Cyclic AMP Efflux Is Regulated by Occupancy of the Adenosine Receptor in Pig Aortic Smooth Muscle Cells* 

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Cultured pig aortic smooth muscle cells respond to extracellular adenosine by activating adenylyl cyclase and by initiating the efflux of cAMP. In the presence of extracellular adenosine, efflux is first order with respect to intracellular cAMP concentration up to at least 125 pmol/10^6 cells. The apparent first-order rate constant for the efflux of cAMP increases in a dose-dependent manner in response to extracellular adenosine or 5-N-ethylcarboxamide adenosine. The EC50 for adenosine for promoting cAMP efflux is 12 μM. For cells stimulated with 5-N-ethylcarboxamide adenosine, the EC50 is 5 μM. When extracellular adenosine is removed, efflux stops abruptly. Cellular cAMP content falls but is still in a range that supports CAMP-dependent protein kinase. These data suggest that in pig aortic smooth muscle cells, the efficiency of cAMP efflux is regulated by A2 receptor occupancy.

Efflux of cAMP occurs in many different cell types (reviewed by Barber and Butcher (1) and Brunton and Heasley (2)). Several properties of efflux as it occurs in avian erythrocytes were presented in the original report by Davoren et al. (3), namely it continued in the presence of sufficient antagonist to cause a sharp drop in cellular cAMP, it was inhibited by probenecid and by caffeine, and it did not occur at 0 °C. Adherent cell lines as well as avian erythrocytes have been used to characterize further the properties of efflux. Efflux is energy-dependent (4-6) and unidirectional (7). It was shown that in avian erythrocytes efflux is not linked to band 3 function, nucleoside transport, hexose uptake, sodium-dependent amino acid uptake, ouabain-sensitive cation transport, or sodium/potassium exchange (8). Efflux is inhibited by several agents that inhibit microtubule formation (7). It is inhibited by certain prostaglandins (4), and this effect is probably due to interaction at the intracellular cAMP efflux site (9). In the case of inhibition by prostaglandin A1, it has been demonstrated that inhibition is due to a glutathione adduct formed intracellularly (10).

In avian erythrocytes the release of cAMP was found to be a significant mechanism for the control of intracellular cAMP levels (1, 5). However, in studies of cAMP release in cultured fibroblasts, cAMP efflux contributed little to the overall control of intracellular cAMP levels (11, 12). Extracellular cAMP is an important signal in Dictyostelium discoideum for the transition from the ameboid to the multicellular form. Cyclic AMP may act as an extracellular messenger in mammalian systems as well. Boxer et al. (13) demonstrated that adherence of polymorphonuclear leukocytes to endothelial cell monolayers is diminished by β-adrenergic receptor stimulation. This is apparently due to extracellular cAMP since inclusion of antibody to cAMP abolishes this effect. Westhead et al. (14) have shown that externally added cAMP potentiates the rise in 2,3-bisphosphoglycerate caused by CO2 in human red cells. T51B cells, a rat liver epithelial cell line (15) and Y-1 mouse adrenal tumor cells (16) have been shown to display cAMP binding sites on their surfaces. Kubler et al. (17) have recently demonstrated that HeLa, Chinese hamster ovary, and S49 cells have an ecto-protein kinase activity that is cAMP-dependent (17).

Adenosine is a potent vasodilator. It has been shown to cause the relaxation of smooth muscle both in vivo and in tissue preparations (18-21). In vascular smooth muscle adenosine stimulates the A1 subcella of adenosine receptors causing a rise in intracellular cAMP (22, 23). There is evidence that cAMP plays some role in adenosine-induced relaxation (18-21), although this does not appear to be the only mechanism by which adenosine causes relaxation.

Several earlier studies, including the original report by Davoren Sutherland, and Maxwell, have suggested that efflux of cAMP is hormonally regulated, but none of these studies has measured efflux in a way that accounts for the effect of the hormonally induced change in cellular cAMP, which is the substrate for efflux (1, 3). In some systems efflux initiated by agonist persists after the agonist is removed or neutralized by application of an antagonist (8). In pig aortic smooth muscle cells, efflux of cAMP occurs during the adenosine-stimulated rise in intracellular cAMP (22, 23). In this report we show that in pig aortic smooth muscle cells, cAMP efflux requires that agonist be present, and the efficiency of cAMP efflux from adenosine-stimulated cells is itself regulated by occupancy of the adenosine receptor.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum was obtained from Sterile Systems, Inc., Logan, UT. Collagenase was purchased from Worthington. All other tissue culture chemicals and solutions were obtained from GIBCO. NECA1 was obtained from Boehringer Mannheim. H8 was obtained from Seikagaku America, Inc., St. Petersburg, FL. Phenylboronate matrix gel was purchased from Amicon Corp., Danvers, MA, and Dowex 1-X4 (200-400 mesh) was purchased from Sigma. 125I-cAMP was obtained from Biomedical Technologies, Inc., Stoughton, MA. RO 20-1724 was a gift of Hoffmann-La Roche and was subse-

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1 The abbreviations used are: NECA, 5-N-ethylcarboxamide adenosine; H8, N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; RO 20-1724, (±)-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid.

