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REVIEW ARTICLE

The role of calcium ions in the mechanism of action of α-adrenergic agonists in rat liver

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Introduction

This Review sets out to describe current information about the action of α-adrenergic agonists in rat liver. The evidence examined supports a mechanism whereby the interaction of α-adrenergic agonists with receptors on the plasma membrane induces a redistribution of cellular calcium which in turn promotes many of the physiological responses of these agonists.

Discrete areas of research relevant for the analysis of α-adrenergic action include the characterization of the hormone receptor, studies on the generation and function of 'second messengers' or 'signals', the examination of cellular calcium redistributions, and the range of physiological responses that are induced by the hormone.

The primary focus of this Review is to examine the role played by the redistribution of calcium in the mechanism of α-adrenergic agonist action in rat liver. Information about receptors for these agonists, about the chemical nature of the transmitted signal, about basal cellular calcium homeostasis and about the hormone-induced physiological responses will be described only briefly. Aspects of this general topic have been discussed in several recent reviews (Exton, 1981, 1982; Williamson *et al.*, 1981; Taylor *et al.*, 1983*a*; Bygrave *et al.*, 1983).

Receptors and messengers for α-adrenergic agonists

α-Receptors have been classified into α_1- and α_2-types on the basis of both agonist (adrenaline > phenylephrine > clonidine) and antagonist (prazosin > yohimbine) potency series (Doxey *et al.*, 1977; Langer, 1977; Starke *et al.*, 1977). More recently, studies involving the partial purification of adrenergic receptors (Guellaen *et al.*, 1979; Graham *et al.*, 1982; Kunos *et al.*, 1983) and structural analyses using specific affinity labels, monoclonal antibodies, auto-antibodies and radiation inactivation, have confirmed the distinct nature of α_1- and α_2-receptors (see Venter & Fraser, 1983). Of these it is the α_1-subclass of receptors that appears to mediate the physiological responses to adrenergic agonists (see El-Refai *et al.*, 1979; Aggerbeck *et al.*, 1980; Hoffman *et al.*, 1980; Tolbert *et al.*, 1980; Prpic *et al.*, 1982; Reinhart *et al.*, 1982*a*,*b*). Some authors have further suggested that the α_1-receptor classification may need to be extended to account for the possibility that these receptors may exist either in precursor form (i.e. uncoupled) or as coupled receptors (El-Refai *et al.*, 1979; Geynet *et al.*, 1981).

It is relevant to point out that much of the currently available information about these receptors has been derived from experiments *in vitro* using membrane fragments enriched in plasma membranes incubated under non-physiological conditions, and takes no account of tissue-related factors (Kenakin, 1983). Thus hormone–receptor interactions in intact cells or tissues may be very different from those employed in binding assays.

To overcome some of these problems we recently have made a direct comparison of hormone–receptor interactions with physiological responses in the intact perfused rat liver (Reinhart *et al.*, 1984*b*). It was shown that adrenaline-induced responses such as glucose output and respiration were associated with the rapid and continuous receptor-mediated uptake of [^{3}H]adrenaline. The presence of a large number of receptors not coupled to the transducing signal was inferred from data showing that the complete inhibition of physiological responses could be correlated with only a partial inhibition of prazosin-sensitive [^{3}H]-adrenaline uptake. Thus it was concluded that most hepatic responses to adrenaline appear to be mediated by a relatively small population of 'coupled' receptors.

The notion that an α_1-receptor-linked transducing signal is generated in or near the plasma membrane following the interaction of the hormone with the α_1-receptor seems to be generally accepted (see Dehaye *et al.*, 1980). While considerable effort currently is being expended on attempts to identify such a signal(s) for α-adrenergic agonists, to date no single proposal has proved to

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be entirely satisfactory. Candidates so far considered include Na^+ ions, redox ratio changes, natural Ca^{2+} ionophores, cyclic GMP and metabolites associated with inositol phospholipid turnover such as diacylglycerol, phosphatidate or arachidonic acid metabolites (see, e.g., Taylor *et al.*, 1983*a*).

Studies examining inositol phospholipid turnover (Jones & Michell, 1978; Michell, 1979; Berridge, 1981; Takai *et al.*, 1981; Exton, 1982; Prpic *et al.*, 1982) led to the postulate that this event was in some way generating a signal that gave rise to the redistribution of cellular Ca^{2+}. However, a major factor hindering acceptance of this theory has been the difficulty of showing that phosphatidylinositol breakdown or resynthesis can occur within the necessary time constraints (i.e. within 1–3s of hormone–receptor binding).

More recent studies indicate that the preoccupation with examining only phosphatidylinositol turnover may have been misplaced and that the initial event following hormone–receptor occupancy may be the breakdown of phospholipids other than phosphatidylinositol. For example, agents such as α-adrenergic agonists, vasopressin and angiotensin, which have been shown to stimulate phosphatidylinositol breakdown in a range of tissues, have been found also to induce the breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate within seconds of hormone administration (see Creba *et al.*, 1983; Berridge *et al.*, 1983; Berridge, 1983, and, for a recent review, Berridge, 1984).

While this seems to be sufficiently rapid to meet the necessary time constraints, the possibility that polyphosphoinositide turnover is a parallel event, and not actually part of any messenger generation, cannot be ruled out (Rhodes *et al.*, 1983). Even so, polyphosphoinositide breakdown (e.g. inositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate) are the strongest candidates yet for the elusive signal, not only for α-adrenergic agonists but also for a range of agents thought to act independently of cyclic AMP. These breakdown products have been implicated as second messengers whose function is the mobilization of intracellular Ca^{2+} pools (Berridge, 1983, 1984). In this regard, very recent work has provided indications that inositol 1,4,5-trisphosphate is able to release Ca^{2+} from non-mitochondrial ATP-dependent pools when applied to permealized hepatocytes (Burgess *et al.*, 1984).

The review of Berridge (1984) draws attention also to the important second messenger role of protein kinase C in cell-surface signal transduction induced by agents that mobilize Ca^{2+} (see Takai *et al.*, 1981; Nishizuka & Takai, 1981; Kishimoto *et al.*, 1980, and for a recent review, Nishizuka, 1984). These latter workers have shown that the diacylglycerol formed from phosphatidylinositol breakdown activates a Ca^{2+}- and phospholipid-sensitive protein kinase C that appears to be involved in signal generation/transduction. In this way it is envisaged (Berridge, 1984; Nishizuka, 1984) that a large number of cellular events are triggered through the combined action of these two parallel signal pathways. Each begins with the hormone-sensitive hydrolysis of phosphatidylinositol 4,5-bisphosphate to give inositol 1,4,5-trisphosphate and diacylglycerol.

Regulation of cellular calcium

In order to evaluate the role of calcium ions in the hepatic action of α-adrenergic agonists, a brief overview of current ideas about the regulation of cellular Ca^{2+} is warranted. Aspects of this topic have been reviewed recently (Denton & McCormack, 1980; Borle, 1981; Williamson *et al.*, 1981; Barritt, 1982; Akerman & Nicholls, 1983).

