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Membrane-Permeant Derivatives of Cyclic AMP Optimized for High Potency, Prolonged Activity, or Rapid Reversibility

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SUMMARY

A novel membrane-permeant derivative of cAMP, cAMP acetoxymethyl ester (cAMP/AM), was synthesized via silylated intermediates. Its ability to induce Cl^- secretion by T84 cells, a human colon cancer cell line, was compared with that of two other membrane-permeant cAMP derivatives that were recently introduced, $N^6,O^{2'}$-dibutyryl-cAMP acetoxymethyl ester (bt_2cAMP/AM) and S_p-5,6-dichlorobenzimidazole-1-β-D-ribofuranoside 3',5'-cyclic phosphorothioate (S_p-5,6-DCl-cBIMPS). All of these compounds are powerful activators of Cl^- secretion when applied extracellularly, with EC_{50} values of 60 μM, 0.7 μM, and 3 μM, respectively. However, cAMP/AM was expected to be readily degraded inside cells, in contrast to the cyclophosphodiesterase-resistant S_p-5,6-DCl-cBIMPS or the only slowly metabolizable N^6-butyryl-cAMP derived from bt_2cAMP/AM. Reversibility of cAMP/AM action was demonstrated by wash-out experiments; Cl^- secretion induced by high doses of cAMP/AM (100 μM) could be quickly abolished by rinsing of the cells, whereas similar experiments with bt_2cAMP/AM and S_p-5,6-DCl-cBIMPS showed much slower decreases. Even more sensitive to residual cAMP derivatives was the synergistic effect of carbachol, which was applied after the incubation with membrane-permeant derivatives and their subsequent wash-out. Although doses of cAMP derivatives that barely activated Cl^- secretion were readily capable of inducing a synergistic response with carbachol, cells incubated with high doses of cAMP/AM (100 μM) and subsequently washed showed only a nonsynergistic carbachol response, in contrast to cells incubated with bt_2cAMP/AM or S_p-5,6-DCl-cBIMPS. We therefore characterize cAMP/AM as a membrane-permeant derivative of cAMP that is easily metabolizable inside cells and hence is most useful for applications where a transient intracellular cAMP signal is desired. In contrast, completely nonmetabolizable S_p-5,6-DCl-cBIMPS seems to be more useful in longer incubations that require steady levels of cAMP-dependent protein kinase activation. bt_2cAMP/AM combines the advantages of intracellular trapping by ester hydrolysis and reduced cyclophosphodiesterase sensitivity of its active intracellular product, which probably lead to its particularly high potency.

Membrane-permeant derivatives of cAMP have been known since Posternak *et al.* (1) introduced bt_2cAMP in 1962. Since then, many different strategies have been used to increase the lipophilicity of cAMP (2, 3). The best strategies modify the group most responsible for the impermeability of cAMP, namely the negatively charged phosphate diester with its low pK_a. Esterification (4-7), including esterification of the *o*-nitrobenzyl esters to form photolabile ("caged") derivatives (6, 8), amidation (9) of the phosphate moiety, and substitution of an exocyclic oxygen by sulfur (3) have been used to reduce polarity in this region. Substitution by sulfur not only increases the lipophilicity but also leads to extraordinary stability against all subtypes of cPDEs, the major class of metabolic enzymes for cAMP. Although the S_p-diastereomers are cAMP agonists, the corresponding R_p-diastereomers exhibit antagonistic effects (3).

One of the most intriguing compounds in this class of derivatives is S_p-5,6-DCl-cBIMPS (10, 11). It is highly lipophilic, resistant to cPDEs, and a good activator of PKA types I and II, but not of the cGMP-dependent kinases. Whereas the phosphorothioate derivatives act directly, the even more lipophilic cyclic phosphate triesters must be activated within the cell by either chemical hydrolysis (as a result of their relative instability) (7) or a photochemical redox process (in the case of caged

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ABBREVIATIONS: bt_2cAMP, $N^6,O^{2'}$-dibutyryl-cAMP; AM, acetoxymethyl; cAMP/AM, cAMP/acetoxymethyl ester; N^6-bt_1cAMP, N^6-butyryl-cAMP; S_p-5,6-DCl-cBIMPS, S_p-5,6-dichlorobenzimidazole-1-β-D-ribofuranoside 3',5'-cyclic phosphorothioate; PKA, cAMP-dependent protein kinase; cPDE, cyclic nucleotide phosphodiesterase; DIEA, *N,N*-diisopropylethylamine; AM-Br, acetoxymethyl bromide; VIP, vasoactive intestinal peptide; TMS, trimethylsilyl; PD, potential difference; I_{SC}, short circuit current; Ac, acetyl.

derivatives) (6, 8). More recently, a cAMP derivative that is enzymatically cleavable intracellularly has been introduced, i.e., bt_2cAMP/AM, with an AM group masking the phosphate (12). AM esters of phosphates are relatively stable at physiological pH extracellularly but are readily cleaved by acetylesterases intracellularly, regenerating the negative charge of the phosphate. The cAMP derivative is therefore trapped within the cell and, depending on its metabolic rate, may accumulate. Several biological assays have demonstrated that bt_2cAMP/AM is an effective membrane-permeant cAMP derivative with an EC_{50} of approximately 1 μM (12), 2–3 orders of magnitude more potent than traditional cAMP analogs like 8-bromo-cAMP, 8-*p*-chlorophenylthio-cAMP, or bt_2cAMP without the AM group.

