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ROLE OF CALCIUM ION IN HORMONE-STIMULATED LIPOLYSIS

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Abstract—Using the flask-incubated fat cell system, the effects of Ca^{2+} removal from the incubation medium on the lipolytic system were studied. The removal of Ca^{2+} resulted in a total abolition of the lipolytic response and the increased cyclic AMP accumulation produced by ACTH. The lipolytic response to isoproterenol and forskolin were reduced approximately 40% by Ca^{2+} removal, but cyclic AMP accumulation was not altered in the presence of either of these agents using a Ca^{2+}-free medium. The lipolytic response to the dibutyryl analog of cyclic AMP was also reduced by omission of Ca^{2+} from the incubation medium. It is concluded the Ca^{2+} is required for the interaction of ACTH with its receptor and the resultant activation of adenylate cyclase. Ca^{2+} also is required at some step in the lipolytic process distal to cyclic AMP.

Extensive evidence has accumulated to suggest that the hormonal activation of lipolysis in the fat cell is the result of an increase in cyclic AMP levels and activation of the cyclic AMP-dependent protein kinase [1]. The resulting phosphorylation of a hormone-sensitive lipase increases the activity of the enzyme, thus increasing the rate of breakdown of triglycerides into free fatty acids and glycerol [1]. The latter of these two accumulates in direct proportion to the rate of lipolysis.

Although it is recognized that free calcium is involved in hormonal control of lipolysis, the exact nature of this involvement is unclear [2–4]. The activation of adenylate cyclase by ACTH in adipose tissue appears to be a calcium-requiring event [5, 6], and the ability of ACTH to increase lipolytic rates has an almost absolute requirement for the presence of calcium ions [4, 7]. On the other hand, the role of calcium ion in catecholamine-stimulated lipolysis remains unresolved. It has been reported by some investigators [7] that no requirement for calcium ion exists in this process, while other investigators have shown partial but consistent reductions in the lipolytic response to catecholamines in the absence of calcium ion [3, 8, 9].

Even in those investigations suggesting a role of calcium ion in the catecholamine-stimulated lipolysis, the nature of this role is far from clear. Some investigators report a reduced accumulation of cyclic AMP in the absence of calcium ion [10] while other workers suggest that calcium ion exerts an influence on the lipolytic process distal to cyclic AMP accumulation [11].

METHODS

Experiments were carried out on fed, Sprague–Dawley rats weighing 250–350 g. Fat pads were removed and isolated fat cells were prepared by the method of Lech and Calvert [12].

Fat cell incubations. Aliquots of fat cells (approximately 25 μg protein) were placed in polyethylene bottles containing Krebs–Ringer bicarbonate buffer (pH 7.4) with bovine serum albumin (4%, w/v). The final volume was 3.0 ml. In those samples containing calcium chloride, the final concentration was 1 mM. In those samples in which calcium chloride was omitted, ethyleneglycol-bis-(amino-ethyl ether)tetraacetate (EGTA) (1 mM) was added (designated Ca^{2+} free). Isotonicity was maintained with an appropriate amount of sodium chloride. Another aliquot of the fat cells was taken for assay of protein content by the method of Lowry *et al.* [13].

Samples were incubated in a shaking water bath at 37° under an atmosphere of 95% O_2–5% CO_2. Drugs were added and incubations were carried out for 45 min (isoproterenol or forskolin) or 60 min (ACTH) except in the time course experiments [14].

Following incubation, the contents of the flask were transferred to hand-held glass homogenizers containing 1 ml of a solution of isobutyl methylxanthine (0.4 mM), EDTA (40 mM), and NaCl (2.0 M) at 0°. Cells were homogenized with three strokes of a motor-driven pestle and transferred to centrifuge tubes. Samples were centrifuged for 10 min at 0° (2000 *g*), and the fat cake was aspirated off and discarded. A sample of infranatant was added to an equal volume of 10% (w/v) trichloroacetic acid for assay of cyclic AMP and another sample was taken for assay of glycerol content.