The total calcium content of liver (approx. 2 μmol/g wet wt; Borle, 1981), is located in discrete cellular compartments (Claret-Berthon *et al.*, 1977; Barritt *et al.*, 1981). The simplest model of cellular calcium regulation considers that calcium is compartmented into four pools located in the extracellular space, the cytoplasm, the mitochondrial matrix and endoplasmic reticulum lumen (see Fig. 1). Within each of these compartments calcium can exist as two main species, either bound to membranes, macromolecules or other ions (Dawson & Hauser, 1970; Williams, 1974), or as the free ion, Ca^{2+}. Although it is Ca^{2+} which plays an important role in the activation of many enzymes, only a small proportion of the total calcium content of any intracellular compartment may be in this form (Borle, 1975; Coll *et al.*, 1982).

The largest pool of cellular calcium, constituting approx. 800nmol/g of liver, is located on the outside of the plasma membrane (Van Rossum, 1970; Claret-Berthon *et al.*, 1977) bound to the proteins and mucopolysaccharides which form the glyocalyx (see Barritt, 1982). In intact liver, a portion of this bound pool of Ca^{2+} is in equilibrium with free Ca^{2+} (approx. 1 mM), giving liver cells access to a relatively large extracellular Ca^{2+} pool (Pool 1 in Fig. 1). The total size of the intracellular calcium pools is more difficult to determine since these measurements generally rely on the fractionation of the tissue or cells, and this may give rise to the artificial redistribution of Ca^{2+} (Reinhart *et al.*, 1984*a*). Nevertheless there is much evidence to indicate that mitochondria and endoplasmic reticulum contain a major proportion of the intracellular Ca^{2+} (reviewed in Bygrave, 1978*a*; Denton & McCormack, 1980; Akerman & Nicholls, 1983).

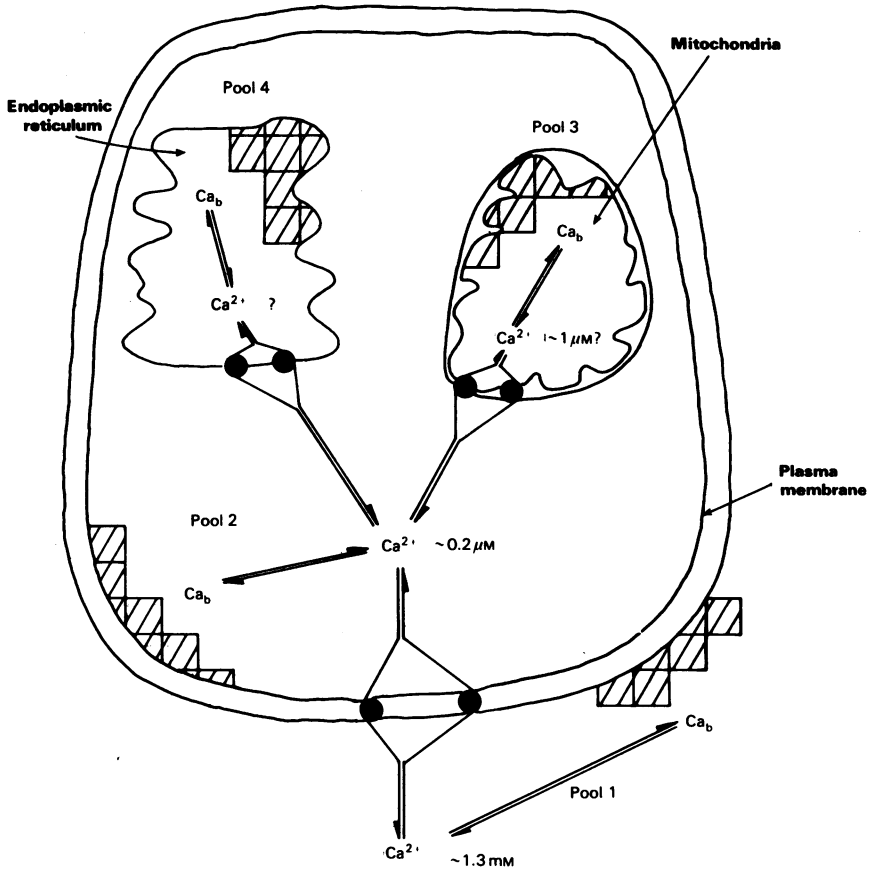

Fig. 1. *Schematic representation of calcium compartmentation in hepatocytes*
The simplest model consistent with current experimental evidence considers four pools of calcium and three Ca^{2+}-translocation cycles. Each pool consists of varying proportions of free (Ca^{2+}) and bound (Ca_b) calcium; bound pools are represented graphically by cross-hatching. Shown are: pool 1, the extracellular space and binding sites on the exterior face of the plasma membrane; pool 2, the cytoplasm and the interior face of the plasma membrane; pool 3, mitochondria; pool 4, the endoplasmic reticulum. Ca^{2+}-translocation cycles may consist of distinct Ca^{2+}-uptake and Ca^{2+}-efflux activities. Further details are discussed in the text.

Considerable interest recently has been focused on the relative contribution of Ca^{2+}-translocation systems located in the plasma membrane, the mitochondrial membrane, and the endoplasmic reticulum, to the regulation of intracellular Ca^{2+} (Becker *et al.*, 1980; Denton & McCormack, 1980; Joseph *et al.*, 1983; Nicholls & Akerman, 1982; Reinhart *et al.*, 1984*a*,*c*,*d*). A model implicating mitochondria in the regulation of Ca^{2+} in the cytoplasmic compartment was first proposed by Drahota *et al.* (1965), and has since been extended by numerous workers (Claret-Berthon *et al.*, 1977; Bygrave, 1978*b*; Nicholls, 1978; Borle, 1981; Coll *et al.*, 1982; Joseph *et al.*, 1983). Consistent with such a model were the findings that mitochondria contain Ca^{2+} transporters for both Ca^{2+} uptake and Ca^{2+} efflux, resulting in the generation of a Ca^{2+}-'translocation cycle' across the mitochondrial inner membrane (see Bygrave, 1978*b*; Nicholls & Akerman, 1982) and that this cycle resulted in the buffering of the extramitochondrial Ca^{2+} concentration at values close to those thought to exist in the cytoplasm (Nicholls, 1978; Becker *et al.*, 1980; Joseph *et al.*, 1983).

