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TREATMENT OF INTACT HEPATOCYTES WITH THE CALCIUM IONOPHORE A23187 PERTURBS BOTH THE SYNTHESIS AND THE DEGRADATION OF THE SECOND MESSENGER CYCLIC AMP

ACTIONS ON ADENYLATE CYCLASE AND CYCLIC AMP PHOSPHODIESTERASE ACTIVITIES

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Abstract—The presence of the calcium ionophore A23187 augmented glucagon's ability to elevate intracellular cyclic AMP concentrations in intact hepatocytes. However, when the cyclic AMP phosphodiesterase inhibitor 1-isobutyl-3-methylxanthine (IBMX) was added to prevent the degradation of cyclic AMP then the presence of A23187 attenuated the ability of glucagon to increase intracellular cyclic AMP concentrations. Treatment of intact hepatocytes with A23187 led to a dose-dependent persistent inhibition of the glucagon-stimulated adenylate cyclase activity expressed by a membrane fraction isolated from such ionophore-treated hepatocytes. In hepatocytes where glucagon-stimulated adenylate cyclase activity was desensitized then A23187-treatment of hepatocytes failed to exert any inhibitory action on adenylate cyclase. Treatment of isolated membranes directly with A23187 did not elicit any changes in glucagon-stimulated adenylate cyclase activity. Such actions of A23187 were blunted when Ca^{2+} (2.5 mM) was not added to the extracellular medium. It is suggested that treatment of hepatocytes with A23187 leads to the functional uncoupling of glucagon-stimulated adenylate cyclase activity in a manner which appears to mimic the desensitization process. A23187-treatment also exerted an overall inhibitory effect on the cyclic AMP phosphodiesterase activity displayed by intact hepatocytes. Thus treatment of hepatocytes with A23187 exerted a profound effect on cyclic AMP metabolism in these cells.

The Ca^{2+}-ionophore A23187 finds widespread use as a vehicle for manipulating intracellular Ca^{2+} concentrations in attempts to attribute specific cellular functions to changes in free Ca^{2+} concentrations (see e.g. Refs 1–4). However, it has been demonstrated [3, 4] that A23187-treatment of hepatocytes can elicit a rise in the intracellular concentration of the important second messenger diacylglycerol, which will lead to the activation of protein kinase C [5]. Thus, A23187, as well as eliciting direct actions by virtue of altering the intracellular Ca^{2+} concentrations, may also trigger effects through the activation of protein kinase C. However, the source of the diacylglycerol, liberated by the action of A23187, appears to be phosphatidic acid rather than polyphosphatidyl inositol [3]. Thus, in view of the heterogeneity of protein kinase C isoenzymes and their presumed differences in specificity for diacylglycerol species [6], the biological potency of diacylglycerol produced through the action of A23187-treatment remains to be ascertained.

We have demonstrated [7] that hormones which stimulate inositol phospholipid metabolism in hepatocytes, such as vasopressin and angiotensin, can trigger the rapid and transient uncoupling of glucagon-stimulated adenylate cyclase. This leads to a state which is very akin to that seen in glucagon desensitization in hepatocytes. Indeed, glucagon itself appears to mediate the desensitization process by a cyclic AMP independent pathway involving stimulation of inositol phospholipid metabolism [7, 8]. The site of the lesion affected has yet to be ascertained, although it is located at the point of coupling between the glucagon receptor and the stimulatory guanine nucleotide regulatory protein, G_s [7, 8].

In this study we show that treatment of intact hepatocytes with A23187 led to profound alterations in the glucagon-stimulated accumulation of cyclic AMP. The results are consistent with A23187 causing both an inhibition of glucagon-stimulated adenylate cyclase activity, akin to that seen in the desensitized state, and overall attenuation of intracellular cyclic AMP phosphodiesterase activity.