The two butyryl groups in bt_2cAMP and bt_2cAMP/AM greatly modify the pharmacological properties, in opposite ways. The 2′-*O*-butyryl group must be cleaved off to yield biological activity, whereas the N^6-butyryl group reduces susceptibility to cPDEs. Butyrate itself can have complicating side effects. Can the butyryl groups be avoided so that cAMP itself is released in essentially one simple step? The previous methodology for synthesizing bt_2cAMP/AM (12) required the butyryl groups to confer solubility in anhydrous organic solvents. Without such solubility, the yields from the esterification reaction were very low. A major goal of the present work was to develop a general procedure for preparing AM esters of phosphate-containing messengers with amino and hydroxy groups left free.

Once cAMP/AM was prepared, we wished to compare its biological activity with that of two of the most potent cAMP agonists described previously, namely S_p-5,6-DCl-cBIMPS and bt_2cAMP/AM. The assay system involved the induction of Cl^- secretion across monolayers of T84 cells, a human colon epithelial cell line. T84 cells were originally derived from the lung metastasis of a colonic carcinoma and form polarized monolayers capable of carrying out vectorial chloride secretion when grown on permeable supports. They have been very widely used as a model of the intestinal chloride secretory mechanism, as well as providing information about other intestinal physiological and pathophysiological mechanisms (13). Although intracellular cAMP obviously has a wide range of biological effects that could be used in assays for the activity of membrane-permeant derivatives, we have found the T84 cell system to be a useful model for two reasons. The first of these is that chloride secretion can be directly assessed in real time. The second is that the cell line displays markedly synergistic secretory responses when cells are stimulated with a combination of a cAMP-mobilizing agonist and an agent that increases cytoplasmic calcium levels. This significantly enhances the sensitivity of the assay for detecting effects of small increments in intracellular cAMP levels above basal conditions, such as might exist after membrane-permeant derivatives have been washed from the bathing fluid. We now report that the three cAMP derivatives studied here have differing properties in this assay system, which may indicate their utility in other cell systems to produce cAMP increases of controlled magnitude and duration.

Experimental Procedures

General methods for synthesis. Proton and ^{31}P NMR spectra were obtained in $CDCl_3$, CD_3OD, or toluene-d_8, with residual $CHCl_3$ (δ = 7.26), CD_2HOD (δ = 3.50), or C_6D_6-CD_2H (δ = 2.30), respectively, acting as the internal standards for ^1H spectra. Phosphoric acid (85%) was used as an external standard for ^{31}P spectra. All NMR spectra were recorded on either a Varian Gemini-200 (200 MHz) or a General Electric QE-300 (300 MHz) spectrometer. Fast atom bombardment mass spectroscopy was performed by the mass spectroscopy facility of the University of California, Riverside.

Materials. Acetonitrile and toluene were stored over activated molecular sieves (type 3A) for at least 3 days. All other solvents were of the highest available purity and were used as received. DIEA was distilled from CaH_2. AM-Br was prepared according to known procedures (14). S_p-5,6-DCl-cBIMPS was synthesized as described previously (15), starting from 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. Nucleotides were from Sigma, and all other reagents were from Aldrich.

Synthesis of 2′-*O*-TMS-cAMP (I). The free acid of cAMP (50 mg, 0.15 mmol) was suspended in 3 ml of dry DIEA. Hexamethyldisilazane (1 ml) and TMS chloride (0.5 ml) were added under argon and the mixture was heated to 100° for 3 hr. After cooling to room temperature, all volatile components were removed under high vacuum. The residual oil was extracted with 2 × 2 ml of dry toluene. A sample of the extract was evaporated to dryness and shown by NMR to consist of N^6,2′-*O*-bis(TMS)-cAMP TMS ester [^1H NMR (toluene-d_8, 200 MHz): diastereomer 1 (80%), δ 0.32 (s, 9 H), 0.45 (s, 9 H), 0.47 (s, 9 H), 4.15–4.61 (m, 3 H, H4′, H5′$_{eq}$, and H5′$_{ax}$), 4.98 (d, 1 H, J = 4.7 Hz, H2′), 5.74 (ddd, 1 H, J = 1.6, 4.7, and 9.5 Hz, H3′), 5.83 (s, 1 H, H1′), 7.64 (s, 1 H, H2), and 8.60 (s, 1 H, H8); diastereomer 2 (20%), δ 4.65 (d, 1 H, J = 4.2 Hz, H2′), 5.20 (m, 1 H, H3′), 6.05 (s, 1 H, H1′), 7.77 (s, 1 H, H2), and 8.58 (s, 1 H, H8)]. The toluene extract was treated with 12 μl of methanol (0.3 mmol) for 3 min, followed by rapid evaporation of the solvents. The remaining white solid was pure 2′-*O*-TMS-cAMP (I) [^1H NMR (CD_3OD, 200 MHz): δ 0.80 (s, 9 H, TMS), 4.32 (m, 3 H, H4′ and H5′), 4.65 (d, 1 H, J = 8.2 Hz, H2′), 4.95 (m, 1 H, H3′), 6.12 (s, 1 H, H1′), 7.16 (broad s, 2 H, NH_2), and 8.40 and 8.45 (2 s, 1 H each, H2 and H8)].