Biochemical assays. Samples for assay of cyclic AMP were extracted with diethyl ether and assayed for cAMP content by the radioimmunoassay of Harper and Brooker [15] as described previously [14]. Glycerol content of samples was determined by the fluorometric method of Chernick [16].

Statistics. Data were analyzed by Student's *t*-test for paired comparisons. A P value of <0.05 was

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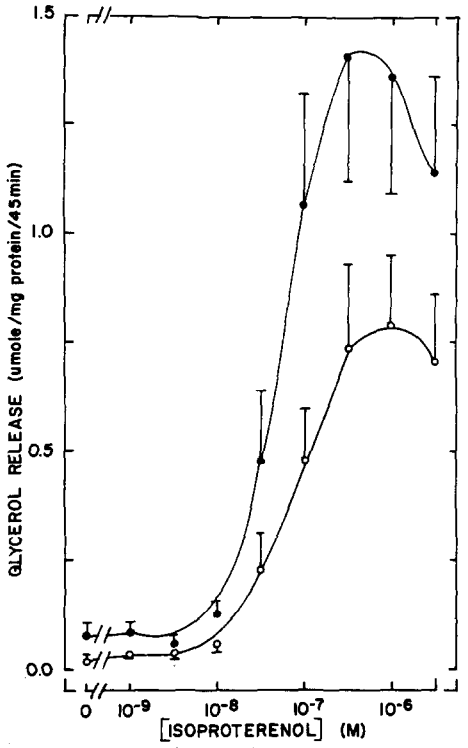

Fig. 1. Dose–response relationship for isoproterenol and glycerol release. Closed circles represent results in the presence of Ca^{2+} in the incubation medium and open circles represent results in the Ca^{2+}-free incubation medium. The results are expressed as the mean ± S.E.M. of five experiments.

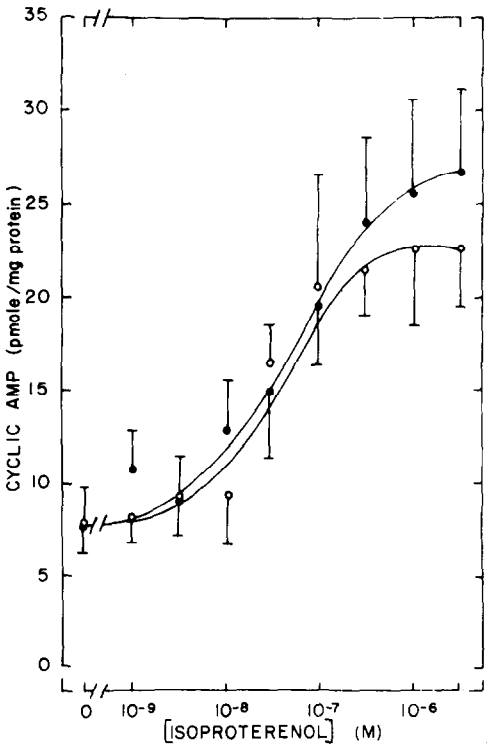

Fig. 2. Dose–response relationship for isoproterenol and cyclic AMP levels. For details see Fig. 1.

considered to be significant. Unless otherwise indicated, data are expressed as mean ± S.E.M.

RESULTS

Dose–response relationships with and without Ca^{2+}. In dose–response experiments, isoproterenol significantly increased rates of lipolysis (Fig. 1) and cyclic-AMP content (Fig. 2) in fat cells at 10^{-8} M and above in the presence of Ca^{2+}. In the absence of Ca^{2+}, significant increases in lipolysis occurred at 10^{-8} M and above, while cyclic AMP increases were significant at 3.3×10^{-8} M and above.

The lipolytic response to isoproterenol was less in the Ca^{2+}-free medium than in that containing the Ca^{2+} (Fig. 1). The absence of Ca^{2+} resulted in significantly reduced responses at 10^{-8} M isoproterenol and above. The maximum response was reduced approximately 45% whereas the EC_{50} was unchanged (5.2×10^{-8} M with Ca^{2+}; 5.6×10^{-8} M without Ca^{2+}). No significant difference in cyclic AMP content was seen between samples containing Ca^{2+} and those without Ca^{2+} at any concentration of isoproterenol.

