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The effect of ionophore A23187 on calcium ion fluxes and α-adrenergic-agonist action in perfused rat liver

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The effect of ionophore A23187 on cellular Ca^{2+} fluxes, glycogenolysis and respiration was examined in perfused liver. At low extracellular Ca^{2+} concentrations (<4 μM), A23187 induced the mobilization of intracellular Ca^{2+} and stimulated the rate of glycogenolysis and respiration. As the extracellular Ca^{2+} concentration was elevated, biphasic cellular Ca^{2+} fluxes were observed, with Ca^{2+} uptake preceding Ca^{2+} efflux. Under these conditions, both the glycogenolytic response and the respiratory response also became biphasic, allowing the differentiation between the effects of extracellular and intracellular Ca^{2+}. Under all conditions examined the rate of Ca^{2+} efflux induced by A23187 was much slower than the rate of phenylephrine-induced Ca^{2+} efflux, although the net amounts of Ca^{2+} effluxed were similar for both agents. The effect of A23187 on phenylephrine-induced Ca^{2+} fluxes, glycogenolysis and respiration is dependent on the extracellular Ca^{2+} concentration. At concentrations of less than 50 μM-Ca^{2+}, A23187 only partially inhibited α-agonist action, whereas at 1.3 mM-Ca^{2+} almost total inhibition was observed. The action of A23187 at the cellular level is complex, dependent on the experimental conditions used, and shows both differences from and similarities to the hepatic action of α-adrenergic agonists.

The study of Ca^{2+} fluxes across membranes was greatly facilitated by the finding that the carboxylic antibiotic A23187 is a bivalent cation ionophore with a high association constant for Ca^{2+}, catalysing the electroneutral exchange of Ca^{2+} for two protons through the formation of a charge-neutral lipid-soluble complex (Reed & Lardy, 1972; Deber & Pfeiffer, 1976). Although initially used to examine Ca^{2+} fluxes in isolated mitochondria (Reed & Lardy, 1972; Wong *et al.*, 1973; Schuster & Olson, 1974; Sordahl, 1974), A23187 is now extensively employed in more complex experimental systems involving the use of whole cells (Kleineke & Stratman, 1974; Chen *et al.*, 1978; Akerman & Nicholls, 1981; Peaucellier *et al.*, 1982) or tissues (Friedmann *et al.*, 1979; Hoye *et al.*, 1979; Bihler *et al.*, 1980; Sugden, 1980; Hasper *et al.*, 1982).

Although the ionophore has been used extensively in studies with perfused liver (Friedmann *et al.*, 1979), or with hepatocytes (Blackmore *et al.*, 1978; Foden & Randle, 1978; Chen *et al.*, 1978), its effect on the redistribution of cellular Ca^{2+} has not been clearly resolved, with some investigators reporting Ca^{2+} efflux (Kleineke & Stratman, 1974; Chen *et al.*, 1978), whereas others report Ca^{2+} uptake (Friedmann *et al.*, 1979) or biphasic responses with efflux following uptake (Burgess *et al.*, 1979) or uptake following efflux (Blackmore *et al.*, 1978).

The interpretation of results from experiments of this type, however, is often difficult, since, although the basis for the action of A23187 is usually ascribed to the dissipation of the Ca^{2+} gradient across the plasma membrane and hence an elevation in the concentration of cytosolic Ca^{2+}, this has rarely been verified by direct measurement.

Variability may be due also to the particular experimental conditions used by different groups, such as the pretreatment of livers or cells, the Ca^{2+} content in incubation media, the ionophore concentration, the length of time for which cells are exposed to the ionophore and the technique used to assay Ca^{2+} fluxes.

Recently we developed a technique (Reinhart *et al.*, 1982*a*) that allows the continuous and quantitative measurement of Ca^{2+} fluxes in rat liver perfused with Ca^{2+} concentrations ranging from only several μM to 1.3 mM. In the present study we have used this technique to examine the effect of A23187 on the redistribution of Ca^{2+} in the perfused rat liver under a variety of experimental conditions. Additional information about the effects of this

agent on cell metabolism was obtained by monitoring the rate of glycogenolysis and respiration, each of which, in liver, appears to be sensitive to the redistribution of intracellular Ca^{2+} (Khoo & Steinberg, 1975; Shimazu & Amakawa, 1975; Assimacopoulos-Jeannet *et al.*, 1977; Sakai *et al.*, 1979). Furthermore, in light of suggestions that A23187 alters Ca^{2+} fluxes in a manner similar to flux changes induced by α-adrenergic agonists (Whiting & Barritt, 1982), we have compared the ability of these two agents to induce changes in Ca^{2+} fluxes and metabolism in the perfused rat liver.