MATERIALS AND METHODS

Bovine serum albumin, theophylline and 1-isobutyl-3-methylxanthine (IBMX) were from Sigma (UK) (Poole, Dorset, U.K.). Creatine kinase,

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phosphocreatine, triethanolamine HCl, cyclic AMP, collagenase, guanine nucleotides, A23187 and all other biochemicals were from Boehringer (UK) Ltd. (Lewes, East Sussex, U.K.). Glucagon was a kind gift from Dr W. W. Bromer, Eli Lilley & Co., Indianapolis, IN. All general biochemicals were of A. R. quality and obtained from BDH Chemicals (Poole, Dorset, U.K.). All radiochemicals were from Amersham International (Amersham, Bucks., U.K.).

Hepatocytes from male Sprague–Dawley rats (200–300 g) were prepared [9] and incubated [7, 8, 10] as described before. The Krebs–Henseleit incubation medium contained 2.5% bovine serum albumin and, where stated, $CaCl_2$ at a final concentration of 2.5 mM.

A membrane fraction was obtained from these hepatocytes as before [11, 12] and used for the assay of adenylate cyclase activity. Briefly, this involved the centrifugation of 1 ml samples of hepatocytes (3–5 mg dry wt/ml) at 14,000 g_{av} for 6 min at 4° in a microcentrifuge. The pellets were resuspended in 150 μl of 1 mM $KHCO_3$ (pH 7.2) and disrupted by repeatedly (×12) syringing them using a 1 ml plastic syringe and 25 G needle. The disrupted pellet was resuspended to 1 ml and recentrifuged again prior to resuspending in 150 μl of $KHCO_3$ (pH 7.2). In some instances a further round of dilution and recentrifugation was applied. This membrane preparation was assayed for adenylate cyclase activity as described by us previously [8, 13]. Briefly, the incubation mixture contained final concentrations of 1.5 mM ATP, 5 mM $MgSO_4$, 10 mM theophylline, 7.4 mg/ml phosphocreatine, 1 mg/ml creatine kinase and 25 mM triethanolamine HCl at a final pH of 7.4. Membranes were present at 1 mg/ml and rates were determined from linear time courses obtained over 10 min incubation at 30°. Measurement of cyclic AMP produced utilized a muscle binding protein assay performed as described by us previously [10, 14]. Intracellular cyclic AMP concentrations were determined as described previously by us [10, 14]. Cyclic AMP phosphodiesterase activity was assayed in the presence of 1 μM cyclic AMP as described before by us [10].

RESULTS

Treatment of intact hepatocytes with glucagon (10 nM) yielded (Fig. 1a) a transient rise in the intracellular concentration of cyclic AMP, as has been shown previously by us [7, 10] and others (see e.g. Refs 15 and 16). If, however, hepatocytes were treated for 5 min with A23187 (5 μM) prior to the addition of glucagon (10 nM), then the ability of glucagon to elevate intracellular cyclic AMP concentrations was dramatically increased (Fig. 1a). Treatment of hepatocytes with A23187 (5 μM) alone had little effect on the "basal" cyclic AMP concentrations over a 20 min period, being 1.6 ± 0.3 at the start and 2.3 ± 0.2 at the end.

