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REGULATION OF CYCLIC AMP METABOLISM IN BOVINE ADRENAL MEDULLARY CELLS

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Abstract—The capacity of cultured bovine adrenal medullary cells to metabolize and export cyclic AMP has been studied. Basal cellular cyclic AMP levels were increased 50% by 100 μM 3-isobutyl-1-methylxanthine (IBMX) and rolipram, a class IV (cyclic AMP-specific) phosphodiesterase (PDE) inhibitor. They were not affected by inhibition of class I (Ca^{2+}/calmodulin-dependent), class III (cyclic GMP-inhibited) or class V PDE (cyclic GMP-specific) with vinpocetine or 3-isobutyl-8-methoxymethyl-1-methylxanthine (8-methoxymethyl-IBMX), SK&F 94120, or MB 22,948, respectively, all at 100 μM. Furthermore, only IBMX and rolipram enhanced the cyclic AMP response to 0.3 μM forskolin. Rolipram had an EC_{50} of ≤1 μM and was equally effective at 100 μM and 1 mM. IBMX enhanced cyclic AMP levels significantly more at 1 mM than at 100 μM. Neither vinpocetine nor 8-methoxymethyl-IBMX (100 μM) enhanced the Ca^{2+}-dependent cyclic AMP response to K^+ depolarization. Elevation of cyclic GMP levels with sodium nitroprusside (10 or 100 μM), to activate any cyclic GMP-stimulated class II PDE and to inhibit any cyclic GMP-inhibited class III PDE, also had no effect on basal or forskolin-stimulated cyclic AMP levels. In the presence of IBMX (1 mM), forskolin (5 μM) caused a rapid and large increase in cellular cyclic AMP levels which was maximal after about 5 min and declined slightly over 3 hr. Over this period, extracellular cyclic AMP levels rose almost linearly reaching levels 2–3 times those in the cells. The results indicate bovine adrenal medullary cells have a high capacity for sustained cyclic AMP export. Furthermore, two PDE isozymes appear to degrade cyclic AMP in these cells, a rolipram-sensitive, cyclic AMP-specific, class IV isozyme and a rolipram-insensitive isoform.

A variety of cellular processes in adrenal chromaffin cells are regulated by the cyclic nucleotides, cyclic AMP and cyclic GMP. These include catecholamine secretion, the integrity of the cytoskeleton, properties of voltage-sensitive Ca^{2+}-channels, tyrosine hydroxylase activity and the expression of specific genes (see Refs 1–3). Receptors for a number of neurotransmitters and hormones that act on the adrenal medulla have been shown to control the levels of cyclic AMP or cyclic GMP in chromaffin cells, and these second messengers are likely to play a physiological part in at least some of the cellular responses to these agents.

Recently, it was reported that atrial natriuretic factor (ANF†) is synthesized and released by adrenal chromaffin cells [4]. Chromaffin cells possess high affinity ANF binding sites [5], and ANF was found to regulate cyclic GMP levels in bovine chromaffin cells [5–7], resulting in the phosphorylation and activation of tyrosine hydroxylase in these cells [7]. Another possible function of the cyclic GMP generated by ANF stimulation of chromaffin cells has been suggested from studies on the related PC12 tumour cell line. In these cells, ANF receptors stimulate cyclic GMP formation [8–10] and this allosterically activates a cyclic nucleotide phosphodiesterase (PDE), accelerating the degradation of cyclic AMP [10]. The result is that ANF can reduce and shorten the cyclic AMP response to agents that stimulate adenylate cyclase, such as adenosine [10].

In normal adrenal chromaffin cells, a number of agents giving cyclic GMP responses have been characterized, including ANF, muscarinic agonists and nitric oxide [5–7, 11, 12]. It is possible that these agents may also regulate cyclic AMP responses by controlling its degradation by a cyclic GMP-stimulated PDE.

Previous biochemical studies have found a number of different PDE activities in extracts of whole adrenal medulla (for review of PDE isozymes and their pharmacology, see Refs 13 and 14). In addition to a cyclic GMP-activated cyclic nucleotide PDE and mRNA for this isoform [15–18], these include a low K_m cyclic AMP-specific PDE [15, 16] that may be particulate [15], a Ca^{2+}/calmodulin activated PDE [16, 19, 20] some of which may be cyclic GMP-specific [16], and a cyclic GMP-specific isozyme that is Ca^{2+}-independent [17]. However, these studies have not examined which isozymes are responsible for the degradation of cyclic AMP in intact chromaffin cells, either under resting conditions or under conditions where cyclic AMP levels are elevated.