Experimental

Animals and perfusions

Male Wistar-strain albino rats weighing between 230 and 270g and having free acess to food were used for all experiments. Rats were anaesthetized with sodium pentobarbitone (50mg/kg body wt.), and the livers were perfused with Krebs–Henseleit (1932) bicarbonate medium equilibrated with O_2/CO_2 (19:1) as described previously (Reinhart *et al.*, 1982*b*). Livers were perfused at a flow rate of 3.5ml/min per g wet wt. of liver, at 35°C. All livers were preperfused for at least 10min with medium containing 1.3mM added $CaCl_2$. At 10min, the free Ca^{2+} concentration in the perfusate was altered either by decreasing the concentration of added $CaCl_2$, or by adding low concentrations of EGTA to the perfusate, and 5min later A23187 was administered by infusion syringe at a flow rate of 0.05% of the total flow, at the final concentrations indicated. The ionophore was solubilized in either ethanol or dimethyl sulphoxide. Preliminary experiments had shown that at concentrations above 0.1% dimethyl sulphoxide significantly alters both mitochondrial respiration and the basal rate of glycogenolysis, and ethanol stimulates the rate of respiration (P. H. Reinhart, W. M. Taylor & F. L. Bygrave, unpublished work). Hence with ethanol as solvent corrections were made for minor ethanol-induced respiratory changes. When dimethyl sulphoxide was used, this agent was infused at less than 0.002% (v/v), at which concentration effects on respiration and glycogenolysis were insignificant. All experiments were performed between 08:00h and 12:00h to minimize diurnal fluctuations of basal metabolism.

Perfusate Ca^{2+} determinations

For most experiments the perfusate Ca^{2+} concentration was measured continuously with a Radiometer F2112 Ca^{2+}-selective electrode as described previously (Reinhart *et al.*, 1982*a*). For each experiment the electrode was calibrated by infusing known amounts of $CaCl_2$, at a constant flow rate, between the liver and the electrode. Neither oxygen- nor glucose-concentration changes in the perfusate interacted with the Ca^{2+}-electrode response (results not shown).

The ionophore induced a slight decrease in the Ca^{2+}-electrode baseline signal (results not shown), and all data shown have been corrected for this response. The electrode response to standard $CaCl_2$ solutions was not altered by the presence of A23187 (results not shown). Electrode membranes were discarded if voltage changes deviated more than 15% from theoretical (Nernstian) values. Other control experiments are detailed by Reinhart *et al.* (1982*a*). As the logarithm of the electrode response becomes non-linear at Ca^{2+} concentrations below 1μM, atomic-absorption spectroscopy was used to determine total Ca^{2+} concentration changes in experiments where EGTA was used to lower the Ca^{2+} concentration below 1μM. For this procedure, samples of perfusate (4ml) were assayed in 0.1% KCl with a N_2O/acetylene flame (medical grade gases).

Calculations of free Ca^{2+} concentration

This was done with an algorithm of the program (Comics) developed by Perrin & Sayce (1967). The logarithms of the total formation constants (Sillen & Martell, 1971) for the following complexes were considered: (pH = 7.4): $PO_4^{3-} + H^+$ (11.8), $EGTA^{4-} + Ca^{2+}$ (11.0), $CO_3^{2-} + H^+$ (10.25), $EGTA^{4-} + H^+$ (9.54), $HEGTA^{3-} + H^+$ (8.93), $HPO_4^{2-} + H^+$ (7.15), $HEGTA^{3-} + Ca^{2+}$ (5.3), $EGTA^{4-} + Mg^{2+}$ (5.2), $HEGTA^{3-} + Mg^{2+}$ (3.4), $HCO_3^- + Mg^{2+}$ (3.4), $CO_3^{2-} + Ca^{2+}$ (3.2), $HPO_4^{2-} + Ca^{2+}$ (2.77), $H_2EGTA^{2-} + H^+$ (2.73), $HPO_4^{2-} + Mg^{2+}$ (2.5), $H_2PO_4^- + Ca^{2+}$ (1.5).

Perfusate glucose and oxygen determinations

Glucose release by the liver was determined by the glucose oxidase/peroxidase method as previously described (Reinhart *et al.*, 1982*b*) and perfusion-circuit lag-times were corrected for also as described in that paper.

Chemicals and materials

A23187 was obtained from Calbiochem-Behring Corp., La Jolla, CA, U.S.A. Phenylephrine and the glucose assay kit (510-A) were obtained from Sigma. Ca^{2+}-electrode membranes (F2002) and filling solution S43316 were obtained from Radiometer, Copenhagen, Denmark. Dimethyl sulphoxide was obtained from Ajax Chemicals, Sydney, N.S.W., Australia. Other chemicals used were of analytical grade.

