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Acetoxymethyl Esters of Phosphates, Enhancement of the Permeability and Potency of cAMP*

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Acetoxymethyl esters of alkyl or aryl phosphates can be prepared by reacting their trialkylammonium or silver salts with acetoxymethyl bromide. Because acetoxymethyl esters are rapidly cleaved intracellularly, they facilitate the delivery of organophosphates into the cytoplasm without puncturing or disruption of the plasma membrane. In addition, acylation of free hydroxyls, for example with butyryl groups, is useful both for synthetic convenience and increased hydrophobicity of the permeant derivatives. The highly polar intracellular messengers cAMP and cGMP were thus converted into uncharged membrane-permeant derivatives. Extracellularly applied N^6,2′-*O*-dibutyryl cAMP acetoxymethyl ester (Bt_2cAMP/AM) is shown to simulate intracellular cAMP in three model systems, namely dissociation of cAMP-dependent protein kinase in fibroblasts, activation of Cl^- secretion of monolayers of the human colon epithelial cell line T_{84}, and dispersion of pigment granules in angel fish melanophores. Bt_2cAMP/AM is effective at concentrations two or three orders of magnitude less than those required for commonly used membrane-permeant cAMP derivatives such as Bt_2cAMP, 8-Br-cAMP, and 8-pCPT-cAMP lacking the acetoxymethyl ester. This methodology should be of general utility for the intracellular delivery of phosphate-containing second messengers.

Second messengers are ions or small molecules that carry information from the cell membrane to targets in the inside of the cell. They play a major role in biological signal transduction and amplification (1). A common feature of most of the known second messengers, such as adenosine 3′,5′-cyclic monophosphate (cAMP) (2, 3), guanosine 3′,5′-cyclic monophosphate (4) (cGMP), *myo*-inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3), or *myo*-inositol-1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) (5), is the presence of phosphates. The correct number and position of these phosphates is essential for biological specificity and also confers extreme hydrophilicity (6, 7). This hydrophilicity prevents endogenously generated molecules from leaking out of cells and thereby maintains high sensitivity within the responding cell and freedom from cross-talk between neighboring cells. However, membrane impermeability also makes deliberate extracellular application ineffective (2, 6, 8), even though such intervention would often be very useful for research or therapeutic reasons. We have therefore sought a general method to convert organic phosphates into uncharged, lipophilic, membrane-permeant derivatives that could enter cells and then hydrolyze back to the original biologically active molecules. Lipophilic, intracellularly hydrolyzable derivatives have been useful for amino, hydroxyl, and carboxylate moieties (9–12). Acetoxymethyl (AM) esters of polycarboxylate cation indicators and chelators are in common use (12–14). Analogous acyloxyalkyl esters applied to phosphates have been less widely exploited but were introduced by Farquhar and co-workers (15). On simple model phosphates, subsequent applications seem to have been limited to potential therapeutic drugs such as phosphonoformate (foscarnet) (16), antiviral nucleotides such as 5-fluoro-2′-deoxyuridine monophosphate (17, 18), and a phosphonate-containing inhibitor of the insulin receptor kinase (19). The phosphonoformate esters proved not to be biologically useful due to failure to hydrolyze to the correct products (16), but esterification did greatly enhance the effectiveness of the antiviral nucleotides and kinase inhibitor (17–19). Considerable work has been done on *o*-nitrobenzyl esters as photolyzable ("caged") derivatives of ATP (20), cyclic nucleotides (21, 22), and inositol phosphates (23), but the emphasis has been on producing a kinetically fast and complete transition from a monoester to the active freed phosphate metabolite (24, 25), rather than as a general means of achieving membrane permeability. Nitrobenzyl esters become cumbersome if more than one are required to mask negative charges because multiple groups add considerable bulk and require high doses of UV to cause cleavage of all the groups. There has been much pharmaceutical interest in antisense oligonucleotides that are taken up by cells, but these applications do not require intracellular reversion to the native structure as may be needed to mimic phosphate-bearing second messengers. Thus, regeneration of negative charges is not crucial to hybridizing with a complementary strand but seems essential for a second messenger analogue to activate its highly specific intracellular receptors as well as for normal handling by breakdown enzymes (6, 7).