In the present study, we have characterized the contributions of five clases of PDE isozymes to the control of cyclic AMP metabolism in bovine adrenal chromaffin cells, by use of selective activators and inhibitors of the different isozymes. We have also characterized the capacity of chromaffin cells to

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† Abbreviations: PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine; 8-methoxymethyl-IBMX, 3-isobutyl-8-methoxymethyl-1-methylxanthine; SNP, sodium nitroprusside; ANF, atrial natriuretic factor.

export cyclic AMP as another means of regulating their cyclic AMP levels.

MATERIALS AND METHODS

Incubation of cultured bovine adrenal chromaffin cells. Primary cultures of bovine adrenal medullary cells were prepared and incubated exactly as described in detail elsewhere [1, 2, 21]. Primary cultures of chromaffin cells were maintained in the presence of mitotic inhibitors, to prevent the proliferation of dividing non-chromaffin cells. Cultures were used on the 3rd day after preparation. Cells received two 5 min washes followed by a 5 min incubation, except where indicated. Drugs [PDE inhibitors, sodium nitroprusside (SNP), forskolin, K^+ or solvent controls] were only present during the incubation period. Cells and incubation medium were then extracted separately, dried down and acetylated, as described [2], prior to cyclic nucleotide assay.

Determination of cyclic AMP and cyclic GMP. Cyclic nucleotides were assayed in cell and medium extracts by two specific radioimmunoassays. The assay for cyclic AMP using antiserum Ab5102 was as described [2]. The assay for cyclic GMP followed identical methods, using a polyclonal antiserum raised in rabbits immunized with 2′-*O*-monosuccinylguanosine-3′:5′-cyclic monophosphate conjugated to keyhole limpet haemocyanin with 1-ethyl-(3-diethylaminopropyl)-carbodiimide. The cyclic GMP antiserum was used at a final dilution of 1 in 51,000. The tracer was prepared by radioiodination of 2′-*O*-monosuccinyl-guanosine-3′:5′-cyclic monophosphate tyrosylmethylester by the chloramine T method and purified as for the cyclic AMP tracer [2]. Acetylated standards were prepared as for the cell and medium extracts [2], using synthetic guanosine-3′:5′-cyclic monophosphate. The standard was calibrated by its UV absorption at 254 nm with a molar extinction coefficient of 12,950.

Data presentation and statistics. All results are presented as means (±SEM) for the stated number of observations from a single cell preparation, and are representative of similar data on the stated number of cell preparations. The data presented in each figure for different treatment conditions are not necessarily from the same cell preparation as other treatment conditions shown in that figure. Furthermore, although basal cyclic AMP levels were very consistent for cells from the same cell preparation, they varied greatly between cell preparations. Basal levels were in the range 65–950 fmol/10^6 cells (mean ± SEM = 308 ± 52 fmol/10^6 cells for 22 cell preparations; see Ref. 2). For these reasons, the data for each treatment have been normalized to control conditions to facilitate comparison with the other treatment groups. Statistical significance for multiple comparisons was assessed by the modified *t*-statistic provided by Fisher's least significant difference (LSD) test, protected by a one-way analysis of variance.

Materials. 3-Isobutyl-1-methylxanthine (IBMX) and SNP were from the Sigma Chemical Co. (St Louis, MO, U.S.A.), and forskolin was from Calbiochem (La Jolla, CA, U.S.A.). 3-Isobutyl-8-methoxy-methyl-1-methylxanthine (8-methoxy-methyl-IBMX) was a generous gift of Dr J. Wells (Vanderbilt University). MB 22,948 (zaprinast: 2-*O*-propoxyphenyl-8-azapurin-6-one) was a generous gift from Dr S. Schofield (May and Baker Pharmaceuticals, Rhône-Poulenc, Australia). Rolipram (ZK62711: racemate of 4-(3′cyclo-pentyloxy-4′-methoxylphenyl)-2-pyrrolidone) was a gift from Dr G. D. Riisfeldt (Schering Pty, Australia). SK&F 94120 [(5-acetamidophenyl)pyrazin-2(1*H*)-one)] was a generous gift of Dr J. Skidmore (Smith Kline & French Research, Welwyn Garden City, U.K.). Vinpocetine (TCV-3B) was a generous gift of Dr Y. Oka (Takeda Chemical Industries, Japan). IBMX, MB 22,948, 8-methoxymethyl-IBMX, rolipram and SK&F 94120 were dissolved in dimethyl sulphoxide; vinpocetine was dissolved in acetone; and forskolin was dissolved in ethanol, before dilution to final concentrations in buffer. Appropriate solvent controls were performed.