Expression of data

Data are expressed as means ± S.E.M. for the numbers of independent experiments described in the legends to the Figures.

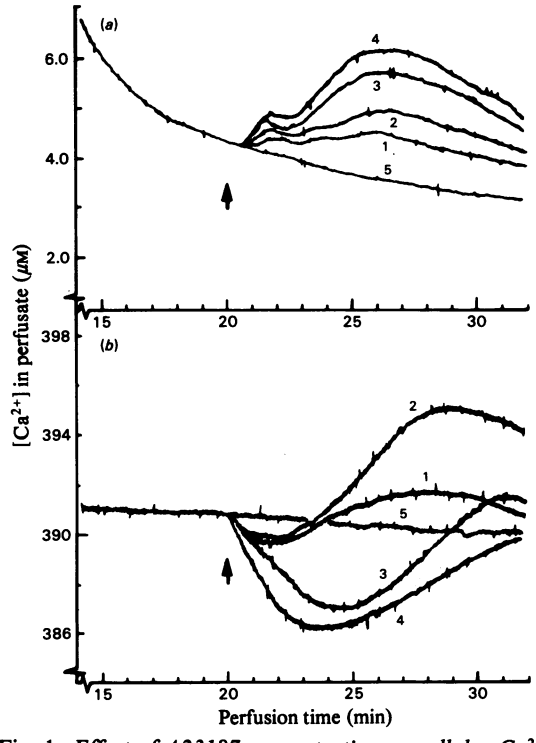

Fig. 1. *Effect of A23187 concentration on cellular Ca^{2+} fluxes*

Livers of fed rats were perfused with medium containing 1.3mM-Ca^{2+} for 12min as described in the Experimental section. Changes in the perfusate Ca^{2+} concentration were measured continuously by using a Ca^{2+} electrode modified for a flow-through mode of operation. At 15min the perfusate Ca^{2+} concentration was decreased to either 4.0μM (*a*) or 390μM (*b*). At 8min thereafter (arrowed), A23187 was infused at final concentrations of 0.25μM (1), 0.5μM (2), 1.0μM (3) or 2.0μM (4). Trace 5 is a recording in which no infusions were performed. Traces shown are typical of those obtained for between 5 and 11 independent experiments performed for each concentration of the ionophore.

Results

Effect of A23187 on cellular Ca^{2+} fluxes, glycogenolysis and respiration

Previous studies with either isolated organelles (Reed & Lardy, 1972) or intact cells (Babcock *et al.*, 1976; Blackmore *et al.*, 1978; Akerman & Nicholls, 1981) have indicated that a number of responses mediated by A23187 show differential sensitivities to either the concentration of the ionophore or the ambient Ca^{2+} concentration. Hence in the present investigation of the action of A23187 in perfused liver we have systematically altered both of these variables.

The data in Fig. 1 show the effects of a range of A23187 concentrations on the net movement of cellular Ca^{2+}. The effects of the ionophore were examined at two different extracellular Ca^{2+} concentrations, firstly to separate predominantly intracellular effects of A23187 from effects owing to the inflow of extracellular Ca^{2+}, and secondly to examine whether or not the A23187 dose–response curve is sensitive to the extracellular Ca^{2+} concentration. With approx. 4.0μM free Ca^{2+} (4.4μM total Ca^{2+}) (Fig. 1*a*) in the perfusate, A23187 induces a net efflux of Ca^{2+} at all ionophore concentrations examined (0.25–2.0μM). A dose of 2μM-A23187 induces a loss of 92 ± 9.7 ($n = 5$)nmol of Ca^{2+}/g of liver, at a maximal rate of 21 ± 4.2 ($n = 5$)nmol of Ca^{2+}/min per g of liver (Fig. 1*a*). The onset of Ca^{2+} efflux is rapid, occurring at 24 ± 3.9s ($n = 4$) after administration, with maximal rates of efflux occurring between 5 and 7min of treatment. The rate of Ca^{2+} efflux remains above control values for at least 10min. At ionophore concentrations below 2μM the rate of Ca^{2+} efflux decreases; however, the efflux response appears to become more prolonged. Hence, although the exact duration of Ca^{2+} efflux is difficult to determine, owing to the low rates of Ca^{2+} efflux involved, the total net loss of Ca^{2+} may be similar for all ionophore concentrations examined. Similar data were obtained when the Ca^{2+} concentrations were increased to 50μM (results not shown).