Synthesis of 2′-*O*-TMS-cAMP/AM (II). 2′-*O*-TMS-cAMP (I) (25 mg, 0.062 mmol) was dissolved in 0.5 ml of dry CH_3CN containing 0.05 ml of DIEA (0.28 mmol) under argon, and 0.028 ml (0.28 mmol) of AM-Br was added. The mixture was stirred for 3 days at room temperature and then evaporated to dryness. The crude product was purified on a Si60 column (4 × 1.5 cm), with dry CH_3CN saturated with hexane as the eluent. Before the separation the column was washed with the same eluent containing 0.1% acetic acid, followed by the eluent alone. Most of the 2′-*O*-TMS-cAMP/AM (II) eluted just before $HDIEA^+Br^-$, to yield 20.5 mg (0.042 mmol, 68%). The product consisted of 90% of one of the R_p/S_p-diastereomers, as determined by ^{31}P NMR [^1H NMR ($CDCl_3$, 300 MHz): δ 0.22 (s, 9 H, TMS), 2.16 (s, 3 H, -$OCCH_3$), 4.43 (m, 2 H, H4′ and H5′$_{ax}$), 4.64 (m, 1 H, H5′$_{eq}$), 4.85 (d, 1 H, J = 5.2 Hz, H2′), 5.34 (m, 1 H, H3′), 5.74 (d, 2 H, J = 13.1 Hz, -CH_2-OAc), 5.91 (s, 1 H, H1′), and 7.87 and 8.41 (2 s, 1 H each, H2 and H8); ^{31}P NMR ($CDCl_3$, 121.5 Hz): δ −7.58 (90%) and −4.62 (10%)].

Synthesis of cAMP/AM (III). Fourteen milligrams (0.029 mmol) of **II** were dissolved in 1 ml of a 1:1 (v/v) $CHCl_3/CH_3CN$ mixture, and 2 μl of HF (49%) were added. The mixture was gently swirled for 2 min before the solvents were evaporated. The product was washed with dry $CHCl_3$ to give cAMP/AM (**III**) in 80% yield as a white solid [^1H NMR (CD_3OD, 300 MHz): δ 2.17 (s, 3 H, -OAc), 4.48 (ddd, 1 H, J = 3.6, 9.5, and 9.5 Hz, H4′), 4.50 (m, 1 H, H5′$_{ax}$), 4.74 (m, 1 H, H5′$_{eq}$), 5.33 (ddd, 1 H, J = 1.0, 4.8, and 12.3 Hz, H3′), 5.75 (m, 2 H, -CH_2-OAc), 6.15 (s, 1 H, H1′), and 8.41 and 8.42 (2 s, 1 H each, H2 and H8); ^{31}P NMR (CD_3OD, 121.5 MHz): δ −6.85; mass spectrometry (positive mode): m/z 402 $(M+H)^+$].

I_{SC} measurements of Cl^- secretion by T84 cells. Cells of the human colonic epithelial cell line T84 were cultured and grown to confluence on rat tail collagen-coated polycarbonate membrane filters (5-μm pore size; Nuclepore, Pleasanton, CA) (16, 17). The filters were glued to Lexan rings (internal area, 1.98 cm^2), which were mounted

into modified Ussing chambers to measure Cl^- secretion (18). Each half of the chamber contained 5 ml of warmed Ringer's solution, gassed continuously with 95% O_2/5% CO_2. The spontaneous PD, measured via calomel electrodes, was continuously short-circuited with an automatic voltage clamp (W.P. Instruments, New Haven, CT) and Ag/AgCl electrodes, but the open-circuit PD was also measured for brief periods (2–5 sec at each time point). After mounting, the cells were allowed to equilibrate for about 20 min. One dose of a cAMP derivative or VIP (10 nM) was then added to the basolateral half of the chamber. Each dose was applied in at least two parallel chambers. The I_{SC}, which was wholly reflective of Cl^- secretion, was monitored continuously throughout the experiment. Data points for the dose-response experiments were taken 12 min after the addition of the cAMP derivative and were expressed as a change of I_{SC}. After 50 min, both sides of one of the parallel chambers were washed with 3 × 5 ml of warmed Ringer's solution. Data points for the decrease of Cl^- secretion were taken 12 min after the wash. Twenty-five minutes after the wash, 100 μM carbachol was added to the basolateral side of the cells. The peak of the response minus the level of I_{SC} at the moment of addition minus the standard response of T84 cells to carbachol alone (43 ± 5 μA/2 cm^2) was considered the synergistic Cl^- secretion. In cases where the maximum measurable total I_{SC} (200 μA/2 cm^2) was exceeded, the experiment had to be stopped to prevent damage to the voltage clamp. Data points are therefore missing for some experiments, particularly for high doses of cAMP derivatives.