We sought to test whether acetoxymethyl esters could be usefully applied to naturally occurring phosphate-containing

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[1] The abbreviations used are: Ins, *myo*-inositol; AM, acetoxymethyl; FlCRhR, cAMP-dependent protein kinase labeled with fluorescein on the catalytic subunit and rhodamine on the regulatory subunit; I_{sc}, short circuit current; AM-Br, acetoxymethyl bromide; DIEA, diisopropylethylamine; δ, chemical shift downfield from tetramethylsilane (^{1}H spectra) or phosphoric acid (^{31}P spectra), in parts/million (ppm); 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; 8-pCPT-cAMP, 8-(*p*-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate.

second messengers such as cyclic nucleotides and inositol polyphosphates. Cyclic nucleotides were the easier class to start with because they are commercially available in quantities sufficient for organic synthesis, have only one phosphate to be protected, and contain a chromophore helpful in tracking the products during analytical separations. In addition, the ability of some cyclic nucleotide derivatives with unprotected phosphates to be active when applied extracellularly at high concentrations means that the additional effect of the AM ester can be quantified.

We now report the synthesis of acetoxymethyl esters of the second messengers cAMP and cGMP, as well as model compounds. For synthetic convenience and additional lipophilicity, free hydroxyl and amino groups of the nucleotides were already protected by butyrate esters and amides. The potency of the AM ester of Bt_2cAMP is demonstrated on several physiological systems, and shown to improve on the starting Bt_2cAMP and other traditional cAMP analogues by two or more orders of magnitude.

EXPERIMENTAL PROCEDURES

Synthesis: General Methods

Proton and ^{31}P NMR spectra were obtained in $CDCl_3$ with residual $CHCl_3$ (δ = 7.26) being used as the internal standard for ^{1}H spectra. 85% phosphoric acid 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 and are reported with the following abbreviations: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; m, complex multiplet. Fast atom bombardment mass spectroscopy (with glycerol as matrix) and precise mass determinations were performed by the mass spectroscopy facility of the University of California, Riverside. Capillary electrophoresis was performed on a Dionex CES.

Materials

Pyridine and acetonitrile were stored over activated molecular sieve (3 Å) for at least 3 days. All other solvents were purchased in highest purity available and were used as received. *N,N*-Diisopropylethylamine (DIEA) was distilled from CaH_2. Acetoxymethyl bromide (AM-Br) was prepared according to known procedures (26). All nucleotides were from Sigma. Phenylphosphonic acid was from Fluka, Switzerland. 4-Methylumbelliferylphosphate was from Boehringer, Federal Republic of Germany. All other reagents were from Aldrich.

*4-Methylumbelliferyl Phosphate Bis(acetoxymethyl)ester (**1**)*

The dilithium salt of 4-methylumbelliferyl phosphate (200 mg, 0.74 mmol) was dissolved in water, and a concentrated solution of silver acetate was added. The disilver 4-methylumbelliferyl phosphate precipitated immediately and was filtered, washed with water, and dried to a shining silver-white powder (yield: 277 mg, 79%). The silver salt (60 mg, 0.13 mmol) was suspended in 1 ml of dry CH_3CN, and 50 mg (0.33 mmol) of AM-Br was added. At frequent intervals, the mixture was treated for 2 min at a time in an ultrasonic bath (Branson B-220). Frequent monitoring by ^{1}H NMR showed the reaction to be complete after 4 h. The supernatant was evaporated to dryness to yield 38 mg (72%) of 4-methylumbelliferyl phosphate bis-(acetoxymethyl)ester (**1**); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 2.12 (s, 6H), 2.43 (s, ^{3}H), 5.73 (dAB, 4H, J_{AB} = 5.5 Hz, J_{PH} = 14.2 Hz, -CH_2-), 6.27 (s, 1H, H3), 7.17–7.25 (m, 2H, H6, H8), 7.59 (m, 1H, H5); ^{31}P NMR ($CDCl_3$, 121.5 MHz) δ −9.1; MS *m/z* $(M+H)^+$ (calculated, 401.0638; observed, 401.0625).