When the perfusate free Ca^{2+} concentration is increased to 390μM ($\simeq$400μM total Ca^{2+}) (Fig. 1*b*), A23187 induces a different pattern of Ca^{2+}-flux changes. Low concentrations of the ionophore (<0.5μM) induce a slow transient influx of Ca^{2+} lasting between 3 and 4min, and involving the movement of approx. 15nmol of Ca^{2+}/g of liver. This is followed by a more prolonged phase of Ca^{2+} efflux from the liver, similar in magnitude to that observed with higher concentrations of the ionophore at 3.2μM-Ca^{2+} (Fig. 1*a*). When the ionophore concentration is increased to 1μM the initial phase of Ca^{2+} uptake becomes larger and more prolonged, about 40nmol of Ca^{2+}/g of liver entering the cell during the first 5–6min of ionophore administration. This is still followed by a transient efflux of Ca^{2+}; however, the magnitude of this efflux response decreases as the ionophore concentration is increased. Hence at 390μM-Ca^{2+}, a 4-fold change in the concentration of the ionophore is sufficient to reverse what is essentially a Ca^{2+}-efflux response to a Ca^{2+}-uptake response. Results similar to those obtained at 390μM-Ca^{2+} were also observed when the total Ca^{2+} concentration was increased to 1.3mM (results not shown).

A23187-induced changes in hepatic respiration and glycogenolysis were also examined and found to be dependent on both ionophore concentration and

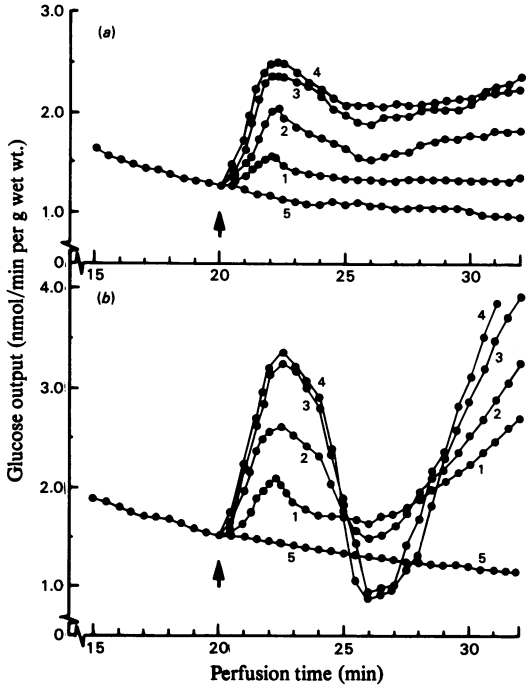

Fig. 2. *Effect of A23187 concentration on the rate of glucose output*

Perfusion details were as described in the legend to Fig. 1. Glucose output was assayed by using glucose oxidase and peroxidase as described in the Experimental section. The extracellular free Ca^{2+} concentration was either 4.0μM (*a*) or 390μM (*b*). Trace numbers are as in the legend to Fig. 1. The arrows indicate the point at which A23187 was infused. Results shown are means for between 5 and 11 independent experiments performed for each concentration of the ionophore; S.E.M. values are omitted for the sake of clarity.

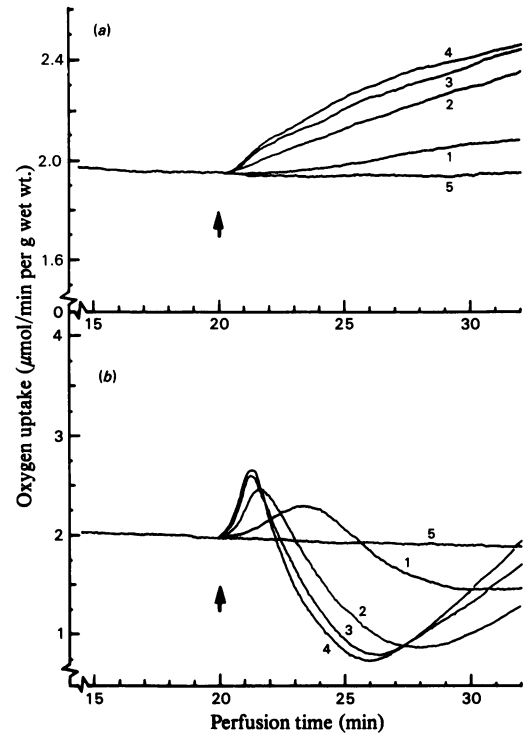

Fig. 3. *Effect of A23187 concentration on the rate of oxygen uptake*

Perfusion details were as described in the legend to Fig. 1. Oxygen uptake was assayed continuously with an oxygen electrode modified for a flow-through mode of operation as described in the Experimental section. The extracellular free Ca^{2+} concentration was either 4.0μM (*a*) or 390μM (*b*). Trace numbers are as in the legend to Fig. 1. The arrows indicate the point at which A23187 was infused. All traces shown have been corrected for the small stimulation of oxygen uptake produced by the ethanol solvent used to solubilize the ionophore. Traces shown are typical of those obtained for between 5 and 11 independent experiments performed for each ionophore concentration.