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where \( P \) is the amount of extracellular CAMP (in picomoles/10^6 cells), \( t \) is time, \( V \) is the maximum velocity of efflux, \( S \) is the intracellular cyclic AMP concentration, and \( K_s \) is the intracellular CAMP concentration at which efflux is half its maximal rate. Equation 1 can be rewritten as follows,

\[
P(t) = \frac{V}{K_s + S} \int_0^t S \, dt
\]

or

\[
P_t = \frac{V}{K_s + S} \int_0^t S \, dt \tag{4}
\]

where \( k_1 (V/K_s) \) is the apparent first-order rate constant for cAMP efflux. The time integral of intracellular CAMP is the area under the curve in the time course of intracellular CAMP accumulation between time = 0 and time = \( t \), as previously described by Barber and Butcher (11). The area in each time segment was estimated by averaging the cellular CAMP between two time points and multiplying by the time interval between those two points. The areas were accumulated with increasing time. No arithmetic integration of the extracellular CAMP was necessary since the sample directly represents CAMP accumulated in the medium.

RESULTS

Fig. 1A shows a time course of change in intracellular and extracellular CAMP in response to 20 \( \mu \)M adenosine. As shown in Fig. 1B, a plot of extracellular CAMP versus the time integral of intracellular CAMP for the time course shown in Fig. 1A is linear, with an apparent first-order rate constant of 0.025/min. In the absence of agonist, there was no change in cellular CAMP and no CAMP appeared in the medium during incubations of from 1 to 90 min in duration. In two experiments, cellular nucleotides were labeled with [3H]adenosine, and the cells were stimulated with 100 \( \mu \)M adenosine; in the first experiment, 1.6%, and, in the second, less than 1%, of total labeled nucleotide plus adenosine appeared in the medium over a period of 15 min. Thus, CAMP exited the cells at about 20 times the rate of efflux of total labeled adenine nucleotide, although it represents less than 1% of cellular adenine nucleotide mass. The time course of change in extracellular CAMP was also observed in dishes to which cAMP

\[
\text{FIG. 1. A, time course of intracellular CAMP (ICAMP) change (\textbullet)} \\
\text{and extracellular CAMP (ECAMP) accumulation in the medium (C)} \\
\text{in response to 20 \( \mu \)M adenosine. B, data plotted from A. Extracellular} \\
c\text{CAMP at time \( t \) is plotted against the time integral of intracellular} \\
c\text{CAMP.}
\]
Regulation of cAMP Efflux

was added at concentrations of 1 nM to 1 μM in the absence of adenosine. As there was no loss of extracellular cAMP over a period of 30 min in these experiments, the amount of cAMP found in the medium after stimulation was taken to represent the total amount extruded during incubation.

Adenosine-stimulated efflux of cAMP was inhibited by probenecid (Fig. 2), an anion transport inhibitor that has been shown to inhibit cAMP efflux in other systems (6, 7, 29). The rate constant for efflux for the time course shown in Fig. 2 was 0.057/min in the absence of probenecid and too low to measure (<0.002/min) in 2 mM probenecid. Table I shows the temperature dependence of cAMP efflux. The efficiency of cAMP efflux has a Q10 of 4.5 between 26 and 37 °C. This steep temperature dependence is similar to that found by Plagemann and Erbe (30) in describing the transport of a synthetic cyclic nucleotide from mouse L-cells.

The time course of change in cellular and extracellular cAMP was observed at adenosine concentrations of 7-180 μM. The apparent first-order rate constants for cAMP efflux were determined at each adenosine concentration. The dependence of k on the concentration of adenosine, the primary agonist, is shown in Fig. 4. Again, the data fit a rectangular hyperbola. The Eadie-Hofstee plot gives an EC50 of 4.2 μM. The lower EC50 obtained with NECA compared with adenosine receptor independent of intracellular CAMP concentration. The dependence of k on the concentration of NECA, an analog of adenosine that is selective for the A2 receptor, is shown in Fig. 4. The Eadie-Hofstee plot of the same data.