Forskolin increased lipolytic rates (Fig. 3) and cyclic AMP content (Fig. 4) in fat cells at concentrations of 10^{-6} M and above in samples containing Ca^{2+}. In the absence of Ca^{2+}, significant increases in both glycerol release and cyclic AMP

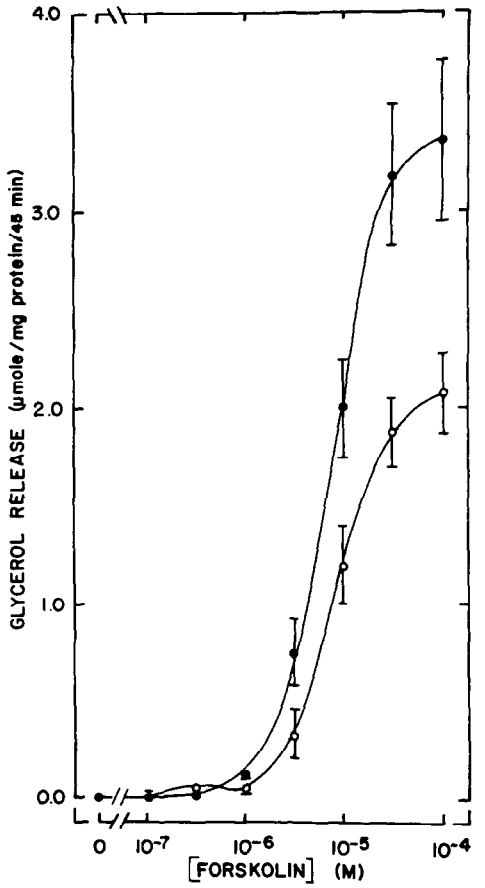

Fig. 3. Dose–response relationship for forskolin and glycerol release. For details see Fig. 1.

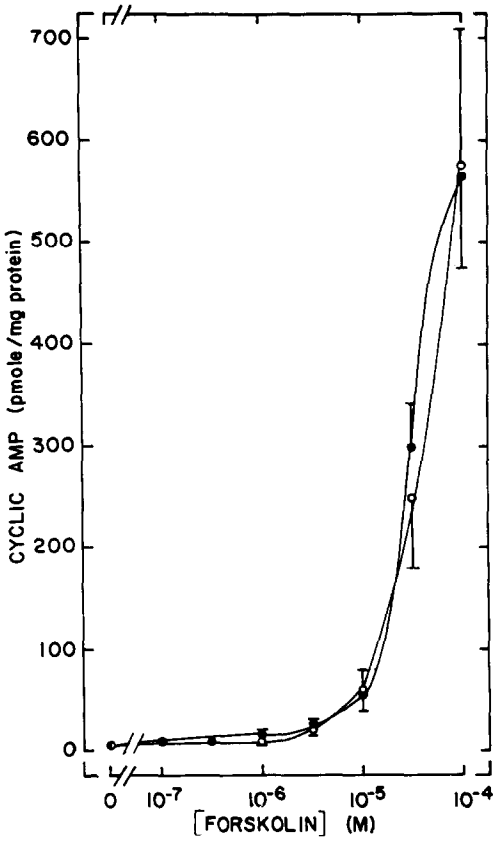

Fig. 4. Dose–response relationship for forskolin and cyclic AMP levels. For details see Fig. 1.