Ca^{2+} concentration. The data in Fig. 2(*a*) show that when the perfusate Ca^{2+} concentration is 4.0μM all concentrations of A23187 examined significantly stimulate the rate of glucose output, maximal effects being observed at an ionophore concentration of 2μM. Maximal rates of glucose efflux are observed 2–3 min after administration, thereafter declining to a constant rate which is maintained for at least 10 min. At a perfusate Ca^{2+} concentration of 390μM, small changes in the ionophore concentration determine both the qualitative and quantitative metabolic responses (Fig. 2*b*). At 0.5μM-A23187 the stimulation of glycogenolysis is very similar to that observed with maximally effective concentrations of A23187 at 4.0μM-Ca^{2+}. As the ionophore concentration is increased to the maximally effective dose of 2μM, the initial glycogenolytic stimulation increases in magnitude and also becomes more transient.

The data in Fig. 3(*a*) show that oxygen uptake by the liver is only slightly stimulated by A23187 at a perfusate Ca^{2+} concentration of 4.0μM. At a perfusate Ca^{2+} concentration of 390μM (Fig. 3*b*) the respiratory responses were larger, more rapid and biphasic, with the concentration of A23187 again determining both the direction and the magnitude of the response. As the ionophore concentration was increased, the stimulatory response became more transient, and the subsequent inhibitory response more prolonged and larger. At the near-maximal concentration of 2μM-A23187, respiration was

inhibited by more than 50% after 5–6 min of ionophore administration.

In all of the experiments described above, it was found that concentrations of A23187 greater than 2 μM did not induce further changes in any of the parameters studied (results not shown).

Comparison of the ability of A23187 and of phenylephrine to induce Ca^{2+}-flux changes, respiration and glycogenolysis in perfused rat liver

Previously we have shown that α-adrenergic agonists rapidly induce large changes in Ca^{2+} fluxes and metabolism in perfused rat liver (Reinhart *et al.*, 1982*a*,*b*). In light of this, and of reports suggesting that α-adrenergic agonists mobilize intracellular Ca^{2+} pools via an undefined natural Ca^{2+} ionophore (Selinger *et al.*, 1974; Dehaye *et al.*, 1980; Blackmore *et al.*, 1982), we have investigated in more detail the relative abilities of A23187 and of the α-adrenergic agonist, phenylephrine, to induce changes in Ca^{2+} fluxes and metabolism. Since maximal effects of A23187 were observed at 2 μM in the previous experiments, this ionophore concentration was used in all experiments described below. Ca^{2+} concentrations of 50, 100 and 400 μM were used, since (*a*) the responses to A23187 were similar in media containing 50 μM- or 4 μM-Ca^{2+} (cf. Figs. 1*a* and 4*a*) and (*b*) the responses to A23187 were near-maximal in media containing 400 μM-Ca^{2+} (Fig. 1*b*).

Data in Fig. 4(*a*) show that when the extracellular Ca^{2+} concentration is 50 μM, pretreatment with A23187 only slightly inhibits the Ca^{2+} efflux induced by phenylephrine. A point of note is that at this concentration of Ca^{2+} variable results were obtained, in terms of both the net amount of Ca^{2+} mobilized by A23187 and the extent to which the presence of the ionophore inhibited phenylephrine-induced Ca^{2+} efflux.

Increasing the extracellular Ca^{2+} concentration further to 100 μM (Fig. 4*b*) significantly alters the pattern of A23187-induced Ca^{2+} fluxes from one of Ca^{2+} efflux to one of biphasic Ca^{2+} fluxes involving the transient uptake and subsequent efflux of Ca^{2+} by the liver. The amount of Ca^{2+} taken up is small (20 nmol/g of liver), and is followed by a more prolonged phase of Ca^{2+} efflux. At this Ca^{2+} concentration, a subsequent administration of phenylephrine induces a rate and amount of Ca^{2+} efflux clearly less than that observed in the absence of A23187.

As the extracellular Ca^{2+} concentration is elevated to 400 μM (Fig. 4*c*) or 1.3 mM (results not shown), the pattern of ionophore-induced Ca^{2+} fluxes changes from one of biphasic responses to one of predominantly Ca^{2+} uptake, with little (400 μM) or no (1.3 mM-Ca^{2+}) Ca^{2+} efflux. Similarly the rate and amount of efflux induced by phenylephrine decreases as the extracellular Ca^{2+} concentration increases.