Recent data indicate that the ability of mitochondria to buffer cytosolic Ca^{2+} may be dependent on the total mitochondrial calcium content. Isolated mitochondria containing less than 10 nmol of calcium/mg of protein buffer the extramitochondrial Ca^{2+} concentration poorly (Joseph *et al.*, 1983). This incomplete buffering is believed to be due to an increase in the rate of mitochondrial Ca^{2+} efflux, in parallel with increased total calcium content (Joseph *et al.*, 1983; Nicholls &

Akerman, 1982). The finding that the free mitochondrial Ca^{2+} concentration increases linearly as a function of the total calcium content *in vitro* (Coll *et al.*, 1982) is consistent with such a proposal. Hence to buffer the cytoplasmic Ca^{2+} concentration effectively, mitochondria *in vivo* need to contain sufficient total calcium to maintain the rate of the Ca^{2+}-efflux pathway constant for a range of total calcium contents. A recent re-examination of the total calcium content in mitochondria (Reinhart *et al.*, 1984*a*) by using a rapid, single-step isolation procedure (Reinhart *et al.*, 1982*c*), showed that large movements of Ca^{2+} can occur during isolation of the organelle, and that inhibitors of mitochondrial Ca^{2+} fluxes, such as Ruthenium Red and nupercaine, are only partially effective in preventing these movements. Only by adjusting the Ca^{2+} concentration of all isolation media close to the mitochondrial 'set point' were these Ca^{2+} movements minimized. Mitochondria isolated under these conditions, i.e. those approximating to the state *in vivo*, may contain only 2nmol/mg of protein. This finding is not consistent with the view that these organelles play a predominant role in regulating cytosolic Ca^{2+}. However, it should be stressed that the relationship between the rate of Ca^{2+} efflux and the total calcium content *in vivo* may be significantly different from that *in vitro*, as endogenous Ca^{2+}-complexing ligands, like phosphate, may effectively buffer the matrix free Ca^{2+} concentration, thereby making the rate of Ca^{2+} efflux less dependent on the total calcium content (Akerman & Nicholls, 1983). The role of mitochondria in regulating the cytoplasmic Ca^{2+} concentration therefore remains to be established.

An alternative role for the mitochondrial Ca^{2+}-translocation cycle has been proposed by Denton & McCormack (1980). These authors suggest that this cycle may regulate the matrix free Ca^{2+} concentration, and hence the activity of numerous Ca^{2+}-sensitive, matrix-located enzymes (McCormack & Denton, 1980, 1981; Hansford & Castro, 1981, 1982). Although the Ca^{2+} sensitivity of such enzymes has been documented for both the isolated enzymes, and for intact, uncoupled mitochondria (McCormack & Denton, 1980; Denton *et al.*, 1980) the question whether or not such regulation is operative *in vivo* is still a controversial issue (Hansford, 1981; Coll *et al.*, 1982; Nicholls & Akerman, 1982; Joseph *et al.*, 1983; Reinhart *et al.*, 1984*c*,*d*).

Regulation of matrix enzymes according to the mechanism proposed by Denton & McCormack (1980) could occur if the free Ca^{2+} concentration in this compartment was in the range of 0.1–1.0 μM. Studies *in vitro* by Joseph *et al.* (1983) suggest that the mitochondrial matrix free Ca^{2+} concentration may approximate to 16 μM, based on their finding that the ratio of mitochondrial total calcium to free Ca^{2+} is constant at approx. 7×10^4 for mitochondria containing between 5 and 25nmol of total calcium/mg of protein. However, the more recent findings of Reinhart *et al.* (1984*a*) indicate that mitochondria *in vivo* may contain only 2nmol of total calcium/mg of protein and matrix free Ca^{2+} concentrations may therefore approach 1 μM. Even lower values may arise *in vivo* in the presence of endogenous Ca^{2+}-complexing ligands. Consequently a role for Ca^{2+} in the regulation of matrix enzymes has yet to be resolved.

Regulation of the cytoplasmic Ca^{2+} concentration by non-mitochondrial Ca^{2+}-translocation cycles must also be considered. Vesicles of non-mitochondrial origin may be able to buffer the ambient Ca^{2+} concentration at a value lower than the 'set-point' attained by isolated mitochondria (Becker *et al.*, 1980). Unfortunately neither the orientation of these vesicles nor the intracellular location of this cycle has, as yet, been defined. However the finding that digitonin-treated, intact hepatocytes buffer the ambient Ca^{2+} concentration at a constant value (approx. 1 μM) in either the presence or absence of Ruthenium Red (Coll *et al.*, 1982), indicates that the final cytoplasmic 'set-point' achieved in the absence of a functional plasma membrane Ca^{2+}-translocation cycle may be independent of the mitochondrial Ca^{2+}-transport system.

It should be emphasized that before conclusions concerning the relative role of plasma membrane or endoplasmic reticular Ca^{2+}-translocation cycles can be made, future studies must rigorously establish the purity of subcellular fractions capable of Ca^{2+} translocation. Clearly our understanding of the regulation of basal Ca^{2+} homeostasis is still incomplete and as a consequence hormonal effects on Ca^{2+} homeostasis may be difficult to interpret.

α-Adrenergic agonist-induced redistributions of cellular Ca^{2+}

Evidence implicating calcium

A role for Ca^{2+} in α-adrenergic agonist action was initially inferred from three pieces of data. Firstly, the glycogenolytic effect of α-adrenergic agonists was severely impaired by the prolonged incubation of hepatocytes in media where the extracellular free Ca^{2+} concentration was reduced to micromolar levels (De Wulf & Keppens, 1976; Assimacopoulos-Jeannett *et al.*, 1977; Whitton *et al.*, 1977). Secondly, in a Ca^{2+}-containing medium the ionophore A23187 had been shown to mimic some adrenaline-induced responses (Selinger *et al.*, 1974; De Wulf & Keppens, 1976; Assimacopoulos-Jeannet *et al.*, 1977). Thirdly, it was shown that the

activity of the glycogenolytic enzyme phosphorylase kinase can be regulated by micromolar concentrations of Ca^{2+}, as well as by cyclic AMP (Shimazu & Amakawa, 1975; Khoo & Steinberg, 1975; Van der Werve *et al.*, 1977; Sakai *et al.*, 1979).

These findings led to the formulation of the hypothesis that the glycogenolytic effects of α-adrenergic agonists in rat liver are mediated by an increase in the cytoplasmic Ca^{2+} concentration (De Wulf & Keppens, 1976; Assimacopoulos-Jeannet *et al.*, 1977). Much recent work has been aimed at evaluating this postulate by defining α-adrenergic agonist-induced Ca^{2+} redistributions, particularly in relation to glycogenolysis (see Exton, 1981; Williamson *et al.*, 1981; Bygrave *et al.*, 1983; Taylor *et al.*, 1983*a*).

Measurement of calcium movements

Initial attempts to examine α-agonist-induced Ca^{2+} redistributions were carried out by following the movement of ^{45}Ca added to the extracellular medium (Assimacopoulos-Jeannet *et al.*, 1977; Foden & Randle, 1978). These experiments indicated that α-agonists can stimulate the rate of Ca^{2+} uptake into hepatocytes. However, further studies using hepatocytes prelabelled with ^{45}Ca showed that the rate of ^{45}Ca efflux is also stimulated by adrenaline (Blackmore *et al.*, 1978; Chen *et al.*, 1978; Babcock *et al.*, 1979; Poggioli *et al.*, 1980; Kimura *et al.*, 1982). Hence it is difficult to interpret ^{45}Ca data in terms of net Ca^{2+} movements.