Results

Synthesis of cAMP/AM. Previous attempts (12) to convert the polar reactant cAMP directly into its AM yielded only minute amounts of cAMP/AM (Fig. 1), despite frequent sonication and the presence of a polar solvent (triethyl phosphate). We therefore sought a protecting group to provide a sufficiently nonpolar derivative of cAMP, i.e., a group that would allow alkylation with AM-Br and that could be removed chemically in the presence of the AM group. We chose the TMS group, mainly because of its easy removability under moderately acidic conditions. The synthesis pathway is outlined in Fig. 1. The free acid of cAMP was easily converted into its tris(TMS) derivative by methods described for gas chromatography-mass spectrometry analysis of sugars and nucleosides (19, 20). The product was efficiently extracted with toluene. Treatment with 2 equivalents of methanol in toluene removed all TMS groups except the one at the 2′-OH group. The resulting 2′-*O*-TMS-cAMP (**I**) was sufficiently nonpolar to allow AM alkylation of the phosphate group under regular conditions (AM-Br and DIEA), to give 2′-*O*-TMS-cAMP/AM (**II**) in good yield (80%). The product was purified on a Si60 column with hexane-saturated CH_3CN as the eluent, surprisingly yielding 90% of one of the two diastereomers. Ester **II** was dissolved in CH_3CN and treated with 2 equivalents of HF (49%) for 2 min. After evaporation of the solvents the crude product was washed with $CHCl_3$ and cAMP/AM (**III**) was obtained as a colorless solid in 80% yield. Its solubility in absolute methanol was about 2 mg/ml. ^{31}P NMR showed no cAMP.

Cl^- secretion by T84 cells. The human colon cancer cell line T84 was grown to confluency on collagen-coated membranes as described before. The membranes were mounted into Ussing chambers to allow measurements of Cl^- fluxes through the monolayer. A maximal dose of VIP (10 nM) added to the basolateral half of the chamber caused an immediate increase in Cl^- secretion that reached a plateau within 10–15 min and was sustained, with only a minor decrease, for >60 min (Fig. 2A). This effect was closely reproduced by bt_2cAMP/AM, S_p-5,6-DCl-cBIMPS, and cAMP/AM in the doses indicated in Fig. 2B. In contrast to experiments with VIP, bt_2cAMP/AM, and S_p-5,6-DCl-cBIMPS, the Cl^- secretion induced by cAMP/AM could be rapidly decreased by washing both halves of the Ussing chamber after 50 min. Secretion returned almost to base-line within 25 min. Some decrease could be observed for S_p-5,6-DCl-cBIMPS, but only slight recovery was seen with bt_2cAMP/AM (Fig. 2B) and practically none with VIP (Fig. 2A).

A more sensitive assay for residual cAMP or its derivatives was the synergism with carbachol-induced Ca^{2+} rises, which greatly potentiate Cl^- secretion when carbachol is applied 0.5–60 min after elevation of cAMP (Ref. 21 and references cited therein). This synergism was originally explained by the removal of the rate-limiting step for the two secretory responses, i.e., basolateral Cl^- influx (22). Very recently, the synergism of Ca^{2+}- and cAMP-induced Cl^- secretion has been studied in more detail.[2] Pretreatment with bt_2cAMP/AM modifies the carbachol-induced Ca^{2+} response by abolishing Ca^{2+} influx without affecting Ca^{2+} release from internal stores. Ca^{2+} influx normally seems to be an inhibitory signal for Cl^- secretion, so suppression of influx by cAMP would enhance secretion, as observed. Whatever the mechanism, the synergism is useful to amplify the response to low levels of cAMP or an analog. In this experiment carbachol (100 μM) was added 25 min after the wash. All activators except cAMP/AM showed strong synergism (Fig. 2B). This result confirms the previous finding that cAMP/AM can be completely washed away with no residual cAMP after 25 min, which is probably due to rapid enzymatic hydrolysis of the AM group and the cyclic phosphate ring of the resulting cAMP.

All three parts of the experiment shown in Fig. 2B, i.e., activation, wash-out, and synergism, exhibited strong dose dependencies for all cAMP derivatives tested (Figs. 3 and 4). The dose-response curves for the activation of Cl^- secretion induced by bt_2cAMP/AM, S_p-5,6-DCl-cBIMPS, and cAMP/AM are shown in Fig. 3. The EC_{50} values were 0.7 μM, 3 μM, and 60 μM, respectively (see also Table 1). Remarkably, bt_2cAMP/AM activity was detectable in the submicromolar range, with a threshold concentration for significant Cl^- secretion of 10 nM with an incubation time of 50 min (Table 1). The threshold concentration for S_p-5,6-DCl-cBIMPS was about 10-fold higher, whereas that for cAMP/AM was approximately 10 μM.