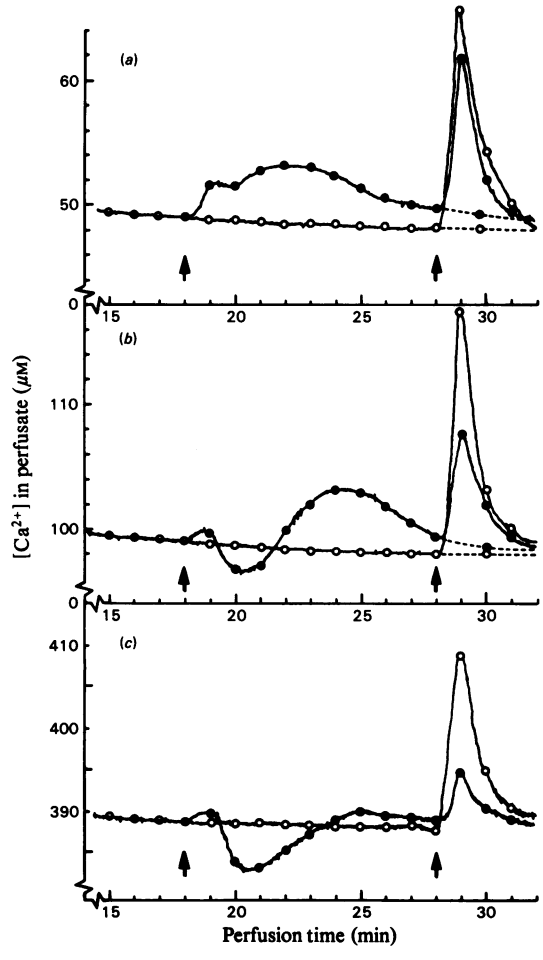

Fig. 4. *Effect of A23187 on phenylephrine-induced cellular Ca^{2+} fluxes at various extracellular Ca^{2+} concentrations*

Livers of fed rats were perfused with medium containing 1.3 mM-Ca^{2+} for 10 min, and perfusate Ca^{2+} was measured continuously, as described in the Experimental section. At 10 min the perfusate Ca^{2+} concentration was decreased to 50 μM (*a*), 100 μM (*b*) or 400 μM (*c*). At 18 min some livers were infused with A23187 (final concn. 2 μM) (●), while others served as controls (○). Then 10 min later phenylephrine (final concn. 2 μM) was infused into some animals, while others served as controls (dashed traces). Traces shown are typical of those obtained for between four and seven independent experiments performed for each condition.

Increases in glycogenolysis and respiration induced by phenylephrine were also inhibited by prior treatment with A23187. The inhibition of phenyl-

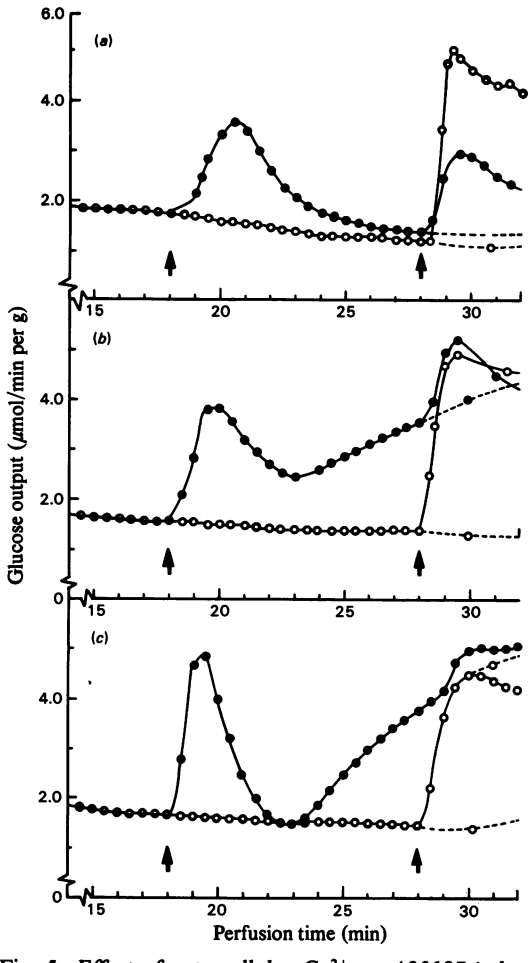

Fig. 5. *Effect of extracellular Ca^{2+} on A23187-induced rates of glucose output*

Perfusion details were as described in the legend to Fig. 4. The perfusate Ca^{2+} concentration was 50μM (*a*), 100μM (*b*) or 400μM (*c*). At 18 min some livers were infused with A23187 (final concn. 2μM) (●), while others served as controls (○). Then 10 min later phenylephrine (final concn. 2μM) was infused into some animals, while others served as controls (dashed traces). The data shown represent means for between four and seven independent experiments performed for each condition.

