + propranolol treatment of the heart did not activate phosphorylase. In addition, two other Ca²⁺-channel blockers were tried (results not shown); methoxyverapamil (compound D-600) and verapamil (1 μM) had no effect on their own (phosphofructokinase activity ratios of 0.19 and 0.21 respectively), but partly prevented the activation of phosphofructokinase by 1 μM-adrenaline + 10 μM-propranolol (from 0.59 to 0.32 and 0.44 respectively).

Because of the possibility that some Ca²⁺-channel blockers may act as α-receptor antagonists [Karlner *et al.* (1982) and references cited therein], it was considered necessary to test whether nifedipine was an α-antagonist. Fig. 4 shows that nifedipine inhibited the binding of [³H]prazosin to heart membranes. However, the concentration of nifedipine at which half-maximal inhibition of α- and β-agonist activation of phosphofructokinase occurred (0.1 μM) had only a marginal effect on the binding of 0.86 nM-prazosin. Nevertheless, the observation that nifedipine inhibited prazosin bind-

Table 2. Effect of K^+ concentration on the activation of phosphofructokinase and phosphorylase by isoprenaline and by adrenaline + propranolol

Hearts were perfused for 10 min in a non-recirculating Langendorff mode with medium containing 0.5 mM-EDTA and 3.0 mM- $CaCl_2$. The medium was then changed to one containing 123 mM-NaCl with 6 mM-KCl, or 107 mM-NaCl with 22 mM-KCl, or 73 mM-NaCl with 56 mM-KCl. Agonists were added to the same medium, and the perfusion continued for a further 4–5 min, at which time the hearts were freeze-clamped. Other experimental details were as given for Fig. 1. Each heart was assayed for phosphofructokinase and phosphorylase activity. Means $\pm$ S.E.M. were calculated and the numbers of hearts are given in parentheses. Abbreviation: n.b. indicates that the hearts were not beating at the time of freeze-clamping.

Perfusate K^+ concn. (mM)	Addition	Activity ratio	
		Phosphofructokinase	Phosphorylase
6	Saline	0.22 ± 0.02 (6)	0.18 ± 0.01 (6)
6	1 μ M-Isoprenaline	0.37 ± 0.04 (6)	0.49 ± 0.03 (6)
6	1 μ M-Adrenaline + 10 μ M-propranolol	0.60 ± 0.06 (5)	0.21 ± 0.02 (4)
22	Saline	0.24 ± 0.01 (5)	0.20 ± 0.03 (4) n.b.
22	1 μ M-Isoprenaline	0.34 ± 0.02 (4)	0.58 ± 0.02 (5)
22	1 μ M-Adrenaline + 10 μ M-propranolol	0.28 ± 0.03 (4)	0.23 ± 0.01 (4) n.b.
56	Saline	0.20 ± 0.03 (6)	0.22 ± 0.01 (6) n.b.
56	1 μ M-Isoprenaline	0.21 ± 0.01 (5)	0.38 ± 0.02 (5) n.b.
56	1 μ M-Adrenaline + 10 μ M-propranolol	0.28 ± 0.02 (5)	0.21 ± 0.03 (5) n.b.

Fig. 5. Effect of nifedipine on the activation of phosphofructokinase and phosphorylase by 9 mM- $CaCl_2$

Hearts were perfused for 8 min in a non-recirculating Langendorff manner with medium containing 0.5 mM-EDTA, 3.0 mM- $CaCl_2$ and the concentration of nifedipine shown. After this time the perfusion medium was changed to one containing 0.5 mM-EDTA, 9 mM- $CaCl_2$ and the same concentration of nifedipine. The hearts were freeze-clamped 4 min after the change to 9 mM- $CaCl_2$. Heart extracts were prepared and assayed for phosphofructokinase and phosphorylase activities. Experimental details were as given for Fig. 1. Means of duplicate determinations of individual hearts are shown. Control values (medium with 3 mM- $CaCl_2$) are also shown (■, □).

ing suggested that the effects of this Ca^{2+} blocker to inhibit isoprenaline- and adrenaline + propranolol-mediated activation of phosphofructokinase (Fig. 3) may in part be due to antagonism at the receptor level. To assess this possibility, the effectiveness of nifedipine to block Ca^{2+} -mediated activation of phosphofructokinase was examined. Fig. 5 shows that this agent completely blocked the activation of phosphofructokinase by 9 mM- Ca^{2+} , with a half-maximal effect at 0.1 μ M. Only marginal activation of phosphorylase occurred at 9 mM- $CaCl_2$, and an effect by nifedipine was difficult to assess. Overall, these findings suggest that a primary effect of nifedipine was to block Ca^{2+} entry into the cell across the sarcolemma. These findings weaken the argument that nifedipine acts predominantly as α -antagonist.