The wash-out is expressed in Fig. 3 as remaining activity for each dose of the three cAMP derivatives, measured 12 min after the wash procedure. In all cases the reduction of Cl^- secretion was dose dependent. bt_2cAMP/AM and S_p-5,6-DCl-cBIMPS showed roughly the same dose response curves for the residual Cl^- secretion as measured for activation, whereas cAMP/AM exhibited a washout dose-response curve that was significantly shifted to higher concentrations (Fig. 3). The difference is probably related to (a) the ability of the cell to metabolize (and therefore inactivate) cAMP or its derivatives and (b) the exit permeability or efflux of the derivative. A qualitative estimate can be extracted by determining the ratio of the EC_{50} values of the wash-out to the EC_{50} values for the activation. This ratio, here called α, is approximately 1 for

[2] M. Vajanaphanich, C. Schultz, R. Y. Tsien, A. E. Traynor-Kaplan, S. J. Pandol, and K. E. Barrett. Cross-talk between calcium and cAMP-dependent intracellular signalling pathways: implicaitons for synergistic secretion in T_{84} colonic epithelial cells and rat pancreatic acinar cells. Submitted for publication.

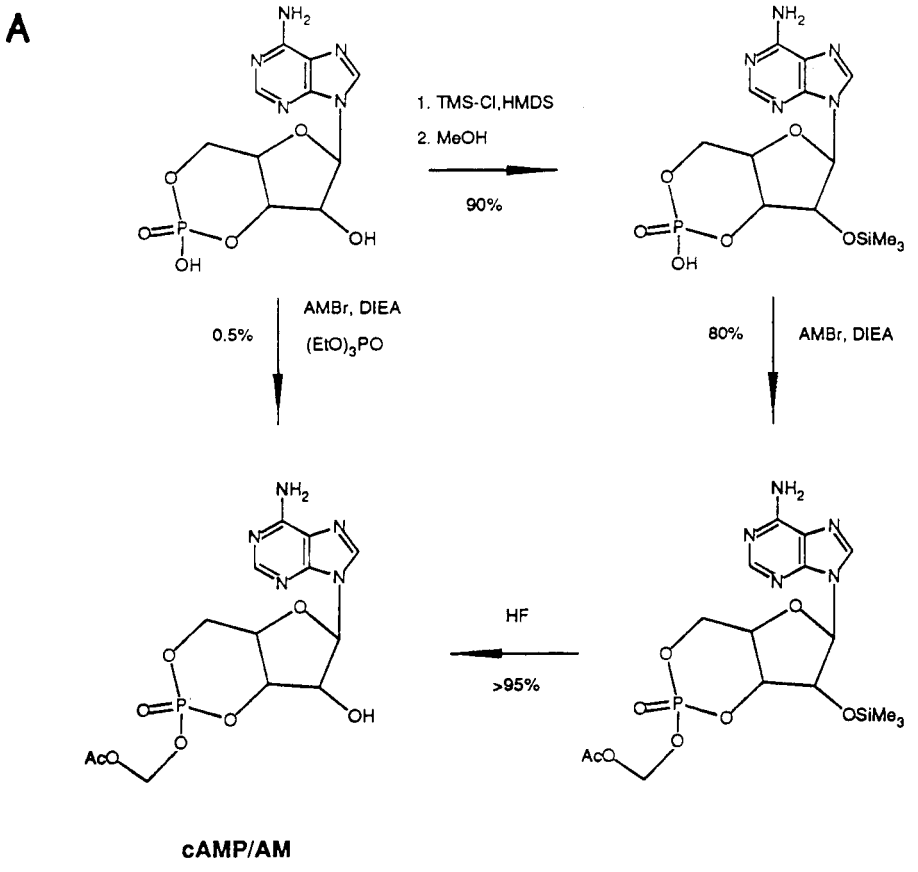
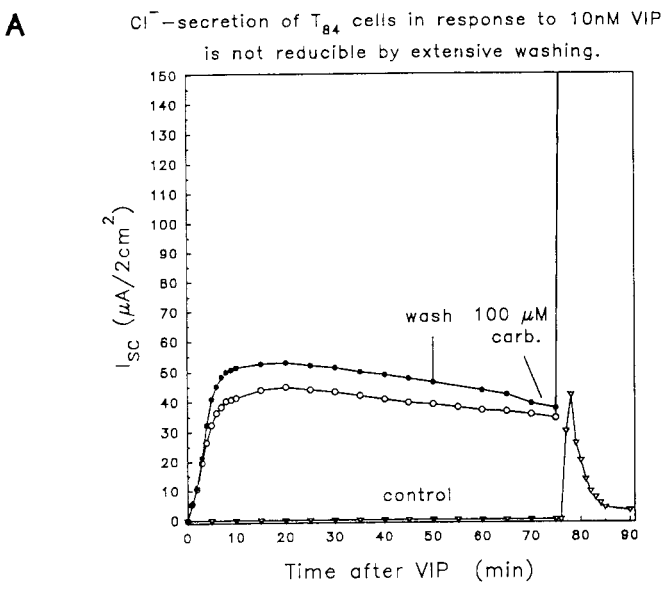