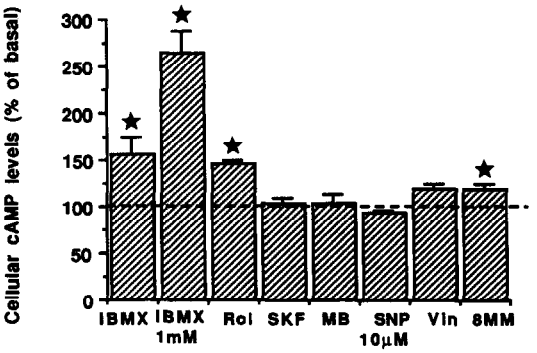

Fig. 1. Effect of selective PDE inhibitors and activators on basal cellular cyclic AMP levels over 5 min in cultured bovine adrenal medullary cells. Drugs were used at 100 μM unless indicated. Values are means (±SEM) for N = 4–6, and are representative data from a single cell preparation for each drug. Abbreviations (number of cell preparations tested in parentheses): IBMX (four cell preparations for 100 μM, 18 for 1 mM); Rol, rolipram (8); SKF, SK&F 94120 (4); MB, MB 22,948 (5); SNP (2); Vin, vinpocetine (10); 8MM, 8-methoxymethyl-IBMX (8). SNP at 10 and 100 μM increased cyclic GMP levels over 5 min from 16(±2) to 308(±41) and 1260(±70) fmol/10^6 cells, respectively; neither treatment affected cyclic AMP levels. 8MM had no significant effect overall in data pooled from eight cell preparations. The representative median result shown for 8MM is from a single cell preparation and did just reach statistical significance. Basal cyclic AMP levels varied between cell preparations from 65 to 950 fmol/10^6 cells; see Materials and Methods. *$P < 0.01$ compared to basal, Fisher's LSD test.

RESULTS

Effects of isozyme-selective PDE inhibitors on cyclic AMP metabolism in bovine adrenal medullary cells

PDE inhibitors were used at 100 μM, which is a high concentration compared to their reported IC_{50} values (Ref. 13, see discussion). Of the inhibitors used, only rolipram, an inhibitor of class IV cyclic AMP-specific PDE, and IBMX, an unselective PDE

inhibitor, increased basal cyclic AMP levels (Fig. 1).The other drugs used had no effect: these were vinpocetine and 8-methoxymethyl-IBMX (inhibitors of Ca^{2+}/calmodulin-dependent class I PDE), SK&F 94120 (an inhibitor of cyclic GMP-inhibited class III PDE), and MB 22,948 (an inhibitor of cyclic GMP-specific class V PDE) [13, 14].

As there are no selective inhibitors for the cyclic GMP-stimulated class II PDE [13], the presence of this isozyme was tested for by increasing cellular cyclic GMP levels with SNP (10 or 100 μM) to activate this isozyme. SNP, however, had no effect on basal cyclic AMP levels (Fig. 1), even though it caused large increases in cyclic GMP levels (see legend to Fig. 1).

The K_m of some of the PDE isozymes may be too high for them to contribute significantly to degradation of cyclic AMP under resting conditions, but they may contribute when cyclic AMP levels are raised by activation of adenylate cyclase. Previous studies have shown that cyclic AMP levels in bovine chromaffin cells can be raised 2–5-fold by stimulation for 5 min with acetylcholine, adenosine, vasoactive intestinal polypeptide, histamine or K^+ depolarization [1, 2, 21–23]. A concentration of forskolin (0.3 μM) was therefore selected that increased cellular cyclic AMP levels by a similar amount, and the contribution of different PDE classes to cyclic AMP degradation under these stimulated conditions was tested. Once again, only rolipram and IBMX were able to inhibit cyclic AMP degradation (Fig. 2A and B). Activation of cyclic GMP-stimulated class II PDE by SNP treatment failed to reduce the cyclic AMP response to forskolin.