Elevation of extracellular $[K^+]$ above 10 mM depolarizes the myocardial cell membrane and decreases the voltage-dependent fast Na^+ current of the action potential. This is associated with loss of electrical and mechanical activity induced by electrical stimulation. At extracellular $[K^+]$ between 10 and 35 mM, the addition of a catecholamine can restore propagated action potentials and contractile activity by virtue of the activation of a slow inward Ca^{2+} current (see Sperelakis & Schneider, 1976). On this basis two concentrations of K^+ were used. Table 2 show that activation of phosphofructokinase by isoprenaline and by adrenaline + propranolol was significantly decreased as

the concentration of K⁺ was increased. No activation occurred at 56mM-KCl. Isoprenaline restored contractility at 22mM-KCl and activated phosphorylase at both 22mM- and 56mM-KCl, although the effect at 56mM-KCl was less than at either 6mM- or 22mM-KCl (Table 2).

Discussion

The present findings support a role for Ca²⁺ in the mechanism by which adrenaline treatment of the isolated perfused rat heart leads to the activation of phosphofructokinase (Patten *et al.*, 1982). Further, the findings show that high extracellular concentrations of Ca²⁺ (>5mM) can activate phosphofructokinase without the addition of adrenaline. As shown previously by other types of experiments (Patten *et al.*, 1982), there was no evidence for the direct interaction of Ca²⁺ with phosphofructokinase leading to its activation. Thus the addition of Ca²⁺ to cell-free extracts was without effect on the activity of phosphofructokinase. Ca²⁺-dependent reactions of unknown identity are clearly responsible, but these can only be activated with the intact organ.

There are two mechanisms by which adrenaline can promote Ca²⁺ transport across the sarcolemma. The first is that mediated via β -adrenergic receptors, and which is most evident when the β -agonist isoprenaline is used. The β -adrenergic actions of catecholamine and the intracellular messenger, cyclic AMP, alter several aspects of the excitation-contraction process in the myocardium [e.g. see reviews by Tsien (1977) and Katz (1979)], in particular the plateau height and duration of the action potential (Reuter, 1973, 1974). This is achieved by increasing the number of available Ca²⁺ channels (Reuter, 1979; Reuter & Scholz, 1977). Cyclic AMP-dependent phosphorylation of a membrane protein to increase the number of Ca²⁺ channels during each action potential is one possibility (Sperelakis & Schneider, 1976; Reuter & Scholz, 1977; Rinaldi *et al.*, 1981).

The second mechanism that adrenaline may use involves the α -adrenergic receptor, and this appears to be independent of cyclic AMP (Jakobs & Schultz, 1982). Our previous findings (Patten *et al.*, 1982) suggest that this is the predominant mechanism, since the activation of phosphofructokinase by adrenaline (0.5 μ M) treatment of the heart was completely blocked by phenoxybenzamine (0.1–10 μ M) and was unaffected by propranolol (10 μ M). However, the mechanism by which α -adrenergic receptors may promote Ca²⁺ entry into the heart is not yet clear. An effect by the agonists to alter the biophysical and/or biochemical properties seems probable. α -Agonist-mediated alterations of mem-

brane potential, membrane protonation, transmembrane redox systems and phosphoinositol turnover are all possibilities.

We have previously argued that Ca²⁺ is probably not the second messenger for the α -adrenergic receptor mechanism in heart (Patten *et al.*, 1982). This view was based on two observations. First, not all α -agonists (e.g. naphazoline) that activated phosphofructokinase produced any significant effect on the rate or intensity of beating (increases in the intracellular concentration of Ca²⁺ might be expected to alter either or both of these parameters). Naphazoline was one of the weaker α -agonists in terms both of activating phosphofructokinase (Patten *et al.*, 1982) and of increasing glucose uptake by rat heart (Clark *et al.*, 1982c); thus the important effects on intensity of contraction may not have been readily discernible by visual inspection. The second observation was based on known properties of phosphorylase kinase and the changes noted in phosphorylase activation ratio when hearts were treated with α -agonists. Muscle phosphorylase kinase is known to be Ca²⁺-sensitive (Brostrom *et al.*, 1971). Thus α -agonists that alter Ca²⁺ concentration within the cell might also be expected to activate phosphorylase. Ca²⁺ activates phosphorylase of perfused rat hearts (Friesen *et al.*, 1967; Namm *et al.*, 1968), and the small, relatively slow-developing, activation of phosphorylase in hearts treated with phenylephrine + propranolol has been proposed to result possibly from increased Ca²⁺ concentrations within the cell (Osnes & Øye, 1975). As reported by Namm *et al.* (1968) and shown in Figs. 1 and 2, the activation of phosphorylase by 12mM-CaCl₂ represents only 20–25% of that maximally attained by β -agonists and is only transient. Such findings imply that cyclic AMP-dependent mechanisms additional to the phosphorylation and activation of phosphorylase kinase are required to augment and sustain the message for glycogenolysis. The phosphorylation and activation of inhibitor-I, an inhibitor of protein phosphatase-1 (Cohen, 1982), might provide such a mechanism.