*Phosphate Tris(acetoxymethyl)ester (**2**)*

Silver phosphate (30 mg, 71 μmol) was suspended in 0.5 ml of dry CH_3CN, and AM-Br (22 mg, 145 μmol) was added. After frequent sonication for 20 h at room temperature, another 15 mg (100 μmol) of AM-Br was added. When the suspended solid had lost its yellow color, the mixture was centrifuged (1000 revolutions/minute, 1 min), the supernatant was evaporated to dryness, and the residue was washed with dry toluene to give phosphate tris(acetoxymethyl)ester (**2**) as a clear oil (yield 98%); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 2.15 (s, 9H), 6.45 (d, 6H, J_{PH} = 13.5Hz); ^{31}P NMR ($CDCl_3$, 121.5MHz) δ −2.25; MS *m/z* 241 $(M\text{-}CH_2OAc)^-$.

*Phenylphosphonate Bis(acetoxymethyl)ester (**3**)*

Phenylphosphonic acid (31.6 mg, 0.2 mmol) and DIEA (130 mg, 1.0 mmol) were dissolved in 1 ml of dry CH_3CN. AM-Br (77 mg, 0.5 mmol) was added, and the solution was stirred at room temperature overnight. After evaporation of the solvent, the solid residue was extracted with dry toluene. Purification of the crude product **3** was performed on a Si 60 column (10 × 40 mm) with 75% toluene, 25% ethyl acetate to yield 52 mg **3** (86%) as a clear oil. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.95 (s, 6H), 5.66 (dAB, 4H, J_{AB} = 5.3 Hz, J_{PH} = 13.8 Hz, -CH_2-), 7.30–7.55 (m, 3H), 7.70 (m, 2H). ^{31}P NMR (toluene-d_8, 121.5 MHz) δ 18.70.

*N^6,$O^{2'}$-Dibutyryl Adenosine 3',5'-Cyclic Monophosphate Acetoxymethyl Ester (**4a**/**4b**)*

Method A—The sodium salt of N^6,$O^{2'}$-dibutyryl cAMP (12.5 mg, 25 μmol) was dissolved in 1 ml of MeOH-H_2O (1:1) and passed through a Dowex 50W-X8 column (10 × 40 mm, H^+ form). The free acid was eluted with 15 ml of 50% MeOH. After evaporating to dryness, DIEA (6 mg, 50 μmol) and 1 ml of dry CH_3CN were added. The reaction was started by the addition of AM-Br (16 mg, 94 μmol). After stirring the solution at room temperature for 4 days, the reaction mixture was chromatographed directly on a Si 60 column (10 × 40 mm, 230–400 mesh) with 95% CH_3CN, 5% hexane as the eluent under slight pressure. The eluant was collected in 5-ml fractions. Fractions 5–7 contained 5.3 mg (38% yield) of the faster eluating diastereomer of dibutyryl cAMP acetoxymethyl ester (**4a**) in high purity. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.05 (t, 3H, J = 7.0 Hz), 1.12 (t, 3H, J = 7.0 Hz), 1.74 (m, 2H), 1.84 (m, 2H), 2.20 (s, 3H), 2.51 (m, 2H), 2.95 (t, 2H, J = 7.0 Hz), 4.36 (ddd, 1H, J = 2.7, 10.1, 10.1 Hz, H4'), 4.49 (dd, 1H, J = 10.0, 10.0 Hz, H5'$_{ax}$), 4.66 (dddd, 1H, J = 2.7, 10.0, 10.0, 22.1 Hz, H5'$_{eq}$), 5.67–5.95 (m, 4H, -CH_2-, H2', H3'), 6.04 (s, 1H, H1'), 8.01 (s, 1H, H2), 8.49 (broad s, 1H, N^6H), 8.78 (s, 1H, H8); ^{31}P NMR ($CDCl_3$, 121.5 MHz) δ −5.0 ppm. Fractions 8 + 9 yielded 8.7 mg of a clear oil which contained diisopropylethylammonium bromide and the slower eluting diastereomer of **4b** (2:1 (w/w) as determined by NMR, yield 2.9 mg **4b**, 21% from dibutyryl-cAMP). ^{1}H NMR ($CDCl_3$, 200 MHz), δ 0.99 (t, 3H, J = 7.0 Hz), 1.05 (t, 3H, J = 7.5 Hz), 1.70 (m, 4H), 2.18 (s, ^{3}H), 2.45 (t, 2H, J = 7.0 Hz), 2.89 (t, 2H, J = 7.5 Hz), 4.40–4.70 (m, 3H, H4',H5'$_{eq}$,H5'$_{ax}$), 5.62–5.78 (AB part of ABX, 2H, J_{AB} = 5.1 Hz, -CH_2-), 5.83 (m, 2H, H2', H3'), 6.01 (s, 1H), 8.02 (broad s, 1H, H2), 8.51 (broad s, 1H, N^6H), 8.69 (s, 1H, H8); ^{31}P NMR ($CDCl_3$, 121.5 MHz) δ −8.0 ppm; MS (**4a**/**4b** 1:1 mixture) *m/z* $(M+H)^+$ (calculated, 542.1652; observed, 542.1681).