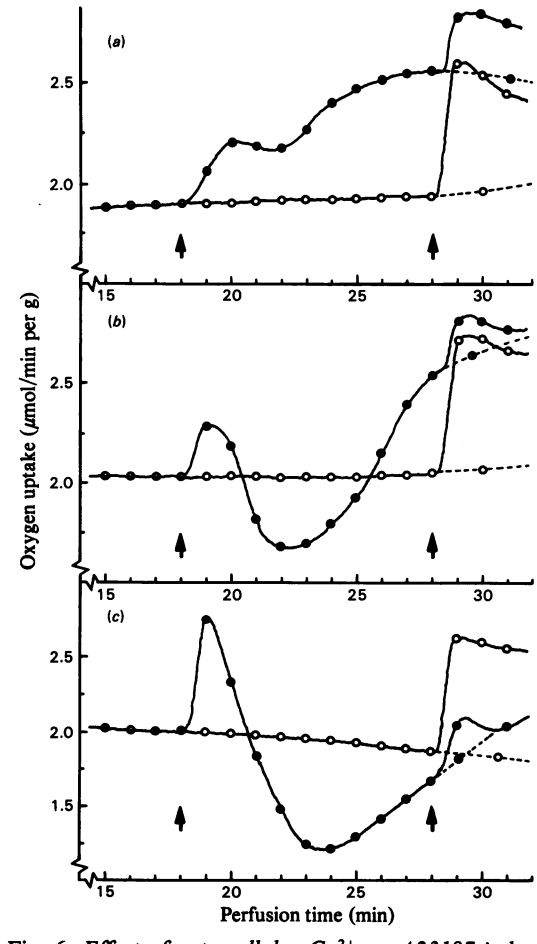

Fig. 6. *Effect of extracellular Ca^{2+} on A23187-induced oxygen uptake*

Perfusion details were as described in the legend to Fig. 4. Oxygen uptake by livers was measured with an oxygen electrode modified for a flow-through mode of operation and described in the Experimental section. The perfusate free Ca^{2+} concentration was 50μM (*a*), 100μM (*b*) or 400μM (*c*). At 18 min some livers were infused with A23187 (final concn. 2μM) (●), while others served as controls (○). Then 10 min later phenylephrine (final concn. 2μM) was infused into some animals, while others served as controls (dashed traces). Traces shown are typical of those obtained for between four and seven independent experiments performed for each condition.

ephrine-induced responses was more severe at higher Ca^{2+} concentrations (Figs. 5 and 6).

Discussion

A major point revealed by this study is that the action of A23187 at the cellular level is both complex and dependent on the particular experimental conditions used. Hence to facilitate the assessment as to whether or not the use of A23187 is justified for a particular experimental regime requires an analysis of how this ionophore alters the cellular Ca^{2+} compartmentation. Such an analysis should at least involve the measurement of both the net cellular Ca^{2+} fluxes and the activity of Ca^{2+}-sensitive

enzymes and pathways in the compartment of interest.

In the present study we have separated those actions of A23187 attributable to the equilibration of Ca^{2+} gradients across intracellular compartments from those attributable to an influx of intracellular Ca^{2+} into the cell. This was achieved by altering the magnitude of the Ca^{2+} gradient across the plasma membrane of liver cells and concomitantly measuring net Ca^{2+} fluxes, glycogenolysis and respiration.

When the extracellular concentration of Ca^{2+} was decreased to approach that thought to exist in the cytoplasm (Murphy *et al.*, 1980), A23187 effectively acted to equilibrate Ca^{2+} gradients across the membranes of intracellular organelles, and, through the action of the plasma-membrane Ca^{2+}-efflux mechanism, induced a net efflux of Ca^{2+} from the cell. At least part of this Ca^{2+} may have originated from the mitochondrial matrix, since (*a*) a significant portion of the total intracellular Ca^{2+} is associated with mitochondria, (*b*) previous work has shown that A23187 induces Ca^{2+} efflux from isolated mitochondria (Reed & Lardy, 1972; Whiting & Barritt, 1982), and (*c*) in hepatocytes the ionophore decreases chlortetracycline fluorescence (Babcock *et al.*, 1979), thought to reflect a decrease in mitochondrial membrane-bound Ca^{2+} (Chandler & Williams, 1978).