Several mechanisms are believed to exist for the transfer of extracellular Ca²⁺ to the cytosol of the heart cell. These include Ca²⁺ channels, which may be potential-dependent or receptor-operated. It is also possible that intracellular Ca²⁺ is mobilized to sustain these Ca²⁺-dependent processes and that the relative extent of use of intracellular and extracellular sources is both tissue- and stimulant-dependent (Bolton, 1979; Putney, 1979; Casteels, 1980; van Breemen *et al.*, 1980). The agents designated as Ca²⁺-channel antagonists apparently exert their actions primarily at the potential-dependent Ca²⁺ channels (Triggle, 1981). These agents inhibit the slow inward Ca²⁺ current in car-

diac tissue in the concentration range at which they produce a negative inotropic effect (Triggle, 1981). Two of the widely used Ca^{2+} antagonists, nifedipine and verapamil, use different mechanisms to produce effects. Nifedipine appears actually to block influx through Ca^{2+} channels. Its effects can be explained as resulting from a decrease in the number of Ca^{2+} channels that can be opened during myocardial depolarization. By contrast, verapamil (and compound D600) alter the kinetics of the slow current. The agents slow down activation and more prominently the recovery from inactivation. If the cell is stimulated early, the channels will not open to allow Ca^{2+} entry. Thus verapamil alters the kinetics of Ca^{2+} turnover, whereas nifedipine does not (Henry, 1980). In the present work, the activation of phosphofructokinase by adrenaline + propranolol, isoprenaline and by high Ca^{2+} concentration were each inhibited by nifedipine. The activation of phosphorylase by isoprenaline was unaffected by nifedipine (Fig. 3) and verapamil (Hess *et al.*, 1975).

The present findings tend to support the argument that influx of Ca^{2+} through nifedipine-sensitive slow channels is required as part of the mechanism by which the α -adrenoceptor (e.g. adrenaline + propranolol) and the β -adrenoceptor (e.g. isoprenaline) lead to the activation of phosphofructokinase. An increase in myoplasmic Ca^{2+} concentration may arise from alterations in the rate of either influx or efflux. If nifedipine acts in the rat heart to block influx of Ca^{2+} into the cell, a constant rate of efflux via the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase Ca^{2+} transporter would lower myoplasmic Ca^{2+} concentration. Thus an effect of adrenergic agonists to increase the myoplasmic Ca^{2+} concentration by promoting influx or by inhibiting efflux of Ca^{2+} would be nullified by nifedipine. Similarly, depolarization of the heart with high concentrations of extracellular K^{+} would prevent opening of the Ca^{2+} channels, and myoplasmic Ca^{2+} concentrations might be expected to decrease. Under these conditions (of high external K^{+}), adrenergic mechanisms that promote influx or inhibit efflux of Ca^{2+} would also have no effect on raising myoplasmic Ca^{2+} concentration. Thus Ca^{2+} -dependent mechanisms would not change (Table 2). However, if adrenergic agents act to inhibit Ca^{2+} efflux, the effects of submaximal concentrations of adrenaline and Ca^{2+} might be expected to be additive. Table 1 shows that this was not the case. Thus the present studies favour, in small part, the proposal that α - and β -adrenergic agents act to promote influx of Ca^{2+} into the cell, which directly, or by a 'trigger' mechanism (Naylor & Grinwald, 1981), leads to an increase in myoplasmic Ca^{2+} concentration. The β -adrenergic activation of phosphorylase does not

require Ca^{2+} and is not inhibited by nifedipine (Fig. 3).

One of the disadvantages with the use of nifedipine and Ca^{2+} -channel blockers has been their property to act as adrenergic-receptor antagonists. Blackmore *et al.* (1979) have shown that verapamil acts predominantly in rat liver as an α -adrenergic antagonist, inhibiting the binding of [^3H]adrenaline to liver plasma membranes. It also blocked adrenaline-mediated activation of phosphorylase and Ca^{2+} efflux in hepatocytes. More recently verapamil has also been shown to inhibit competitively α_1 -adrenergic receptors in rat myocardium (Karliner *et al.*, 1982). There are indeed structural resemblances between verapamil and some of the α -antagonists, notably phenoxybenzamine. Thus the effects of verapamil, which are varied (Blackmore *et al.*, 1979) and all attributable to Ca^{2+} -channel blockade, may be due to its effects as either a Ca^{2+} -channel blocker or an α -antagonist. Since our findings suggest that the myocardial effects of α -agonists involve an initial movement of Ca^{2+} through the Ca^{2+} channels of the sarcolemma, any blocking effect of Ca^{2+} -channel blockers may be misleading. To circumvent this problem our approach has been to use nifedipine. Although binding studies showed that nifedipine displaced prazosin (Fig. 4) from α_1 -binding sites of myocardial membrane preparations in a competitive manner, it blocked not only α -agonist activation of phosphofructokinase but also the activation produced by the β -agonist and by high Ca^{2+} concentration.

In summary, both α - and β -adrenergic mechanisms for the activation of rat heart phosphofructokinase appear to involve movement of Ca^{2+} from outside the cell to the inside via Ca^{2+} channels that can be inhibited by nifedipine. The initial step in the α -adrenergic-receptor-mediated activation of phosphofructokinase may involve promotion of Ca^{2+} transfer across the sarcolemma, although inhibition of Ca^{2+} efflux cannot be dismissed. Intracellular Ca^{2+} -dependent reactions, of unknown identity, are then initiated, leading to the activation of phosphofructokinase.

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