A detailed kinetic analysis of exchangeable Ca^{2+} pools in hepatocytes, performed by Barritt *et al.* (1981), revealed that under steady-state conditions the administration of adrenaline for 15min stimulated both the rate of inflow, exchange, and the loss of ^{45}Ca from hepatocytes. The effects of adrenaline on ^{45}Ca exchange rates were interpreted as indicating a decrease in the size of the mitochondrial plus endoplasmic reticulum calcium pool, and an increase in the cytoplasmic exchangeable calcium pool, mediated by both the inflow of extracellular Ca^{2+} and the release of intracellular Ca^{2+}.

An alternative approach used to define adrenaline-induced Ca^{2+} redistributions is the determination of net movements of Ca^{2+} across the plasma membrane of liver cells or intact tissue. Using either atomic absorption spectroscopy (Blackmore *et al.*, 1978, 1979) or Ca^{2+}-sensitive electrodes (Chen *et al.*, 1978; Althaus-Salzmann *et al.*, 1980; Sies *et al.*, 1981) to measure Ca^{2+} movements continuously, it was shown that adrenaline induced a net loss of Ca^{2+} from hepatocytes, apparently ruling out a contribution of extracellular Ca^{2+} in the mediation of adrenaline-induced responses. A major problem in measuring net Ca^{2+} fluxes is that small and transient net Ca^{2+} movements are difficult to detect. Previous workers have overcome this problem by lowering the extracellular Ca^{2+} concentration to between 5 and 50 μM (Blackmore *et al.*, 1978, 1979; Althaus-Saltzmann *et al.*, 1980; Sies *et al.*, 1981). However, such non-physiological conditions have been shown to alter significantly adrenaline-induced Ca^{2+} fluxes (Barritt *et al.*, 1981) and physiological responses (Reinhart *et al.*, 1984*c*).

The determination of adrenaline-induced net Ca^{2+} movements in intact liver perfused with media containing 1.3mM added Ca^{2+} was made possible by the development of a sensitive Ca^{2+}-electrode technique (Reinhart *et al.*, 1982*b*). A resolution of ± 1 μM-Ca^{2+}, against a background of 1.3mM-Ca^{2+}, was achieved by coupling the electrode output to a microprocessor-ion analyser linked in turn to a computing integrator through a bucking voltage device similar to that described by Madeira (1975). Using this approach it was shown that α-adrenergic agonists rapidly stimulate the transient efflux of Ca^{2+} from the liver. The onset was rapid, occurring at approx. 4–7s after phenylephrine administration; maximal rates of efflux were observed at 35–45s of treatment, thereafter declining to be no longer detectable after 2–3min. The total amount of Ca^{2+} lost (approx. 120nmol of Ca^{2+}/g of liver; Table 1) constitutes between 5 and 10% of total cellular calcium. Within seconds of terminating infusion of the α-agonist, Ca^{+} was rapidly accumulated, the total amount of Ca^{2+} entering the liver being similar to the amount initially lost. The re-administration of phenylephrine resulted in another cycle of Ca^{2+} efflux which was again followed by Ca^{2+} uptake. This process can be repeated more than 20 times without any decrease in the rate of Ca^{2+} efflux or the stimulation of glycogenolysis. Hence even at 1.3mM-Ca^{2+}, adrenaline initially stimulates the net efflux of Ca^{2+} rather than uptake.

When these experiments were repeated in the presence of only approx. 6 μM-Ca^{2+} in the perfusage (Reinhart *et al.*, 1982*b*), it was found that during the first administration of phenylephrine, neither the rate nor the amount of Ca^{2+} efflux is diminished by reducing the perfusate Ca^{2+} concentration. However, after removal of the α-agonist no re-uptake of Ca^{2+} can be detected. Further administrations of the hormone under these conditions result in much reduced rates of Ca^{2+} efflux until by the fifth 'pulse' of phenylephrine very little Ca^{+} efflux can be detected. Hence the repeated administration of α-adrenergic agonists to liver perfused with media containing only approx. 6 μM-Ca^{2+}, together with sensitive Ca^{2+} electrode measurements, represents a power-

Table 1. *α-Adrenergic agonist-sensitive pool of intracellular calcium*

Total pool size	160nmol/g of liver
Amount mobilized by a single administration of α-agonist	120nmol/g of liver
Source	(*a*) Mitochondria (70nmol/g of liver) (*b*) Plasma membrane/endoplasmic reticulum (?) (50nmol/g of liver)
State	Probably bound calcium
Temporal details of mobilization	(*a*) Onset 4–7s* (1s?)† (*b*) Maximal rate 45s (*c*) Complete 180s
Maximal rate of efflux	120nmol/min per g of liver
Refilled by	Extracellular calcium
Depleted by	(*a*) Repeated α-adrenergic administrations in media containing only μM Ca^{2+} concentrations (90–95%) (*b*) EGTA + A23187 (80–90%) (*c*) Antimycin A (10–50%)

* Measured by Ca^{2+} electrode in perfused liver (Reinhart *et al.*, 1982*b*).
† Measured by fluorescence spectrofluorometry with quin2-loaded hepatocytes (Charest *et al.*, 1983).

ful approach to deplete selectively the hormone-sensitive pool of intracellular Ca^{2+}. By quantifying the total amount of Ca^{2+} efflux the size of the hormone-sensitive pool of intracellular Ca^{2+} can be assessed (Table 1). Furthermore, an assessment of the role played by this pool in mediating adrenaline-induced responses can be made.

Characterization of the intracellular pool of Ca^{2+}

Although many properties of the hormone-sensitive pool of Ca^{2+} have been determined (see Table 1), considerable controversy still exists with respect to the intracellular location of this pool. In large part this appears to be related to the limitations of currently available experimental approaches. The preincubation of cells or tissue with ^{45}Ca in either the presence or absence of α-adrenergic agonists, followed by cell fractionation and the determination of the ^{45}Ca content in a mitochondrial and/or microsomal pellet (Foden & Randle, 1978; Althaus-Salzmann *et al.*, 1980; Poggioli *et al.*, 1980; Barritt *et al.*, 1981; Berthon *et al.*, 1981; Kimura *et al.*, 1982), has given rise to variable results, possibly due to the redistribution of Ca^{2+} during tissue fractionation.

An alternative approach to identifying the location of the hormone-sensitive pool of intracellular Ca^{2+} is to use atomic absorption spectroscopy to measure the total calcium content in liver fractions isolated before or after α-adrenergic agonist challenge (Babcock *et al.*, 1979; Blackmore *et al.*, 1979; Murphy *et al.*, 1980; Taylor *et al.*, 1980; Reinhart *et al.*, 1982*b*). Although, again, the experimental conditions used by different workers vary significantly, and possibly redistributions of Ca^{2+} during homogenization and fractionation of the tissue cannot be ruled out (Reinhart *et al.*, 1984*a*), all of the studies are in agreement that at least a portion of the hormone-sensitive pool is mitochondrial in origin.