Fig. 1. Synthesis pathway to cAMP/AM (A) and the structures of bt_2cAMP/AM and S_p-5,6-DCl-cBIMPS (B). *HMDS*, hexamethyldisilazane; *MeOH*, methanol; *Me*, methyl; *Et*, ethyl.

bt_2cAMP/AM and S_p-5,6-DCl-cBIMPS (Table 1), meaning that the particular compound enters the cell as quickly as it is able to leave it, without being metabolized, as is expected for the latter, or that the agonist enters the cell as quickly as its active metabolite leaves or is degraded, as should be the case for bt_2cAMP/AM. The active metabolite N^6-bt_1cAMP accumulates rapidly from hydrolysis of the AM and 2′-*O*-butyrate groups[3] but should decay slowly because it is probably a poor substrate for cPDEs (23) or export from the cell (Fig. 5). Therefore, the wash procedure had only little effect (Fig. 2B). Prolonged trapping was not observed in the case of cAMP/AM, where the product of the AM hydrolysis should be cAMP itself, an excellent substrate for the cPDEs. This is expressed as an α value smaller than 1 (estimated to be approximately 0.2), characteristic of agonists whose metabolism or export from the cell (24) can outpace delivery and which need a steady supply of new material entering the cell to maintain the high level of Cl^- secretion.

Synergism with carbachol was used to amplify the responses to small residual activation of the cAMP pathway. In all cases the wash reduced the amount of synergistic Cl^- secretion. The effect was most pronounced for cAMP/AM, for which practically no synergism could be monitored at any concentration when the cells were washed 25 min before the addition of carbachol (Fig. 4). For S_p-5,6-DCl-cBIMPS the washing procedure led to a shift in the dose-response curve of roughly 1 order of magnitude (Fig. 4), whereas in the experiments with bt_2cAMP/AM a smaller shift could be observed, which is consistent with the poor reduction of Cl^- secretion after the wash

[3] Preliminary analysis of T84 cell lysates by capillary electrophoresis showed significant amounts of N^6-bt_1cAMP when cells were incubated with bt_2cAMP/AM under the same conditions as used for measurements of Cl^- secretion.



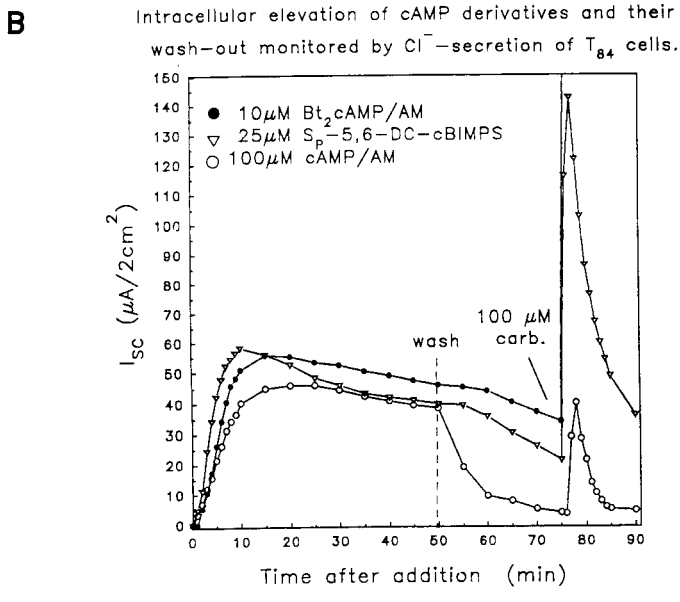


Fig. 2. Activation of Cl^- secretion by T84 cells mounted in Ussing chambers (see text for details). A, Extracellular basolateral application of VIP at time 0 and comparison of experiments with a wash procedure (●), as indicated, or without wash (○) (three experiments each). ▽, Control response to carbachol (*carb.*) alone (control experiments). B, Extracellular basolateral application of various membrane-permeant derivatives of cAMP in submaximal doses. Fifty minutes after activation the cells were washed and the decrease of Cl^- secretion was monitored. Twenty-five minutes after the wash (i.e., 80 min after the activation) 100 μM carbachol was added to induce synergistic Cl^- secretion in all experiments. In the case of bt_2cAMP/AM, the I_{sc} exceeded 200 μA/2 cm^2 and the voltage clamp had to be shut off. All *traces* represent the mean of at least three experiments in duplicate.

for this compound. Interestingly, even very small doses of bt_2cAMP/AM (in the range of 10 nM), which by themselves were barely able to elevate Cl^- secretion even after 50 min, showed a substantial amount of synergism with carbachol (Fig. 4).