We have shown previously that lines of pig aortic smooth muscle cells from different primary cultures vary in their cAMP content and in their fractional efflux of cAMP after stimulation with adenosine (22). How different the time course of change in intracellular CAMP can be from one line to another can be seen from a comparison of Figs. 1 and 2. Within a cell line, the extent and time course of response of confluent cells typically remain similar with passage. Agonist-dependent efflux of cAMP has been observed in all the pig aortic smooth muscle lines we have worked with in various contexts (more than 50). In this study, we found that cells that responded to 20 μM adenosine with at least a 3-4-fold increase in cellular CAMP over basal levels had a higher maximum release rate constant than cells that showed a less than 2-fold increase in cellular CAMP in response to 20 μM adenosine. The same was true of their response to NECA. The line of low responder cells used for the experiments described in Fig. 4 gave a maximum release rate constant of 0.017/min. A high responder line gave a maximum cAMP release rate constant of 0.07/min. However, these cell lines

![Fig. 2. Effect of 2 mM probenecid on the time course of changing intracellular cAMP (IcAMP) (○) and extracellular cAMP (EcAMP) (□) in response to 50 μM adenosine. Cells were preincubated with DMEM containing 2 mM probenecid and then stimulated with 50 μM adenosine.](image)

![Fig. 3. Effect of adenosine on the first-order release rate constant k. Release rate constants were determined for various extracellular adenosine (ADO) concentrations using the transformation shown in Fig. 1B. Each point is the result of a six-point time course of intracellular and extracellular cAMP accumulation in which two dishes were used for each time point and duplicate measurements of cAMP were made for each sample. Inset shows an Eadie-Hofstee plot of the same data.](image)

![Fig. 4. Effect of NECA on the first-order release rate constant k. Release rate constants were determined for various NECA concentrations using the transformation shown in Fig. 1B. Replication is as for Fig. 3. Inset shows an Eadie-Hofstee plot of the same data.](image)

**TABLE I**

Temperature dependence of cAMP efflux

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Cellular CAMP pmol/10^6 cells</th>
<th>k (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>Highest observed after stimulation</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.0</td>
<td>2.3 (10)</td>
</tr>
<tr>
<td>15</td>
<td>5.3</td>
<td>9.7 (15)</td>
</tr>
<tr>
<td>25</td>
<td>9.2</td>
<td>25.8 (2)</td>
</tr>
<tr>
<td>37</td>
<td>8.4</td>
<td>73.5 (2)</td>
</tr>
</tbody>
</table>

*Cellular and extracellular cAMP were measured in samples taken 0, 2, 4, 6, 10, and 15 min after stimulation with 50 μM adenosine. The number in parentheses is the time at which the highest level of intracellular cAMP was observed.
exhibited essentially the same EC₅₀ for NECA stimulation of cAMP efflux: 4.2 μM for the low responders and 5.7 μM for the high responders. These lines responded analogously to adenosine, low responding cells giving EC₅₀ values for efflux of 14 and 13 μM in two separate sets of experiments, and high responding cells giving an EC₅₀ of 10 μM. Thus, the maximum efficiency of efflux observed varied from cell line to cell line, but the dose dependence of efficiency on primary agonist concentration did not vary significantly from cell line to cell line.

We examined the effect of the phosphodiesterase inhibitor RO 20-1724 on intracellular cAMP and on the accumulation of extracellular cAMP, in order to be able to create conditions in which cellular cAMP would remain high after agonist was removed. Table II shows the effect of RO 20-1724 on cellular and extracellular cAMP after 10 min of incubation with 20 μM adenosine. RO 20-1724 alone had no effect on cellular cAMP nor did it cause any efflux. At doses up to 10 μM in the presence of adenosine, it supported substantial efflux of cAMP. A subsequent test of inhibitor concentrations up to 300 μM showed a 2-fold further increase in cellular cAMP between 10 and 300 μM RO 20-1724. However, at RO 20-1724 concentrations above 10 μM there was inhibition of net cAMP efflux. A dose of 5 μM RO 20-1724 was used for subsequent experiments.

To further test the hypothesis that the efflux process requires the continuing presence of agonist, experiments were conducted in which cells were incubated with 20 μM adenosine plus 5 μM RO 20-1724 to prevent rapid loss of cAMP after agonist was removed. The incubation medium was then changed to one containing no adenosine but still containing phosphodiesterase inhibitor and also containing adenosine deaminase. The time course of one such experiment is shown in Fig. 5. Intracellular CAMP was degraded rapidly after the adenosine was removed despite the presence of RO 20-1724 (Fig. 5A). Efflux of CAMP ceased abruptly with the removal of agonist (Fig. 5B). This experiment was conducted four times, twice with low responding lines and twice with high responding lines. In each case, efflux stopped immediately with removal of agonist.