In an effort to minimize such redistributions, a novel fractionation procedure was developed (Reinhart *et al.*, 1982*b*). This procedure allows the complete fractionation of liver homogenate in a single 30s centrifugation step, by utilizing Percoll as an iso-osmotic density-gradient medium. Whereas an earlier conventional fractionation study indicated that phenylephrine decreases the total calcium content of all fractions examined (Blackmore *et al.*, 1979), the rapid-fractionation study indicated that two distinct pools of intracellular Ca^{2+} were being mobilized (Reinhart *et al.*, 1982*b*). Approx. 50% of the total amount of Ca^{2+} mobilized by α-agonists was correlated with a decrease of the mitochondrial calcium content, while the other 50% was derived from a fraction enriched in both plasma-membrane and endoplasmic-reticulum vesicles. Significant decreases in the calcium content of these fractions were observed after 25s of hormone treatment, with near-maximal effects being evident between 45 to 60s of treatment, consistent with the time course of Ca^{2+} efflux detected by Ca^{2+}-electrode measurements in the perfused liver (Reinhart *et al.*, 1982*b*). Although it is impossible totally to rule out Ca^{2+} redistributions during any fractionation study, the above data are consistent with the view that two distinct pools of intracellular calcium are mobilized and that one of these appears to be mitochondrial in origin.

A number of workers have used other approaches, not involving tissue fractionation, to define the hormone-sensitive pool of Ca^{2+} (Chen *et al.*, 1978; Babcock *et al.*, 1979; Barritt *et al.*, 1981;

Blackmore *et al.*, 1982; Kimura *et al.*, 1982; Reinhart *et al.*, 1982*b*; Whiting & Barritt, 1982). Babcock *et al.* (1979) used the fluorescent probe chlortetracycline as an indicator of mitochondrial membrane-bound Ca^{2+} (Luthra & Olson, 1978). Although the data obtained from these studies are consistent with a mitochondrial location of the hormone-sensitive pool of Ca^{2+}, the possibility that in intact cells chlortetracycline fluorescence is in part non-mitochondrial in origin cannot be ruled out.

A number of workers have used inhibitors of mitochondrial function to show that these agents can inhibit the effects of α-adrenergic agonists (Chen *et al.*, 1978; Barritt *et al.*, 1981; Blackmore *et al.*, 1982; Reinhart *et al.*, 1982*a*). Compounds used include the uncoupling agents 2,4-dinitrophenol and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, mitochondrial respiratory chain inhibitors such as amobarbital, antimycin A and rotenone, the mitochondrial ATP synthase inhibitor oligomycin, and the ionophores A23187 and valinomycin. Although the use of ionophores or uncouplers is of little value in identifying the location of the hormone-sensitive pool of Ca^{2+} due to the non-specificity of their action, the use of respiratory chain inhibitors at concentrations similar to those required to inhibit uncoupler-stimulated respiration (Reinhart *et al.*, 1982*a*), is again consistent with a role for mitochondria in α-agonist-induced responses.

Finally, some workers (Althaus-Salzmann *et al.*, 1980; Barritt *et al.*, 1981; Kimura *et al.*, 1982) have attempted to identify the hormone-sensitive pool of Ca^{2+} by correlating the kinetic properties of it with those of one of the kinetically-defined cellular Ca^{2+} pools described by Claret-Berthon *et al.* (1977). Again the data indicate that at least part of the hormone-sensitive pool of Ca^{2+} appears to be mitochondrial in origin. Evidence for a second intracellular pool is increasing, but as yet this has not been clearly defined.

The recent appearance of papers suggesting that α-adrenergic agonists mobilize a non-mitochondrial pool of Ca^{2+} from permeabilized hepatocytes (Burgess *et al.*, 1984; Shears & Kirk, 1984) indicates that the question of the source of intracellular Ca^{2+} is far from resolved.

Role of both intracellular and extracellular Ca^{2+} pools: plasma membrane Ca^{2+}-cycling

Although much interest has been focused on defining the intracellular pools of hormone-sensitive Ca^{2+}, the role of extracellular Ca^{2+} has received little attention. This is due, in part, to observations that the brief removal of extracellular Ca^{2+} does not inhibit the effect of α-adrenergic agonists on phosphorylase activation, Ca^{2+} release, glucose output or mitochondrial respiration (Blackmore *et al.*, 1979, 1982; Reinhart *et al.*, 1982*b*, 1984*c*). Hence, it has been suggested that the mediation of α-adrenergic responses does not require extracellular Ca^{2+} (Blackmore *et al.*, 1982).

The role of extracellular Ca^{2+} has recently been re-examined. By using a combination of ^{45}Ca and Ca^{2+}-electrode techniques it was shown that Ca^{2+} movements across the plasma membrane are regulated by distinct Ca^{2+}-uptake and Ca^{2+}-efflux activities (Reinhart *et al.*, 1984*d*). These activities lead to the generation of a Ca^{2+}-translocation cycle, net Ca^{2+} movements being regulated by the ratio of Ca^{2+} uptake to Ca^{2+} efflux.

α-Adrenergic agonists were shown to alter the activities of both the rate of Ca^{2+} uptake and efflux in a time-dependent manner. The initial effect, occurring within 7s of hormone administration, is an increase in the rate of Ca^{2+} efflux from liver, presumably in response to the mobilization of intracellular Ca^{2+} within 1–2s (Charest *et al.*, 1983). While this movement of Ca^{2+} out of the cell is still occurring, the rate of Ca^{2+} entry into the cell increases, leading to an increased rate of Ca^{2+}-cycling across the plasma membrane (Reinhart *et al.*, 1984*d*). Within 3–4min of hormone administration, net Ca^{2+} efflux has ceased while the rate of Ca^{2+}-cycling has reached a new steady state, significantly higher than the basal rate of cycling. This steady state is maintained for the duration of α-agonist administration. Both the basal and hormone-stimulated rates of Ca^{2+}-cycling were shown to be dependent on the extracellular Ca^{2+} concentration. After removal of the α-adrenergic agonist a large transient net uptake of Ca^{2+} into cells is observed, while the rate of cycling is again transiently stimulated. Within 5–7min of hormone removal, net Ca^{2+} movements have ceased, while the rate of Ca^{2+}-cycling has returned towards basal levels.

Hence extracellular Ca^{2+} appears to fulfil two important roles in the α-adrenergic agonist-induced redistribution of cellular Ca^{2+}. Firstly, it allows the maintenance of a significantly higher rate of plasma membrane Ca^{2+}-cycling, and secondly, extracellular Ca^{2+} repletes the hormone-sensitive pool of Ca^{2+} after removal of the α-agonist. The possible significance of plasma membrane Ca^{2+}-cycling in mediating α-adrenergic agonist-induced responses is discussed further below.