The concentration dependence of rolipram's effects on basal and forskolin-stimulated cyclic AMP levels were investigated. In both cases, the EC_{50} for rolipram was ≤ 1 μM, and 3 μM rolipram had the same effect as 100 μM (Fig. 3).

It is possible that Ca^{2+}/calmodulin-activated class I PDEs are only activated when cytosolic Ca^{2+} levels are elevated. To test this possibility, cells were

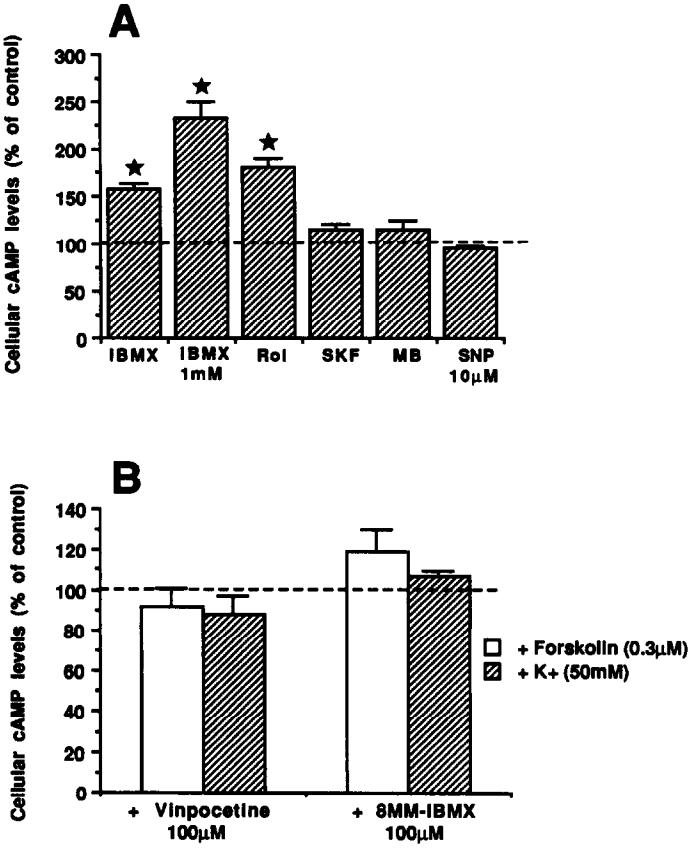

Fig. 2. Effect of selective PDE inhibitors and activators on stimulated cyclic AMP levels over 5 min in cultured bovine adrenal medullary cells. (A) Forskolin 0.3 μM. (B) Forskolin (open columns) or depolarization with 50 mM K^+ (equimolar substitution for Na^+, solid columns). Drugs were used at 100 μM unless indicated otherwise. Values are means (±SEM) for N = 4–6, and are representative data from a single cell preparation for each drug. Forskolin increased cellular cyclic AMP levels to 322(±23)% of basal (range from 20 cell preparations: 194–627%) and K^+ increased them to 237(±36)% of basal (range from five cell preparations: 156–332%). Abbreviations as in legend to Fig. 1. Number of cell preparations: IBMX (2); Rol (3); SKF (2); MB (3); SNP (2); Vinpocetine (4 with forskolin, 5 with K^+); 8MM (4 with forskolin, 3 with K^+). SNP at 100 μM also had no effect on the response to forskolin (not shown). *$P < 0.01$ with respect to basal, Fisher's LSD test.

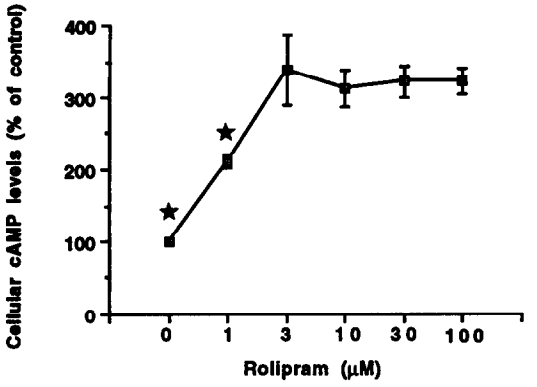

Fig. 3. Concentration dependence of rolipram-induced potentiation of cellular cyclic AMP levels in the presence of 0.3 μM forskolin over 5 min in cultured bovine adrenal medullary cells. Values are means (±SEM) for N = 4, data from a single cell preparation and representative of two such experiments. A similar concentration dependence was seen for the effect of rolipram on basal cyclic AMP levels (i.e. in the absence of forskolin). *P < 0.01 with respect to all other conditions, Fisher's LSD test.