During the ionophore-induced Ca^{2+} mobilization the cytoplasmic Ca^{2+} concentration appears to be transiently elevated, as reflected by a stimulation in the rate of glycogenolysis. This finding contrasts with previous reports, indicating that in the absence of extracellular Ca^{2+} A23187 has only a small effect on the activity of phosphorylase *a*, or on the rate of glycogenolysis (Assimacopoulos-Jeanett *et al.*, 1977; Friedmann *et al.*, 1979). This may have been due in part to the depletion of Ca^{2+} from intracellular stores in these experiments, since cells were exposed to Ca^{2+}-free media for extended periods of time. In the present study such a depletion was minimized by using low concentrations of EGTA (25 μM) and decreasing the duration for which livers were exposed to such low-Ca^{2+}-containing media. In contrast with the rapid and transient effects of the ionophore on Ca^{2+} fluxes and the rate of glycogenolysis, the effect on stimulating oxygen uptake was slow and prolonged (Fig. 3*a*). Since the large stimulation of respiration in isolated mitochondria (Reed & Lardy, 1972) has been related to an increase in the rate of Ca^{2+} cycling across the mitochondrial inner membrane (Reed & Lardy, 1972; Heaton & Nicholls, 1976), the tentative conclusion can be drawn that such cycling is not occurring here to any significant extent. An important corollary to this is that increases in the concentration of cytoplasmic Ca^{2+} sufficient to stimulate glycogenolysis and the plasma-membrane Ca^{2+} efflux mechanism are not sufficient to increase significantly the rate of the mitochondrial Ca^{2+} uniporter.

Even in the presence of the significant gradient of Ca^{2+} across the plasma membrane at 50 μM perfusate Ca^{2+}, A23187 is still able to induce the biphasic efflux of Ca^{2+} from the liver associated with a stimulation in the rate of glycogenolysis similar to that observed at 4 μM-Ca^{2+}. Although it is difficult to interpret these findings with any precision, it appears as though the plasma-membrane Ca^{2+}-efflux system has sufficient reserve capacity to counteract the ionophore-induced inflow of Ca^{2+}. Consistent with such a Ca^{2+} cycle is the significantly higher rate of ionophore-stimulated respiration at 50 μM perfusate Ca^{2+} than at 4 μM. Such complications may be partly responsible for some of the divergent results previously obtained with A23187 in liver, since either sub-maximal doses of the ionophore or small changes in the gradient of Ca^{2+} across the plasma membrane may significantly alter the effect of this agent in liver. In hepatocytes such variations may be magnified by the possibility that the prolonged perfusion of livers with Ca^{2+}-free media during the isolation of the cells may induce significant alterations in the distribution of Ca^{2+} within the cells.

At higher extracellular [Ca^{2+}], A23187 induces Ca^{2+} uptake by the liver. Under these conditions both the rate of respiration and that of glucose output are transiently elevated above those rates observed in the absence of a Ca^{2+} gradient across the plasma membrane. At longer times the rates of both responses are significantly inhibited, raising the possibility that some Ca^{2+}-sensitive enzymes are stimulated only within a tightly defined range of Ca^{2+} concentrations, and increasing the Ca^{2+} concentration above some optimum is inhibitory. Alternatively, in these conditions, A23187 may have toxic effects through decreasing ATP in the liver (Friedmann *et al.*, 1979).

A second major point revealed by the present study is the usefulness of ionophores in the study of α-adrenergic action; similarities as well as differences in the way these two agents induce a redistribution of cellular Ca^{2+} were shown to exist. The ionophore-induced rate of Ca^{2+} efflux, and to a lesser extent the amount of Ca^{2+} effluxed, are very much less than that achieved either by α-adrenergic agonists or by other Ca^{2+}-mobilizing hormones (Reinhart *et al.*, 1982*a*). This is consistent with other reports indicating that *in vivo* the gradient of Ca^{2+} across the inner mitochondrial membrane may be as low as 2–20-fold (Denton *et al.*, 1980; Coll *et al.*, 1982; McCormack *et al.*, 1982). Hence even the total equilibration of such a gradient would result in only a relatively low rate and amount of Ca^{2+} efflux from the cell. The result of the short-term Ca^{2+}-depletion experiments reported in the present paper

indicate that only a portion of the hormone-sensitive Ca^{2+} pool is susceptible to mobilization by A23187 at low Ca^{2+} concentrations. Thus only under conditions where the ionophore has induced a large change in Ca^{2+} fluxes across the hepatic plasma membrane are the effects of α-agonists largely inhibited.

In summary, then, it is clear from this work that considerable caution needs to be exercised when studies are to be undertaken with the ionophore A23187, especially when complex systems such as perfused liver are under examination.

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