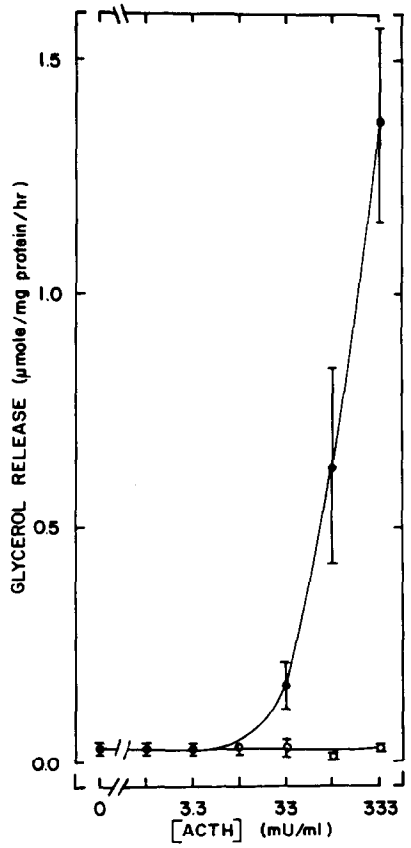

Fig. 5. Dose–response relationship for ACTH and glycerol release. For details see Fig. 1.

content were observed with forskolin at concentrations of 3.3×10^{-6} M and above.

Removal of Ca^{2+} from the incubation medium resulted in a significant reduction in the lipolytic response to forskolin at 3.3×10^{-6} M and above. The maximum lipolytic response was reduced by 40% with no change in the EC_{50} (7.5×10^{-6} M with Ca^{2+}; 8.0×10^{-6} M without Ca^{2+}). No significant difference in cyclic AMP accumulation was noted between samples containing Ca^{2+} and those without Ca^{2+} at any concentration of forskolin.

Following a 60-min incubation in the presence of Ca^{2+} and various concentrations of ACTH, samples were taken for glycerol and cyclic AMP determinations. A dose-dependent increase in both lipolytic activity (Fig. 5) and cyclic AMP content (Table 1) was seen with concentrations of ACTH of 33–333 mU/ml. In the absence of Ca^{2+}, no increase in either glycerol release (Fig. 5) or cyclic AMP content (Table 1) was seen at any concentration of ACTH.

Time courses with and without Ca^{2+}. Fat cells were incubated with 333 mU/ml of ACTH with and without Ca^{2+} for various periods of time following which samples were taken for glycerol determination or cyclic AMP assay. In those experiments in which cyclic AMP was determined, 10^{-3} M theophylline was included to magnify any changes in this cyclic nucleotide.

Following a short lag period, the lipolytic rate increased in the presence of Ca^{2+} (Fig. 6) and remained constant for up to 60 min. In the absence of Ca^{2+} no increase in glycerol release was observed at any time period tested. In the presence of Ca^{2+}, cyclic AMP levels were elevated by the earliest time period sampled (5 min), increasing to maximum values by 15 min and remaining elevated at near maximum levels for the duration of the incubation (Fig. 7). At no time point was there any increase in cyclic AMP levels in the absence of Ca^{2+}.

Dibutyryl cyclic AMP with and without Ca^{2+}. In five experiments, fat cells were incubated for 1 hr in the presence and absence of Ca^{2+} with the dibutyryl analog of cyclic AMP (10^{-3} M) following which samples were taken for glycerol determination. The rate of glycerol release in the presence of Ca^{2+} (4.38 ± 0.32 μmoles/mg protein/hr) was significantly

Table 1. Effects of ACTH on cyclic AMP levels in Ca^{2+}-containing and Ca^{2+}-free incubation medium

ACTH (mU/ml)	Cyclic AMP (pmoles/mg protein)	
	With Ca^{2+}	Without Ca^{2+}
0	9.1 ± 1.3	9.4 ± 1.7
33	10.6 ± 1.3*	10.4 ± 2.2
100	12.7 ± 1.4*	8.9 ± 2.1
333	15.7 ± 2.7*	10.6 ± 2.7

The results are the mean ± S.E.M. of five experiments.
* $P < 0.05$ as compared to the corresponding control.