Discussion

There are three obvious problems to consider when living cells are activated by membrane-permeant derivatives of

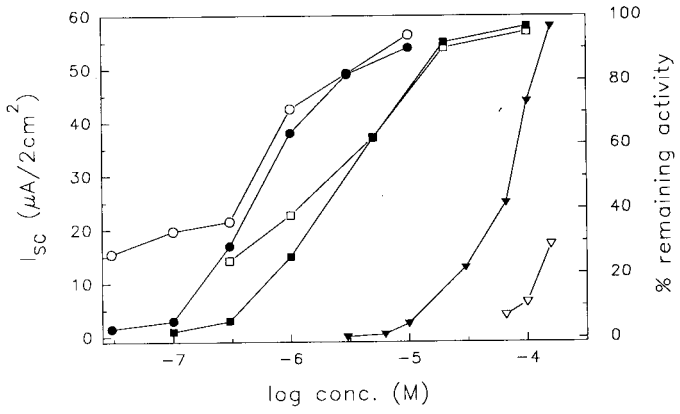


Fig. 3. Dose dependence of Cl^- secretion. Experiments were performed as depicted in Fig. 2. Data points for the dose dependencies of Cl^- secretion were taken 12 min after activation (*closed symbols*) and those for the reduction of Cl^- secretion were taken 12 min after the wash (*open symbols*) for cAMP/AM (*inverted triangles*), S_p-5,6-DCl-cBIMPS (*squares*), or bt_2cAMP/AM (*circles*). All experiments were performed at least three times in duplicate.

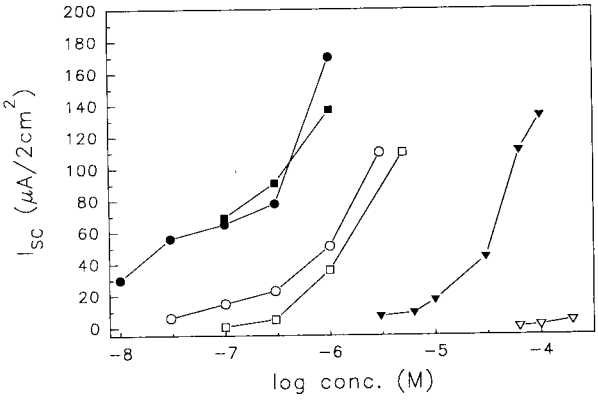


Fig. 4. Dose-response relationship for the net synergistic Cl^- secretion induced by carbachol without wash (*closed symbols*) or 25 min after the wash (*open symbols*) (three to seven experiments each) for monolayers incubated with cAMP/AM (*inverted triangles*), S_p-DCl-cBIMPS (*squares*), or bt_2cAMP/AM (*circles*). The net synergistic Cl^- secretion is defined as the peak response minus the level of Cl^- secretion at the moment of carbachol addition minus the standard response to carbachol alone (43 ± 5 μA/2 cm^2) (three experiments).

TABLE 1

Half-maximally active concentrations of membrane-permeant cAMP derivatives for the induction of Cl^- secretion by T84 cells and for secretion after the extracellular derivative was washed away

The threshold concentrations for the activation of Cl^- secretion at two time points are also included.

	EC_{50}		α^a	Effective Threshold Concentration	
	Activation[b]	Wash[c]		12 min	50 min
	μM			μM	
S_P-5,6-DCl-cBIMPS	3	3	~1	0.25	0.1
bt_2cAMP/AM	0.7	0.5	~1	0.03	0.01
cAMP/AM	60	250	~0.2	10	3

[a] α = activation EC_{50}/wash EC_{50}. See text for details.
[b] EC_{50} for activation of Cl^- secretion, measured 12 min after the addition of the cAMP derivative.
[c] EC_{50} for reduction of cAMP derivative-induced Cl^- secretion, measured 12 min after the wash procedure.