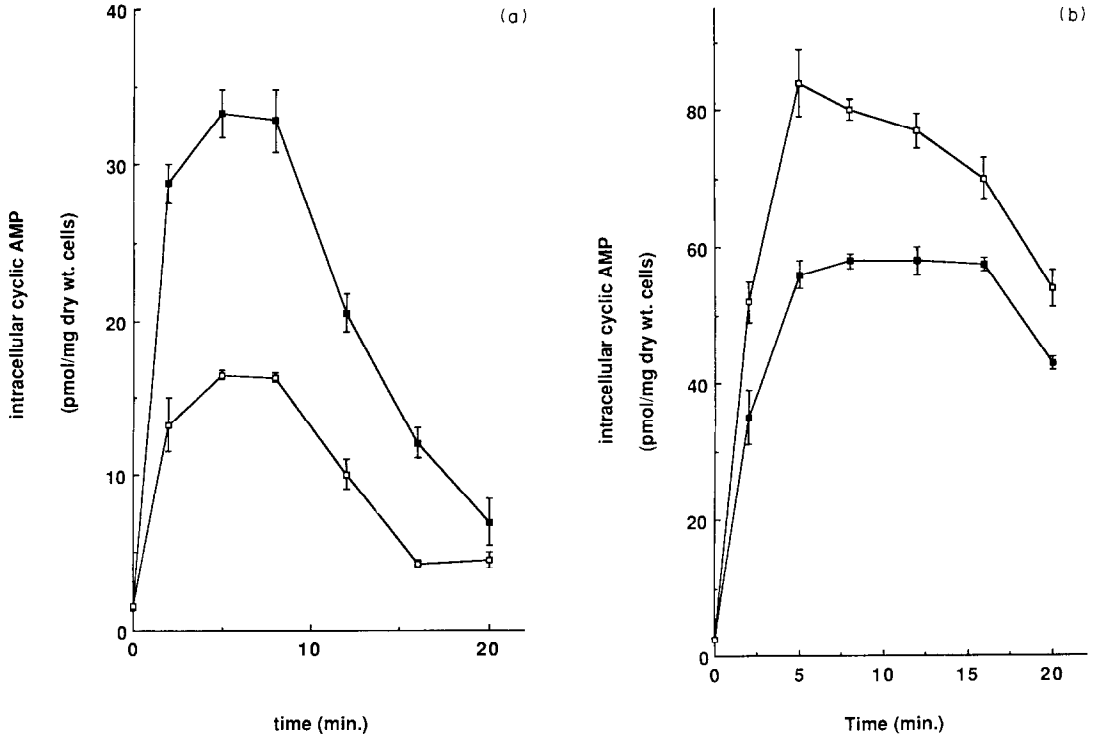

Fig. 1. Action of A23187 on the ability of glucagon to increase intracellular cyclic AMP accumulation in the presence of added external Ca^{2+} (2.5 mM). Cells were challenged with glucagon (10 nM) and then harvested for determination of intracellular cyclic AMP at the shown time intervals. (a) Experiments were performed in the absence of IBMX and either the presence (■) or absence (□) of A23187 (5 μM) added 5 min prior to glucagon. (b) Experiments were performed as in (a) except that IBMX (1 mM) was present. Errors are SD for N = 3 experiments using cell preparations from different animals.