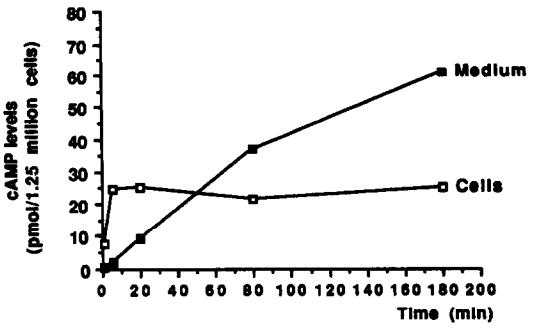

Fig. 4. Effect of 5 μM forskolin in the presence of 1 mM IBMX on cellular and extracellular cyclic AMP levels in cultured bovine adrenal medullary cells over 3 hr. Values are means (±SEM) for N = 6, data from a single cell preparation and representative of two such experiments. Basal levels were in the range 240–400 fmol/10^6 cells for both cellular and medium cyclic AMP and did not change over 3 hr in the absence of drugs.

stimulated by K^+ depolarization, which causes a large influx of extracellular Ca^{2+} and a Ca^{2+}-dependent increase in cellular cyclic AMP levels [1]. Neither vinpocetine nor 8-methoxymethyl-IBMX had any effect on the cyclic AMP response to K^+ (Fig. 2B).

At a high concentration (1 mM), the non-selective PDE inhibitor IBMX enhanced both basal and forskolin-stimulated cyclic AMP levels more than it did at 100 μM (Figs 1 and 2A). Rolipram at a higher concentration (1 mM) had no further effect on basal or forskolin-stimulated cyclic AMP levels than it did at 100 μM (data not shown). A combination of 100 μM rolipram with 100 μM SK&F 94120 in the presence of 0.3 μM forskolin had no further effect than rolipram alone (data not shown).

Export of cyclic AMP from bovine adrenal medullary cells following forskolin stimulation

The contribution of cyclic AMP export to regulating cellular cyclic AMP levels was studied during continuous stimulation of chromaffin cells over 3 hr with a high concentration of forskolin (5 μM). A high concentration of the non-selective PDE inhibitor IBMX was included to block degradation. Under these conditions, cellular cyclic AMP levels rose more than 16-fold within 1 min, and reached a maximum of over 50 times the basal levels within 5 min (Fig. 4). The cellular cyclic AMP levels then remained fairly stable or declined slowly over the next 3 hr. In contrast, extracellular cyclic AMP levels rose less than 2-fold at 1 min and only 6-fold after 5 min. However, the export of cyclic AMP continued almost linearly over the next 3 hr, achieving levels of 150-fold over basal levels. At this time, extracellular cyclic AMP levels were more than twice the cellular levels.

DISCUSSION

The present experiments indicate that in intact bovine adrenal medullary cells, under both basal and stimulated conditions, cyclic AMP is degraded primarily by a rolipram-sensitive (cyclic AMP-specific, class IV) PDE.

It was not expected that cyclic AMP might be degraded by PDE isozymes that are specific for cyclic GMP (class V), and this was confirmed by the lack of effect of MB 22,948. However, a number of studies had indicated that cyclic GMP could activate a PDE that hydrolysed cyclic AMP and cyclic GMP, and that this activity was a major part of the cyclic AMP PDE activity in adrenal medullary extracts [15, 17]. We found no evidence that such a class II PDE isozyme contributed to cyclic AMP degradation in intact chromaffin cells: SNP treatment caused significant increases in cellular cyclic GMP levels but did not reduce basal or forskolin-stimulated cyclic AMP levels (Figs 1 and 2). Furthermore, this PDE isozyme exhibits competitive inhibition of cyclic AMP degradation at high concentrations of cyclic GMP [15, 17]; however, 100 μM SNP which increased cyclic GMP levels almost 80-fold produced no change in basal or forskolin-stimulated cyclic AMP levels (Fig. 2, legend), suggesting cyclic AMP and cyclic GMP are degraded by distinct PDE enzymes. It is possible that a cyclic GMP-activated PDE is present selectively in vascular smooth muscle or endothelial cells within the adrenal medulla and is therefore detected in medullary extracts but not in preparations of purified chromaffin cells.