Fig. 6 shows a plot of extracellular CAMP versus the time integral of intracellular CAMP after the agonist was removed in the experiment described in Fig. 5, in comparison with the efflux observed when the same cell line was incubated with 10 μM adenosine in the absence of RO 20-1724, conditions that span the same range of intracellular CAMP concentration. This comparison makes it clear that the lowered intracellular CAMP level that occurs after agonist is removed is adequate to support substantial efflux when agonist is present.

**Table II**

<table>
<thead>
<tr>
<th>Condition*</th>
<th>RO 20-1724</th>
<th>Adenosine</th>
<th>Cellular</th>
<th>Extracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>pmol/10⁶ cells</td>
<td>pmol/10⁶ cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>10.1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>8.1</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>18.1</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>20</td>
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<td>7.9</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>20</td>
<td>24.0</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>42.8</td>
<td>16.2</td>
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<tr>
<td>3</td>
<td>20</td>
<td>63.6</td>
<td>21.2</td>
<td></td>
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<tr>
<td>10</td>
<td>20</td>
<td>83.6</td>
<td>20.0</td>
<td></td>
</tr>
</tbody>
</table>

*Dishes were preincubated with HEPES-buffered DMEM, with the indicated amount of RO 20-1724, for 5 min. Adenosine was added and incubation continued for 10 min.

As a test of whether cellular CAMP had any role in the regulation of efflux via a protein kinase A-dependent mechanism, cells were incubated with H8, a protein kinase inhibitor shown to be effective in blocking A kinase in other cell systems (32). Fig. 7 shows the time course of change in intracellular and extracellular CAMP after stimulation with 50 μM adenosine for cells incubated with or without H8. Internal CAMP levels were higher in cells incubated with H8, suggesting that A kinase-mediated effects ordinarily serve to damp the intracellular rise in CAMP after stimulation with adenosine. Fig. 8 shows the accumulation of extracellular CAMP versus the time integral of the change in cellular CAMP in the presence and absence of H8. The rate constant for efflux was not significantly different in the presence of the A kinase inhibitor.

**DISCUSSION**

In 1963 Davoren et al. (3) reported that incubation of pigeon erythrocytes with epinephrine caused an efflux of CAMP as
well as a rise in the cellular content of the cyclic nucleotide. Subsequent studies showed that human plasmas cAMP rises sharply in response to β-adrenergic agonists (33) or glucagon (34). Parathyroid hormone prompts the synthesis and secretion of cAMP into the urine by human kidney (35). Perfusion studies or tissue incubations showed efflux of cAMP from glucagon-stimulated rat liver (36) and from fat pads (37), heart (38), and cervical ganglion (39) stimulated with β-adrenergic agonists. Many cultured cell lines have now been shown to carry out efflux of cAMP in response to a variety of agonists (12). We show here that in smooth muscle cells at lower temperatures than initiation of efflux. Another way in which control of efflux differs from activation of adenylate cyclase is that efflux is not down-regulated with time when cyclase activity is. This is suggested by our data in which it can be seen that the efflux rate is constant during intervals in which the cellular CAMP content rises and falls again. Barber and Butcher (11) observed a constant rate of cAMP efflux from WI-38 cells over a time course for which they had previously shown by isotope incorporation that cyclase activity decreased by 50% (12). In some cell types there is a lag of several minutes after maximum activation of cyclase before efflux is fully activated (4, 29, 42). In avian erythrocytes and mammalian reticulocytes, efflux continues once initiated, after agonist is washed away or after antagonist is given (3, 5, 6).

Brunton and Mayer (5) found that a constant fraction of cellular cAMP was extruded by pigeon erythrocytes over a wide range of doses of isoproterenol. The dose dependences for intracellular and extracellular cAMP were superimposable both early (5 min) and late (30 min) in the time course. This suggests that in this cell either the transport is always on or activation of efflux is complete at doses of agonist much lower than those needed for maximum stimulation of synthesis. This is also one of the cell types in which efflux continues after the removal of agonist.

Some prostaglandins are able to both activate adenylate cyclase and inhibit efflux (4). Presumably the activation occurs via surface receptors and activation of the cyclase stimulatory G protein. Brunton and Heasley (9, 10) have shown that the inhibitory effect of the prostaglandins is due to an intracellular effect at the cAMP efflux site. Krupinski et al. (44) have recently determined the amino acid sequence of adenylylcyclase from bovine brain and found that the cyclase is topologically similar to several channels and transporters, most strikingly the multidrug transporter called P glycoprotein. As they suggest, the most likely thing to be transported by cyclase is cAMP itself. It may be that although the cyclase protein is also the transport site, the functions of synthesis and transport are regulated separately, at least in part. A comparison of the time courses, dose dependences, and dependence on the continued presence of agonist observed in the various cell types that have been studied suggests that the extent to which the control of efflux is linked to control of cyclase activity is different in different cell types.