The cytoplasmic free Ca^{2+} concentration

The underlying feature of all models of α-adrenergic agonist-induced Ca^{2+} redistributions in liver is that the cytoplasmic free Ca^{2+} concentration increases during α-agonist administration (see Exton, 1981; Williamson *et al.*, 1981; Bygrave *et*

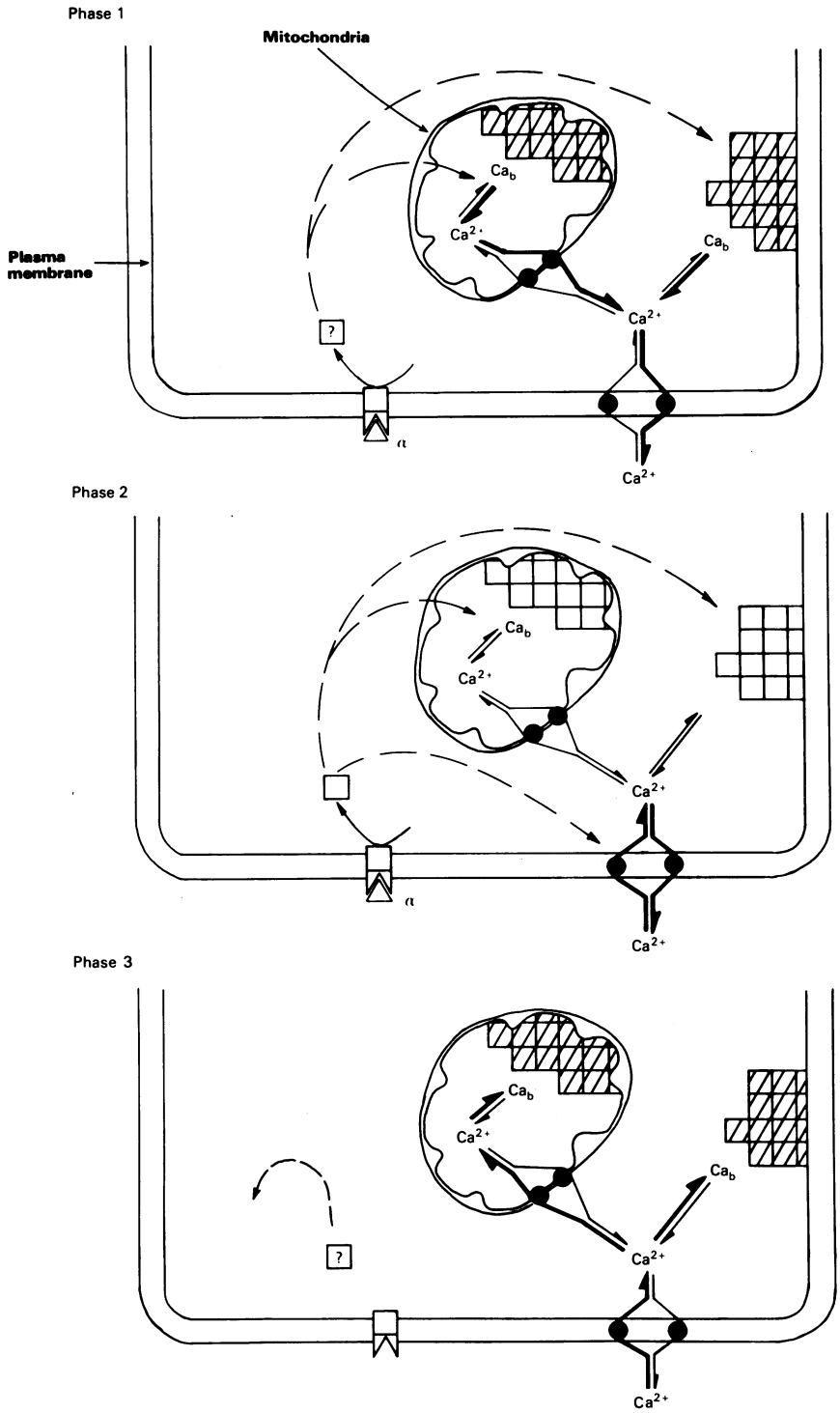

Fig. 2. *Three phases of calcium redistribution induced by α-adrenergic agonists*
Phase 1 is characterized by the rapid mobilization of intracellular, presumably bound calcium (Ca_b) by one or more undefined 'second messengers'. Part of this calcium appears to be derived from the mitochondria, while a second site of mobilization has not been clearly characterized, but may include a plasma membrane or endoplasmic reticular pool (for the sake of simplicity only the former is shown in the Figure). This mobilization may elevate the cytoplasmic Ca^{2+} concentration, stimulate the rate of net Ca^{2+} efflux from the cell, and lead to the depletion of the

al., 1983; Taylor *et al.*, 1983*a*). However, until recently, this has been difficult to determine experimentally due to the lack of suitable techniques. A kinetic analysis of adrenaline-induced Ca^{2+} redistributions revealed that the size of a small intracellular pool was increased (Barritt *et al.*, 1981). However, this pool could not be unequivocally identified as representing cytoplasmic free Ca^{2+}. An alternative approach was adopted by Murphy *et al.* (1980). These workers developed a null-point titration technique whereby hepatocytes were incubated in Ca^{2+}-free medium supplemented with digitonin to permeabilize the plasma membrane. The final free Ca^{2+} concentration attained was assumed to represent the cytoplasmic Ca^{2+} concentration, and it was shown that this concentration does appear to increase transiently from 0.15 μM to 0.45 μM following α-agonist treatment (Murphy *et al.*, 1980).

Recently, the development of a new fluorescent Ca^{2+} indicator, quin2, with high selectivity for Ca^{2+} over Mg^{2+} or H^+ (Tsien, 1980) has allowed the development of a fluorescent technique for cytoplasmic Ca^{2+} determinations (Tsien, 1981; Lew *et al.*, 1982; Tsien *et al.*, 1982*a*,*b*; Pozzan *et al.*, 1982; Hesketh *et al.*, 1983).

The technique has recently been applied to liver by Charest *et al.* (1983), who showed that α-adrenergic agonists can maximally stimulate quin2 fluorescence within 6–10 s of hormone administration. It was concluded that the cytoplasmic Ca^{2+} concentration increases from 0.2 to 0.6 μM in the presence of α-adrenergic agonists.

Current model of α-agonist-induced Ca^{2+} redistributions

The data generated to date give rise to a model of hormone-induced Ca^{2+} redistributions which can be described in three distinct phases (see Fig. 2).

Phase 1. The 'first' phase is characterized by the mobilization of intracellular Ca^{2+}, possibly from two distinct pools. A large proportion of the Ca^{2+} mobilized appears to represent bound Ca^{2+} as judged by the amount of the ion mobilized (see Table 1), and differences between A23187-induced and α-agonist-induced rates of Ca^{2+} efflux (Reinhart *et al.*, 1983). This mobilization may increase the free Ca^{2+} concentration in the cytoplasm (Murphy *et al.*, 1981; Charest *et al.*, 1983) and within other intracellular compartments. Possibly as a result of such an elevation of the cytoplasmic Ca^{2+} concentration, the rate of net Ca^{2+} efflux from the cell is stimulated (Reinhart *et al.*, 1982*b*). As the pool of hormone-sensitive Ca^{2+} is being depleted, net Ca^{2+} efflux diminishes. During this period, the rate of Ca^{2+}-cycling at the plasma membrane increases significantly. Within 3–5 min of hormone administration, Ca^{2+}-cycling has reached a new steady state, while net Ca^{2+} fluxes are no longer detectable (Reinhart *et al.*, 1984*a*).