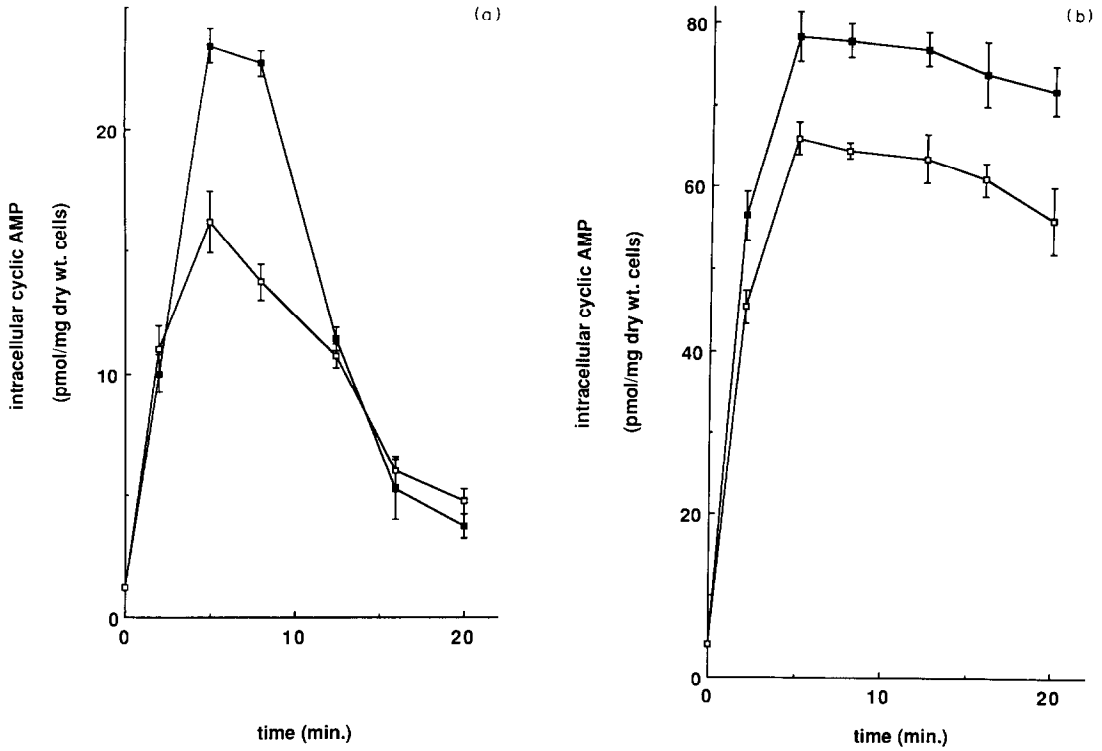

Fig. 2. Action of A23187 on the ability of glucagon to increase intracellular cyclic AMP accumulation in the absence of added Ca^{2+}. (a) Experiments were performed in the absence of IBMX and either the presence (■) or absence (□) of A23187 (5 μM) added 5 min prior to glucagon (10 nM). (b) Experiments were performed as in (a) except that IBMX (1 mM) was present. Errors are SD for N = 3 experiments using cell preparations from different animals.

4-Isobutyl-1-methylxanthine (IBMX) is a non-selective inhibitor of the multiple forms of cyclic AMP phosphodiesterase activity which occur in hepatocytes and at a concentration of 1 mM IBMX has been shown to inhibit over 95% of the cyclic AMP phosphodiesterase activity displayed by hepatocyte homogenate [10, 17]. When measurements of intracellular cyclic AMP concentrations were performed in hepatocytes which had been pre-treated for 15 min with IBMX (1 mM) then, as before [10], we noted an approximate 5-fold enhancement of the rise in cyclic AMP concentrations elicited by glucagon (Fig. 1b). However, under such conditions the addition of ionophore A23187 (5 μM), 5 min prior to the addition of glucagon, now caused a marked attenuation of the ability of glucagon to elevate intracellular cyclic AMP concentrations (Fig. 1b).

IBMX can also act to block R-site adenosine receptors [18, 19]. However, we [20] and others [19] have demonstrated that agonists of such receptors do not affect cyclic AMP accumulation in hepatocytes. Indeed, if the non-methylxanthine cyclic AMP phosphodiesterase inhibitor Ro-20-1724, which does not interact with adenosine receptors (see Ref. 20), was employed to block the degradation of cyclic AMP, then A23187-treatment still attenuated (*ca.* 25–30%) the ability of glucagon to stimulate hepatocyte intracellular cyclic AMP concentrations (data not shown).

In all of our previous studies (see e.g. Refs 7, 8, 10–12), we have routinely incubated intact hepatocytes in the presence of Ca^{2+} (2.5 mM) as described in Ref. 9. The above experiments were thus repeated in the absence of added Ca^{2+}. We noted that the magnitude of the increase in intracellular cyclic AMP concentrations elicited by glucagon and the form of the transient glucagon-mediated elevation in intracellular cyclic AMP exhibited was unaffected whether or not Ca^{2+} (2.5 mM) was added to the external medium (Figs 1a and 2a). Similarly, the absence or presence of added Ca^{2+} (2.5 mM) had little discernible effect on the glucagon-stimulated rise in intracellular cyclic AMP noted in the presence of the cyclic AMP phosphodiesterase inhibitor IBMX (Figs 1b and 2b). Indeed, this could readily be seen in difference plots (Fig. 3) for cyclic AMP accumulation observed in the presence and absence of added exogenous Ca^{2+} (2.5 mM).