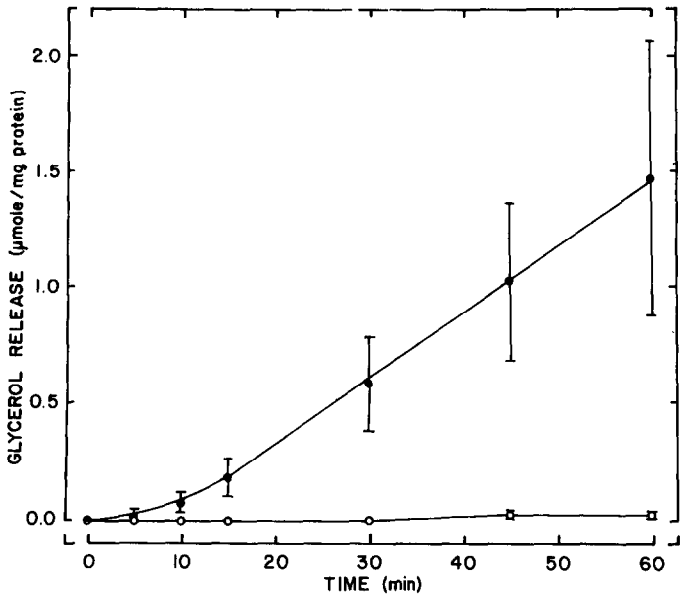

Fig. 6. Time-course of the effect of ACTH (333 mU/ml) on glycerol release. For details see Fig. 1.

greater than the rate of release in the absence of Ca^{2+} (3.04 ± 0.09 μmoles/mg protein/hr).

DISCUSSION

It is well documented that calcium ion is required for the ACTH stimulation of lipolysis [4, 7] in adipose tissue and steroidogenesis in the adrenal cortex [17]. This appears to be related to an obligatory role for calcium ion in the activation of adenylate cyclase and the subsequent increase in tissue levels of cyclic AMP [5, 6].

The present study confirms and extends these observations. Elimination of calcium ion from the incubation medium totally abolishes the lipolytic response to ACTH. The absence of calcium ion also abolished the ability of ACTH to elevate cyclic AMP levels in fat cells at all concentrations of the hormone tested. When theophylline was added to the incubation medium to magnify cyclic AMP changes, ACTH increased cyclic AMP levels greater than 10-fold, an effect which was sustained for a 60-min incubation period. Removal of calcium ion completely abolished this response to ACTH at all time

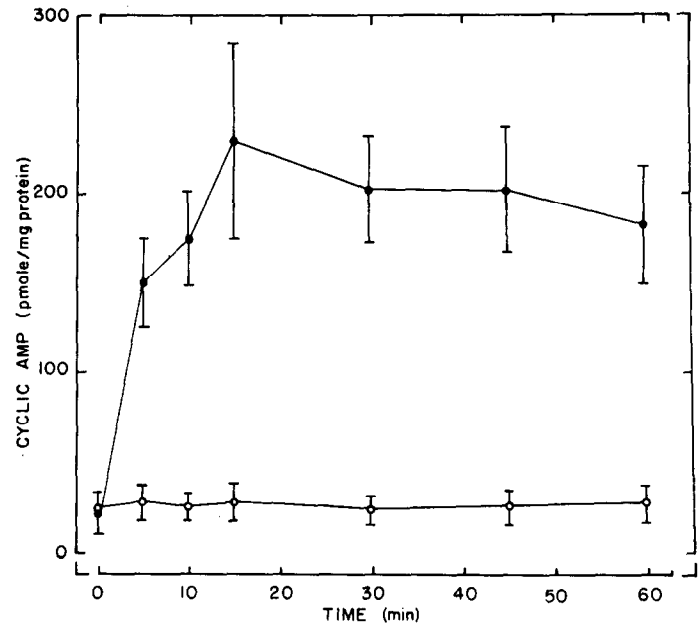

Fig. 7. Time-course of the effects of ACTH (333 mU/ml) on cyclic AMP levels. For details see Fig. 1. Theophylline (10^{-3} M) was included in the incubation medium.

periods tested. It thus appears that the ability of ACTH to elevate cyclic AMP levels in fat cells has an absolute requirement for the presence of calcium ion. This is consistent with the work of Birnbaumer and Rodbell [6] and Bär and Hector [5] which demonstrated a Ca^{2+} requirement for ACTH stimulation of adenylate cyclase in fat cell membranes.