Phase 2. Ca^{2+} movements during this 'phase' are dependent on the extracellular Ca^{2+} concentration. At concentrations above 100 μM the continued presence of the hormone results in an elevated rate of Ca^{2+}-cycling across the plasma membrane, which may contribute to the maintenance of the elevated cytoplasmic Ca^{2+} concentration. This new steady state appears to be sustained for the duration of hormone administration. During this 'phase' the hormone-sensitive pool of Ca^{2+} remains depleted.

Phase 3. The sequence of Ca^{2+} movements during the third 'phase' is triggered by the termination of α-adrenergic agonist administration. The constraints keeping the hormone-sensitive pool of Ca^{2+} in the depleted state are removed, and the pool refills. The net movement of Ca^{2+} into the cell is transiently stimulated, yet the cytoplasmic Ca^{2+} concentration appears to be returning towards basal values (Reinhart *et al.*, 1982*b*). The rate at which the hormone-sensitive pool of Ca^{2+} is refilled is dependent on the extracellular Ca^{2+} concentration. Hence even at 100 μM extracellular Ca^{2+}, the hormone-sensitive pool can be refilled if sufficient time (7–10 min) is allowed (J. G. Altin, P. H. Reinhart & F. L. Bygrave, unpublished work). At the end of this phase the cell is again in a basal state with respect to Ca^{2+} homeostasis.

Role of Ca^{2+} redistributions in mediating α-adrenergic agonist-induced responses

Ca^{2+}-dependent effects

The role played by α-agonist-induced Ca^{2+} redistributions in mediating hormone responses has only recently been examined in detail (Reinhart *et al.*, 1983, 1984*a*,*b*,*c*,*d*; Taylor *et al.*, 1983*b*). This was made possible by the development of techniques to inhibit the rate of plasma membrane

hormone-sensitive pool of calcium. Phase 2 occurs within 2–3 min of hormone administration, by which time cells have returned to a steady-state with respect to net Ca^{2+} movements. During this phase, Ca^{2+}-cycling across the plasma membrane, and possibly across other membranes, appears to be elevated. This cycling may allow the maintenance of an elevated cytoplasmic Ca^{2+} concentration. Phase 3 is initiated by the removal of α-adrenergic agonists from the extracellular space. This results in the net uptake of Ca^{2+} by the cell, leading to the repletion of the hormone-sensitive pool of calcium.

Ca^{2+}-cycling, and to allow the relatively specific depletion of the small intracellular hormone-sensitive pool of Ca^{2+} (Reinhart *et al.*, 1982*b*, 1984*d*). The Ca^{2+}-dependence of a number of adrenergic responses, including the rate of glucose output, lactate, pyruvate, β-hydroxybutyrate and acetoacetate release, alterations in the mitochondrial and cytoplasmic redox ratios, the rate of mitochondrial respiration, plasma membrane K^+ fluxes, [^{3}H]adrenaline uptake, and gluconeogenesis from a range of substrates, was examined (Reinhart *et al.*, 1982*b*, 1984*b*,*c*,*d*; Taylor *et al.*, 1983*b*). It was found that all responses, except the rate of [^{3}H]adrenaline uptake, were obligatorily dependent on the redistribution of the hormone-sensitive pool of Ca^{2+}. Hence the depletion of this intracellular pool, representing only 5–10% of the total cellular Ca^{2+}, was sufficient to inhibit almost totally α-agonist-induced responses. A further feature revealed by these studies is that phenylephrine-induced responses can be separated into 'transient' and 'sustained' responses.

'Transient' and 'sustained' responses

Transient α-adrenergic agonist-induced responses are characterized by a rapid onset of the response, rising to maximal levels within 30–60s of hormone treatment and followed by a decay of the response towards basal values within the next 3–5min, even though the hormone is continuously being administered (Reinhart *et al.*, 1984*c*). Responses classified as transient include changes in both net Ca^{2+} and K^+ movements (Blackmore *et al.*, 1979; Althaus-Salzmann *et al.*, 1980; Reinhart *et al.*, 1982*b*, 1984c) and alterations in the cytoplasmic and mitochondrial redox ratios (Scholz & Schwabe, 1980; Sugano *et al.*, 1980; Reinhart *et al.*, 1984*c*; Taylor *et al.*, 1983*b*).

Sustained responses also appear to be rapid in onset; however, these responses remain near-maximally stimulated for the duration of hormone administration. Examples of such responses include the adrenaline-stimulated rate of glucose output (Reinhart *et al.*, 1981, 1982*b*, 1984*c*; Kimura *et al.*, 1982), mitochondrial respiration (Jacob & Diem, 1975; Sugano *et al.*, 1978; Scholz & Schwabe, 1980; Reinhart *et al.*, 1981, 1982*a*), glycolysis, gluconeogenesis and ketogenesis (Reinhart *et al.*, 1984*c*; Taylor *et al.*, 1983*b*) and the rate of ^{45}Ca exchange across cellular membranes (Barritt *et al.*, 1981; Reinhart *et al.*, 1984*d*).

These two response types are further characterized by differences in their dependence on cellular Ca^{2+} redistributions. Transient responses appear to be independent of extracellular Ca^{2+}, while being obligatorily dependent on the mobilization of intracellular Ca^{2+} (Reinhart *et al.*, 1984*c*). Sustained responses, on the other hand, are dependent on both extracellular and intracellular Ca^{2+}. In the absence of extracellular Ca^{2+}, adrenaline still maximally stimulates sustained responses, but rather than remaining stimulated, the responses return towards basal values within 3–5min of hormone administration. Hence it appears as though the mobilization of the intracellular hormone-sensitive pool of Ca^{2+} is limited to mediating adrenaline-induced responses only during the first 3–5min of hormone treatment, extracellular Ca^{2+} being required to sustain these responses. Consistent with such a scheme is the finding that re-elevating the extracellular Ca^{2+} concentration to 1.3mM, after the decay of sustained responses, results in the rapid re-activation of these responses (Reinhart *et al.*, 1984*c*). Thus a close relationship may exist between extracellular Ca^{2+}, plasma membrane Ca^{2+}-cycling, and the maintenance of sustained responses. If an increased cytoplasmic free Ca^{2+} concentration does indeed mediate some prolonged effects, then it is tempting to speculate that the plasma membrane Ca^{2+}-translocation cycle can regulate the cytoplasmic Ca^{2+} concentration under these conditions.