In smooth muscle cells, efflux does not appear to be significantly regulated by cAMP-dependent kinase. For one thing, such a regulation would lead to a more complicated dependence on cellular cAMP concentration than was observed. Further, efflux was not inhibited by H8 (Fig. 8). Efflux has been shown to occur in the S49 lymphoma line that lacks the catalytic subunit of A kinase (45).

Inhibition of cAMP efflux by probenecid, an inhibitor of transport of organic anions, has been demonstrated in many
cell types (2); efflux is also inhibited by this compound in smooth muscle cells (Fig. 2). Inhibition of efflux by methylxanthines is a commonly observed property (2, 40, 46). We have previously shown that efflux from smooth muscle cells is inhibited by isobutylmethylxanthine (22).

Plagemann and Erbe (30) have described the exit transport of a synthetic nucleotide, tricyclic 7-deazapurine nucleoside monophosphate, in mouse L-cells. This transport appears to be a carrier-mediated process with many properties similar to those described for cAMP efflux. Efflux of the model nucleotide does not appear to require hormonal stimulation of the cells, although it was not examined whether the model nucleoside itself was an agonist for cyclase activation.

The pseudo first-order rate constant, k, which we have measured, is a real operational measure of the efficiency of efflux on a whole cell basis. However, it may not always be a good measure of the ratio $V_{max}/K_e$ of efflux, assuming that efflux is a saturable process. Taking k to be equal to $V_{max}/K_e$ is based on the assumption that the cellular cAMP concentration is low compared with $K_e$ (Equations 1–4). It should be pointed out that the straightness of the line obtained by plotting extracellular cAMP as a function of the time integral of intracellular cAMP is a robust test of this assumption only when the cellular CAMP level traverses a significant range, with several points in both the high and low end during the time course observed. If S in Equation 3 is constant, it may have any relationship to $K_e$, and the plot will still be linear.

The resulting k will have a contribution from S in the denominator, that is, it will underestimate $V_{max}/K_e$, the more so the higher S is. However, this error is opposite in direction to that which might mislead us in concluding that $V_{max}/K_e$ of efflux increases with the concentration of primary agonist. In some experiments done with high responder cell lines, the k observed at 100 μM NECA was lower than that observed at 30 μM NECA (data not shown). We interpreted this as evidence that at the level of cellular CAMP observed in those experiments (200 pmol/10^6 cells at 100 μM NECA), its concentration approached $K_e$.

We have consistently stated intracellular cAMP in units of mass/cell since we have not measured intracellular volume and particularly have no information about the size of the compartment whose CAMP concentration is relevant. If the site for efflux is very near the site for synthesis, so that molecules are captured for efflux quickly compared with their rate of equilibration with the whole compartment to which they have access, the effective concentration driving efflux may increase disproportionately to the whole cell accumulation, and this could give rise to an increase in the efficiency of efflux such as we have observed as the rate of synthesis increased. Such surface kinetic effects would not account for the behavior of the cell types in which efflux lags behind synthesis and/or persists after agonist is removed.

As summarized in the Introduction, only a few cases of any effect of extracellular cAMP have been found in higher animal systems, and none of these has been characterized in detail. Efflux does not seem to have a consistent role in regulation of cellular cAMP. Barber and Butcher (11, 12) showed that cAMP efflux contributes approximately 18% of the turnover of cAMP in WI-38 fibroblasts. As shown in Table II, in the absence of phosphodiesterase inhibitor a total of 3.7 pmol of cAMP was extruded in 10 min, although in the presence of 10 μM RO 20-1724 intracellular cAMP was higher at the end of 10 min by 37 pmol/10^6 cells. As shown in Fig. 5A, even in the presence of 5 μM phosphodiesterase inhibitor, cAMP was eliminated quickly after removal of agonist when no efflux was occurring. Thus, efflux contributes only slightly to elimination of cellular cAMP in smooth muscle cells; rather, elimination is controlled by phosphodiesterase activity. The control of the fractional efflux of cAMP by agonist concentration suggests that cAMP may be part of an extracellular feedback loop. cAMP was not found to affect smooth muscle cell response to adenosine under the conditions tested here. cAMP should perhaps be tested for effect upon the release of agonists that initiate cAMP synthesis and efflux.

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