Hepatocytes were treated with A23187 (5 μM) for 5 min and then a washed membrane fraction prepared for assay of adenylate cyclase activity. We observed no change in the basal ($-1.5 \pm 0.6\%$) or forskolin-stimulated ($-1.0 \pm 0.7\%$) or NaF (15 mM)-stimulated ($-2.2 \pm 0.5\%$) adenylate cyclase activity SD (N = 4 separated experiments). However, the glucagon-stimulated adenylate cyclase activity was reduced markedly ($-48.0 \pm 4.5\%$; SD, N = 4 separate experiments using cells from different animals). Indeed, A23187-treatment of intact hepa-

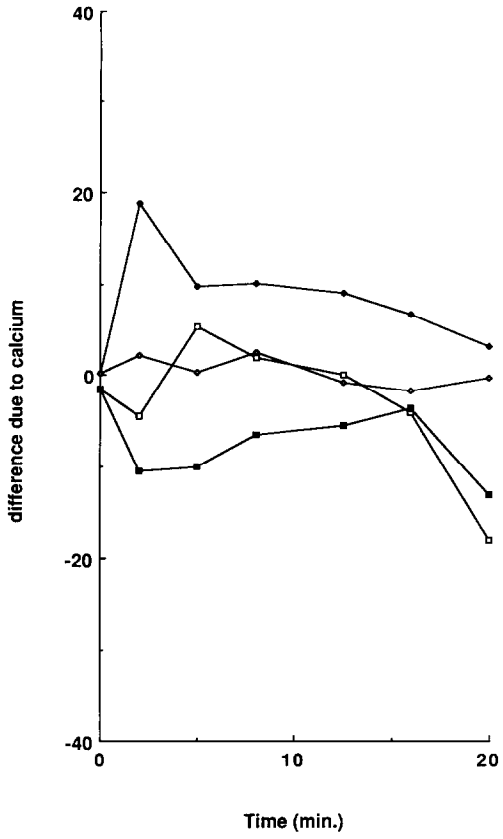

Fig. 3. The effect of elevated external Ca^{2+} on glucagon-stimulated cyclic AMP accumulation. These are difference plots of the intracellular cyclic AMP accumulation noted in the presence and absence of added Ca^{2+} (2.5 mM). Data is presented as (accumulation in presence of Ca^{2+}) − (accumulation in absence of Ca^{2+}). Data are shown for (plus A23187 plus IBMX) = (■), (plus A23187 minus IBMX) = (◆), (no A23187 plus IBMX) = (□) and (no A23187 no IBMX) = (◇) and reflects those given in Figs 1 and 2.

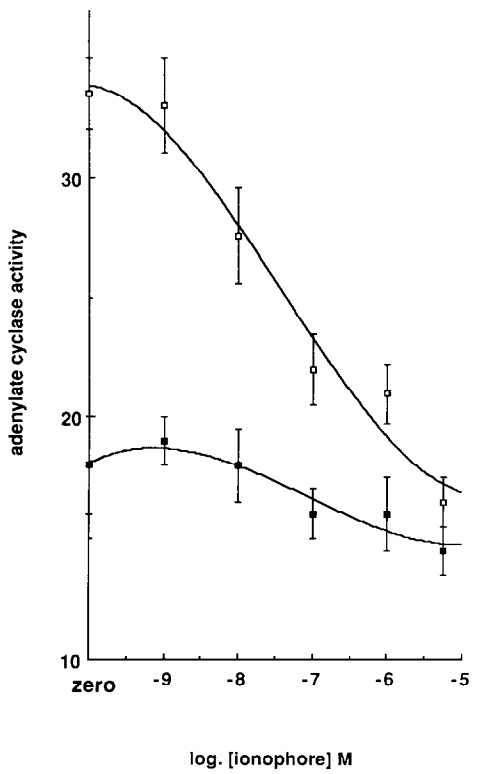

Fig. 4. Treatment of hepatocytes with A23187 causes inhibition of glucagon-stimulated adenylated cyclase. Hepatocytes were treated for 5 min at 37° with increasing concentrations of A23187 prior to harvesting and production of a washed membrane fraction, which was used for the assay of glucagon-stimulated adenylate cyclase activity (□). In some instances, 5 min prior to the addition of A23187, glucagon (10 nM) was added to the cells (■). Data is given with SD for three cell preparations (N = 3) from different animals. There was no significant difference ($P < 0.001$) between the degree of inhibition of membrane glucagon-stimulated adenylate cyclase activity elicited by treatment of hepatocytes with 5 μM-A23187 alone, glucagon (10 nM) alone and glucagon together with 5 μM-A23187. Adenylate cyclase assay were performed in duplicate in each instance.