Although many adrenaline-induced responses are mediated by the redistribution of cellular Ca^{2+}, in most instances the mechanism or site of action of Ca^{2+} is not known. The hepatic response to α-adrenergic agonists examined in most detail is the glycogenolytic effect, where it is believed that activation of phosphorylase kinase activity is mediated by binding of Ca^{2+} to the calmodulin subunit of the enzyme (Shenolikar *et al.*, 1979; Walsh *et al.*, 1980). The mechanistic basis for numerous other Ca^{2+}-dependent effects stimulated by α-adrenergic agonists has not been established, although a general proposal receiving much current attention is that Ca^{2+}-sensitive protein kinases and phosphatases regulate enzyme activities through cycles of protein phosphorylation and dephosphorylation (see, e.g., Greengard, 1978; Cohen, 1982; Nestler & Greengard, 1983).

The current model of α-adrenergic agonist action in rat liver

From the above it should be obvious that many aspects of α-adrenergic agonist action in rat liver remain to be established. Hence any model of α-agonist action needs to be viewed as a working model only, which will need to be modified as new experimental data comes to hand. Such a 'working model' is depicted in Fig. 3.

Of the two types of α-receptors (α_1- and α_2-) most physiological responses appear to be mediated by a subpopulation of coupled α_1-adrenergic receptors. Binding of the agonist to these receptors is believed

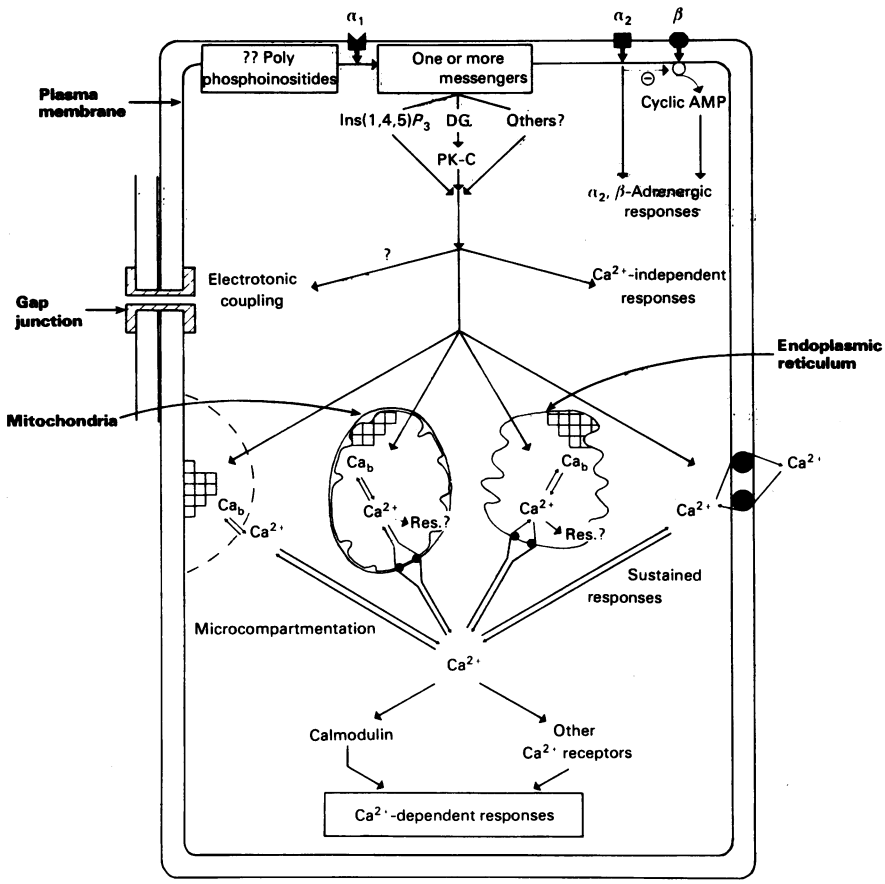

Fig. 3. *A current model for the action of α-adrenergic agonists in rat liver*
Most adrenaline-mediated effects in mature, healthy rats appear to be mediated by coupled α_1-receptors. Hormone–receptor binding may lead to the generation of one or more as yet undefined 'second messengers'. Current evidence indicates that products of polyphosphoinositide hydrolysis such as inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (DG), or protein kinase C (PK-C), may be part of the second messenger system. Under some conditions signal transduction may be by electrotonic coupling through gap junctions. A major response to this messenger(s) appears to involve the redistribution of both intracellular and extracellular calcium, resulting in an elevation of the cytoplasmic Ca^{2+} concentration, and the stimulation of Ca^{2+}-dependent responses. The alteration of the bound/free calcium ratio (Ca_b/Ca^{2+}) within organelles such as mitochondria or the endoplasmic reticulum may induce responses (Res.?) within these organelles. While all transient responses require only the mobilization of intracellular calcium, sustained responses are dependent on the extracellular Ca^{2+} concentration, and the Ca^{2+}-translocation cycle across the plasma membrane of cells.

to induce the generation of one or more 'second messengers', as well as leading to the internalization and metabolism of the hormone. Current candidates fulfilling at least some of the criteria required of 'second messengers' are diacylglycerol, and breakdown products of polyphosphoinositides such as inositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate.

There appear to be at least two intracellular targets for the 'second messenger(s)'. Firstly, a bound pool of intracellular Ca^{2+} is mobilized. At least a portion of this pool appears to represent bound Ca^{2+} in the mitochondrial matrix. This mobilization leads to an increase in the concentration of cytoplasmic Ca^{2+} and the net extrusion of Ca^{2+} from the cell, possibly by a plasma membrane-located Ca^{2+}-sensitive Ca^{2+} pump. Whether or not this mobilization increases the Ca^{2+} concentration in other cellular compartments, such as the mitochondrial matrix and endoplasmic reticulum lumen, has not been clearly defined. Virtually all of the α-adrenergic agonist-mediated effects examined to date appear to be obligatorily dependent on the mobilization of intracellular

Ca^{2+}. Within 3–4min of hormone treatment the intracellular pool of hormone-sensitive Ca^{2+} has become virtually depleted.

The second target for α-agonist induced 'second messenger(s)' appears to be a plasma membrane-located Ca^{2+} gate or channel. Even while net Ca^{2+} is still being extruded, an increase in the Ca^{2+} permeability of the plasma membrane leads to an enhanced rate of Ca^{2+}-cycling. This Ca^{2+}-cycle appears to maintain the elevated cytoplasmic Ca^{2+} concentration, indicating that sustained responses to α-adrenergic agonists are dependent predominantly on extracellular Ca^{2+}.

After removal of α-adrenergic agonists from the extracellular space, a large net movement of Ca^{2+} into the cell occurs, refilling the previously depleted intracellular pool of Ca^{2+}, while the cytoplasmic Ca^{2+} concentration, the rate of plasma membrane Ca^{2+}-cycling and all sustained hormone responses return towards basal values.

While aspects of this 'working model' still require experimental verification, it is hoped that over the next few years significant advances will be made in the identification of α-adrenergic agonist-induced 'second messenger(s)' and the way in which they can induce the redistribution of cellular Ca^{2+}. A further goal is the examination of the mechanism(s) whereby the redistribution of cellular Ca^{2+} induces many α-adrenergic agonist-induced responses.

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