The failure of SNP to enhance basal or forskolin-stimulated cyclic AMP levels, together with the lack of effect of SK&F 94120 either alone or in combination with rolipram, indicate that class III, cyclic GMP-inhibited PDEs do not contribute to cyclic AMP degradation in intact chromaffin cells.

Several biochemical studies have failed to detect any Ca^{2+}/calmodulin-activated PDE activity in

extracts of rat or bovine adrenal medulla [15, 24, 25]. Others did find such an activity [16, 19, 20], although in one case only after very considerable purification [19]. The Ca^{2+}/calmodulin-stimulated PDE is selective or specific for cyclic GMP [16, 19] or has a very high K_m for cyclic AMP [20], such that it is unlikely that it is involved in cyclic AMP metabolism *in situ*. The lack of effect of inhibitors of the Ca^{2+}/calmodulin-activated PDE on basal and forskolin- and K^+-stimulated cyclic AMP levels found here is consistent with these observations (Figs 1 and 2).

Previous studies have found that cyclic AMP responses in bovine chromaffin cells are enhanced by Ro 201724 [22, 23, 26]. Since this compound is a selective inhibitor of the cyclic AMP-specific class IV PDE [13], our present data with rolipram agree with these earlier reports showing that a class IV PDE degrades cyclic AMP in these cells.

It was noteworthy that, at 1 mM, IBMX enhanced cyclic AMP responses more than it did at 100 μM (Figs 1 and 2A). In contrast, rolipram had no further effect at 1 mM over its effects at 100 μM, and its maximum potentiation of the cyclic AMP responses was roughly half that seen with IBMX. IBMX has a high K_i of up to 50 μM for many PDE isozymes [13] and concentrations of 0.5–1 mM are therefore necessary to get good inhibition of these isozymes. The greater potentiation seen with 1 mM compared with 100 μM IBMX was therefore to be expected. In contrast, the other PDE inhibitors used have substantially lower K_i values for their preferred isozymes and most cannot be used as selective inhibitors at high concentrations. A high concentration of the isozyme-selective PDE inhibitors, in comparison to their K_i values, was used in the present study to screen for the possible presence of enzymes with a high V_{max}; however, even at 100 μM only one inhibitor had any effect.

The present data indicates that rolipram was unable to inhibit fully the PDE activity in bovine chromaffin cells even at 1 mM, suggesting that at least two PDE isozymes are present in intact chromaffin cells: one is rolipram-sensitive and the other is insensitive to rolipram, SK&F 94120, MB 22,948, vinpocetine and 8-methoxymethyl-IBMX, and is not activated, inhibited or competed for by cyclic GMP. The nature of this second IBMX-sensitive PDE remains to be established.

Cyclic AMP export from bovine chromaffin cells has been reported previously [1, 2, 21]. When cellular cyclic AMP levels were increased 2–5-fold by nicotine, histamine or K^+ stimulation, we found no change in cyclic AMP export over 5 min, but a slow increase occurred over 90 min with continuous stimulation [1, 2, 21]. The present data indicate that when cellular cyclic AMP levels are increased to much higher levels, cyclic AMP export increases significantly within the first few minutes of stimulation, and over long periods of incubation is responsible for the extrusion of large quantities of cyclic AMP (Fig. 4). The results show that adenylate cyclase in chromaffin cells has the capacity to be activated for very extended periods without desensitizing or experiencing substantial feedback inhibition by cyclic AMP or protein kinase A. Under such conditions, it appears that cyclic AMP export limits the rise in cellular cyclic AMP levels by extruding cyclic AMP from the cell. Although *modulation* of cyclic AMP export from chromaffin cells has not been reported, it is a mechanism that has the potential to regulate cellular cyclic AMP levels very profoundly, especially over long time periods.

The present results indicate that cyclic AMP levels in bovine adrenal chromaffin cells are regulated by metabolism by a rolipram-sensitive, class IV PDE and by export of cyclic AMP from the cell.

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