tocytes caused the inhibition of glucagon-stimulated adenylate cyclase activity in a dose-dependent fashion (Fig. 4). However, if hepatocytes were pretreated with glucagon (10 nM), 5 min prior to exposure to A23187, in order to elicit maximal desensitization [7, 8], then treatment with A23187 did not lead to any further reduction in the glucagon-stimulated adenylate cyclase activity observed in a washed membrane fraction obtained from these cells (Fig. 4). These effects of A23187 on glucagon-stimulated adenylate cyclase were only observed when intact cells were treated with A23187. Thus the treatment of isolated membranes with identical doses of A23187 for periods of 5–10 min did not cause any alterations (less than 5% change) in glucagon-stimulated adenylate cyclase activity.

Intracellular ATP concentrations were not affected (less than 5% change) by the presence of either A23187 (5 μM) or IBMX (1 mM).

The direct addition of A23187 (5 μM) to hepatocyte homogenates did not alter (less than 5%) cyclic AMP phosphodiesterase activity.

Pretreatment of hepatocytes prior to isolation of a washed membrane fraction led to a persistent attenuation of glucagon-stimulated adenylate cyclase activity. This was not affected by varying the dilution of membranes into the assay or by washing the membranes centrifugally, between two and four times.

Maximal effects of Ca^{2+} ionophore on these systems was elicited within 5 min and further pre-incubation up to 10 min did not alter the observations noted here.

DISCUSSION

Glucagon activates adenylate cyclase activity in hepatocytes and causes an increase in the intracellular concentrations of cyclic AMP [10, 15, 16]. This increase, is, however, transient due to the combined effects of desensitization of adenylate cyclase [7, 8, 21] and the activation of the degradative action

of cyclic AMP phosphodiesterases [10, 22, 23]. We have shown [7, 8] that the desensitization of adenylate cyclase is a cyclic AMP-independent process which appears to be elicited through the stimulation of inositol phospholipid metabolism.

We show here (Fig. 1b) that treatment of hepatocytes with the calcium ionophore A23187 inhibited glucagon-stimulated cyclic AMP accumulation measured in the presence of IBMX, which acts to inhibit fully hepatocyte cyclic AMP phosphodiesterase activity [10]. This implies that treatment of intact cells with A23187 led to the inhibition of cellular glucagon-stimulated adenylate cyclase activity.

Elevated Ca^{2+} concentrations have been shown [24] to exert an inhibitory effect on adenylate cyclase by competing with Mg^{2+} both for ATP and by binding to regulatory sites on this hormone-activated enzyme [24]. However, such effects were only evident at high [Ca^{2+}] and, unlike the observations noted here (Fig. 4), were shown to be fully reversible upon washing/dilution of the membranes [24]. Furthermore, treatment of isolated membranes with elevated Ca^{2+} concentrations also rendered a reversible inhibition of fluoride-stimulated adenylate cyclase activity as well as glucagon stimulated adenylate cyclase activity [24]. Such observations contrast strongly then with the pronounced persistent inhibitory effect that is seen solely on the glucagon-stimulated adenylate cyclase activity of membranes prepared from intact hepatocytes which had been treated with A23187. G_s–adenylate cyclase coupling, assessed by stimulation with NaF, was thus unaffected by treatment of hepatocytes with A23187 as was the functioning of the catalytic unit of adenylate cyclase itself. Thus treatment of hepatocytes with A23187 appeared to mimic both the desensitization process than can be elicited by both glucagon [8] and hormones which activate inositol phospholipid metabolism [7]. Certainly, the inability of A23187 to exert a direct action on adenylate cyclase activity displayed by an isolated membrane-fraction and that a washed membrane fraction displaying persistently attenuated glucagon-stimulated adenylate cyclase activity could be isolated from A23187-treated cells would support this conclusion. Indeed, if A23187 was added to hepatocytes subsequent to maximal desensitization being elicited by glucagon then no further inhibitory action ensued (Fig. 4), indicating that identical or complimentary processes were being triggered.