The elimination of the cyclic AMP elevation by the removal of calcium ion may not be adequate to fully explain the lack of lipolytic response under these conditions. The work of Lang and Schwyzer [18], Lang *et al.* [19] and Fain *et al.* [20] suggests that the ACTH stimulation of lipolysis is the result of two concurrent mechanisms, one of which is cyclic AMP dependent, and the other is cyclic AMP independent. Fain [2] has suggested that both of these processes are mediated through a common receptor. Carchman *et al.* [21] suggested a similar concept for the effects of ACTH on adrenocortical tissue in which one type of receptor mediated the effect of ACTH through two concurrent but independent processes.

If two separate processes are involved in the mediation of ACTH stimulation of lipolysis, it is apparent that both are calcium ion dependent, as removal of this divalent ion completely abolished the lipolytic response to this hormone. Based on this concept, the most likely role for calcium ion is at the level of a receptor which is common to both processes. This is consistent with the work of Katocs *et al.* [4] which suggested that calcium ion was necessary for the interaction of ACTH with its adipocyte receptor.

The conclusion that calcium acts at the level of the ACTH receptor is supported by the present work and that of Kuo [22] which show that the elevations in cyclic AMP levels produced by stimulants other than ACTH, such as catecholamines and forskolin, are not calcium ion dependent. According to current concepts, the catalytic subunit of adenylate cyclase is common to the various hormones [2, 6] and is stimulated directly by forskolin [23]. If calcium ion exerted its effect at the level of the catalytic subunit, the absence of this cation should influence the cyclic AMP generated following stimulation by any hormone and by forskolin. This is not the case, as only the response to ACTH is affected.

Thus, it appears that Ca^{2+} is necessary for the interaction of the ACTH molecule and its membrane receptor. Activation of this receptor leads to increased cyclic AMP synthesis and stimulation of lipolysis. The possibility exists that the Ca^{2+}-dependent activation of this receptor also stimulates lipolysis through a cyclic AMP-independent pathway.

Although the role of Ca^{2+} in the lipolytic response to ACTH can be localized to the receptor-adenylate cyclase system, such is not the case for other stimulants of lipolysis. The increase in cyclic AMP following isoproterenol stimulation was not affected by lack of Ca^{2+}, while the lipolytic response was reduced significantly. The same relationship was found for the effects of forskolin on cyclic AMP levels and lipolytic activity. Thus, neither isoproterenol, which increases adenylate cyclase activity through interaction with a beta-adrenergic receptor, nor forskolin, which directly activates the catalytic subunit of adenylate cyclase, was dependent on Ca^{2+} for its ability to increase cyclic AMP levels. This suggests that Ca^{2+} played a role in the lipolytic response distal to the production of cyclic AMP.

Other authors have reported a similar dual role for Ca^{2+} in the adrenal cortex. The ability of ACTH to elevate cyclic AMP levels in this tissue was dependent on the presence of Ca^{2+} and the steroidogenic response to exogenous cyclic AMP was diminished in the absence of Ca^{2+} in the incubation medium [17, 24, 25].

In contrast to the present study, Schimmel [8] reported that the absence of Ca^{2+} resulted in a reduced sensitivity of fat cells to the lipolytic effect of epinephrine without any change in the maximum response. This reduced sensitivity was accompanied by a reduced accumulation of cyclic AMP. Although no explanation is available for these differences from the present results, it should be noted that Schimmel used epinephrine, a mixed alpha and beta adrenergic receptor agonist, while the present study employed isoproterenol, a specific beta receptor agonist. The report of Lawrence and Larner [26] has indicated the presence of both receptor types in rat adipocytes.

In summary, Ca^{2+} was shown to interact with the lipolytic system in at least two ways. The ability of ACTH to elevate cyclic AMP levels in fat cells exhibited an absolute requirement for Ca^{2+}. This most likely represents an interaction of ACTH, Ca^{2+} and the ACTH-receptor. Additionally, Ca^{2+} was required for the full activation of lipolysis by other lipolytic agents, through an interaction at some step in the lipolytic sequence distal to cyclic AMP production.

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