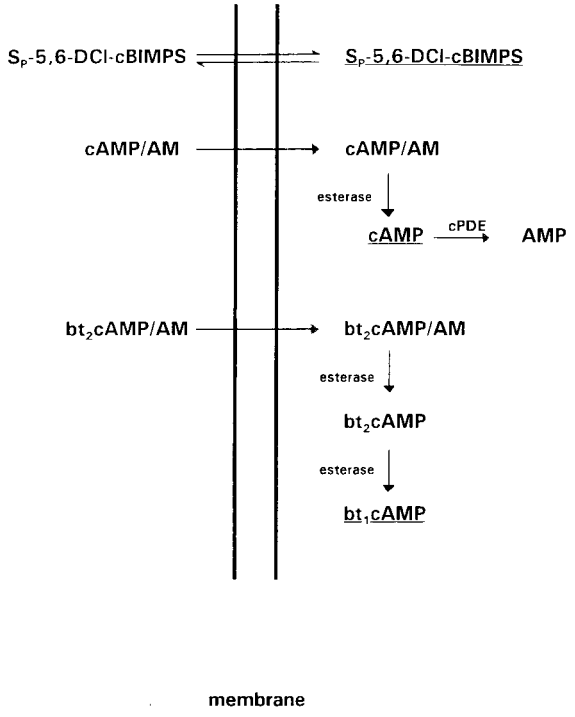


Fig. 5. Proposed mode of action for the three membrane-permeant derivatives of cAMP. Active compounds are *underlined*. Whereas S_p-5,6-DCl-cBIMPS is expected to leave the cell as quickly as it is able to enter it, AM esters are readily cleaved inside cells, thus liberating relatively impermeant phosphoanions. Intracellular hydrolysis of bt_2cAMP/AM yields bt_2cAMP, which is subsequently debutyrylated to N^6-bt_1cAMP, the active metabolite. N^6-bt_1cAMP is neither membrane permeant nor a good substrate for most cPDEs.

cAMP, first, unwanted effects of byproducts derived from intracellular bioactivation, e.g., butyrate; second, the fate of the derivative inside cells; and third, the failure of normal turn-off mechanisms to terminate the stimulation.

Many membrane-permeant cAMP derivatives, such as bt_2cAMP, cAMP/AM, and bt_2cAMP/AM, require intracellular hydrolysis of protecting groups to become active (Fig. 5). Intracellularly these compounds liberate butyric acid or acetic acid and formaldehyde, or all three. At the usual concentrations generated, these side products rarely cause problems, although control administrations are always advisable and antidotes for formaldehyde are available (12).

The metabolism of a particular cAMP analog inside cells is primarily determined by the action of cPDEs (Fig. 5). Whereas S_p-5,6-DCl-cBIMPS is apparently stable against cPDEs (11), N^6-bt_1cAMP derived from bt_2cAMP/AM is expected to be hydrolyzable, albeit severalfold more slowly than cAMP (23). It should be kept in mind, however, that there at least five cPDEs known, with variable distribution in different tissues, and that their activity towards cAMP analogs has not been studied in all cases.

Although most cAMP analogs appear to work through PKA, the question of whether an effect is actually due to the cAMP derivative or one of its metabolites sometimes arises; 8-chloro-cAMP has been particularly controversial (25–27). Some of the necessary control experiments are not easily performed, because the metabolites are formed inside the cells and membrane-permeant derivatives of these metabolites (e.g., 8-chloroadenosine) are not yet available. Use of derivatives that are completely stable inside a particular type of cell, as is S_p-5,6-DCl-cBIMPS in all cases tested so far, avoids this ambiguity, but such stability has to be proven for each new cell type, and the possibility remains that the derivative works through mechanisms unrelated to cAMP.

The metabolism of cAMP/AM is unlikely to generate unnatural nucleosides or nucleotides. If the supply from the extracellular medium is interrupted, exogenous cAMP should be degraded just like endogenous cAMP, although this expectation has not yet been proven by chemical assays. In many applications the generation of a transient cAMP signal may be more physiological and meaningful than the nearly irreversible activation of intracellular targets produced by more traditional derivatives.

Another point worth mentioning regards the binding of the active cAMP derivative to its immediate target, the regulatory subunit of PKA (28). The EC_{50} of S_p-5,6-DCl-cBIMPS is in the same range as that of cAMP (10, 30), whereas that of N^6-bt_1cAMP is somewhat higher. Both isoforms of the regulatory subunit (type I and type II) have two different binding sites (A and B) each for cAMP. Both of them have to be occupied for activation of the enzyme. Extensive studies have shown that cAMP analogs often have preferences for one particular binding site (29–31). With pairs of well discriminating analogs, it is sometimes possible to determine which isoform of PKA is dominant in a particular cell type, although in most tissue both forms are abundant (Refs. 32 and 33 and references cited therein). Because some of the most site-selective analogs tested so far are not particularly membrane permeant or must accumulate in high levels inside the cell because their affinity towards the enzyme is rather low (29–31), AM esters of these analogs might provide powerful tools for selectively modulating the activity of PKA isoforms in intact cells.

In conclusion, cAMP/AM is the least potent cAMP derivative of the three tested here, although it is as potent as 8-*p*-chlorophenylthio-cAMP and more potent than bt_2cAMP and 8-bromo-cAMP (12). Its rapid intracellular turnover makes it particularly useful for applications where a transient intracellular cAMP signal is required. S_p-5,6-DCl-cBIMPS has intermediate potency and enters and exits cells rapidly. Because it seems to be extraordinarily stable towards metabolic enzymes, it is most useful in long term incubations, but one may need to verify that its actions result solely from the cAMP signaling cascade. Finally, bt_2cAMP/AM is the most potent derivative tested, probably because intracellular cleavage of the nonpolar groups traps the active metabolite N^6-bt_1cAMP inside the cell, where it is only slowly hydrolyzed by cPDEs.

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