We have suggested [7] that the mechanism by which desensitization is triggered could involve the activation of protein kinase C by the diacylglycerol produced upon stimulation of inositol phospholipid metabolism. This was based upon our observation that treatment of hepatocytes with either hormones that stimulated inositol phospholipid metabolism [7] or with the phorbol ester TPA, which can stimulate protein kinase C [5], could elicit a state where the coupling of the glucagon receptor to G_s was inhibited [25]. However, we did note that chronic stimulation of protein kinase C by TPA also led to the inhibition of adenylate cyclase itself and to loss of coupling between G_s and adenylate cyclase [26]. This contrasts with desensitization, which effects a transient uncoupling of the glucagon receptor from G_s [7, 8]. Indeed, the timecourse of desensitization appears to follow that of the transient rise in diacylglycerol occurring upon receptor-mediated stimulation of inositol phospholipid metabolism in hepatocytes [3, 4, 7]. As it has been shown that treatment of hepatocytes with A23187 causes a rise in diacylglycerol concentration, which is considerably more pronounced than that elicited by either vasopressin or glucagon [3, 4], it is highly likely that the production of this second messenger allows A23187-treatment of intact hepatocytes to mimic the glucagon desensitization process. Of course, as diacylglycerol activates protein kinase C by increasing its affinity for Ca^{2+} [5, 27, 28], it is possible that the elevation of intracellular Ca^{2+}, by A23187-treatment, would also lead to the enhancement of the activity of protein kinase C.

Treatment of rat prostatic cells with A23187 has been shown [29] to block the desensitization of beta-adrenergic desensitization process observed in these cells. However, unlike glucagon desensitization in hepatocytes, in prostatic cells desensitization involved both receptor loss from the cell surface and also a dramatic reduction in G_s–adenylate cyclase coupling. Thus the mechanism(s) of desensitization employed in the prostatic cells appears to be very different from that seen for hepatocytes. Indeed, beta-adrenergic receptor desensitization can occur through both receptor phosphorylation and also a cyclic AMP dependent process [30].

Despite the fact that A23187-treatment of hepatocytes caused the inhibition of adenylate cyclase activity by mimicking the desensitization process, we made the observation (Fig. 1a) that A23187 actually augmented the ability of glucagon to increase intracellular cyclic AMP in the absence of the phosphodiesterase inhibitors IBMX and Ro-20-1724. This suggests that A23187 can also act to block the degradation of cyclic AMP, presumably by exerting an inhibitory effect on cyclic AMP phosphodiesterase activity. Indeed, we have noted that TPA-treatment of hepatocytes exerted a similar effect [31]. A23187 did not act as a direct cyclic AMP phosphodiesterase inhibitor as no inhibition of activity was seen by treating homogenates with this ligand. Presumably, the inhibitory effect seen in intact hepatocytes was also mediated via C-kinase activation, although A23187 can cause a marked rise in free fatty acids [3] which have been shown [32] to inhibit one of the major phosphodiesterases occurring in rat hepatocytes. In any event, such inhibitory actions, elicited by A23187-treatment of intact cells, must suffice to overcome the expected stimulatory effect of increased intracellular Ca^{2+} on the Ca^{2+}-calmodulin activated species of cyclic AMP phosphodiesterase [33] occurring in hepatocytes. However, as the latter enzyme accounts for less than 5% of the high affinity cyclic AMP phosphodiesterase activity in hepatocytes and its activation by Ca^{2+} and calmodulin is small [MDH, B. Lavan, T. Lakey, unpublished], then the role of this enzyme in regulating hepatocyte cyclic AMP metabolism is unlikely to be of much significance.

The ionophore A23187 clearly perturbs profoundly second messenger systems in hepatocytes

as indicated by actions on diacyglycerol production [3, 4] and on cyclic AMP metabolism as shown here. Such studies emphasize the care that needs to be taken in trying to define the molecular basis of changes on cellular function elicited by using the ionophore A23187 to manipulate intracellular Ca^{2+} concentrations.

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