Method B—58 mg (0.12 mmol) of the sodium salt of Bt_2cAMP was dissolved in 0.5 ml of H_2O, and 300 μl of 1.8 M $AgNO_3$ solution was added. The resulting white precipitate was filtered off, washed with H_2O, and dried to yield 30.5 mg (45%, 54 μmol) of the silver salt of Bt_2cAMP. The white powder was suspended in 1 ml of dry CH_3CN, and 51 mg (330 μmol) AM-Br were added. The suspension was frequently sonicated for 4 h at room temperature. The two resulting diastereomeric acetoxymethyl esters **4a** and **4b** were purified as described under method A to yield 2.8 mg of the fast eluting isomer **4a** (10% yield) and 9.6 mg (35%) of the slow eluting diastereomer **4b**. NMR and MS analysis of the products of both methods were identical.

*N^2,$O^{2'}$-Dibutyryl Guanosine 3',5'-Cyclic Monophosphate Acetoxymethyl Ester (**5a**/**5b**)*

The sodium salt of Bt_2cGMP (24 mg, 47 μmol) was passed through Dowex 50W-X8 (H^+ form), and the free acid was eluted with 15 ml of 50% MeOH. After evaporating to dryness, 1 ml of dry acetonitrile, 13 mg (100 μmol) of DIEA, and 21 mg (135 μmol) of AM-Br were added. The solution was stirred overnight, evaporated to dryness, dissolved in CH_3CN/hexane (95:5, v/v), and eluted over an Si 60 column (10 × 40 mm) to yield 11 mg (40%) of the two diastereomers of dibutyryl cGMP-AM (**5a**/**5b**) as a mixture. ^{1}H NMR (**5a** only, $CDCl_3$, 200 MHz) δ 1.00 (m, 6H), 1.74 (m, 4H), 2.38 (s, 3H), 2.42 (m, 2H), 2.48 (m, 2H), 4.18 (ddd, 1H, J = 4.0, 10.0, 10.0 Hz, H4'), 4.30–4.54 (m, 2H, H5'$_{ax}$, H5'$_{eq}$), 5.13 (ddd, 1H, J = 1.8, 4.1, 10.0 Hz, H3'), 5.56 (dd, 1H, J = 4.1, 4.1 Hz, H2'), 5.71 (dAB, 2H, J = 12.5, 9.1 Hz, -CH_2-), 6.04 (d, 1H, J = 4.0 Hz, H1'), 7.65 (broad s, 1H, N^2H), 10.14 (s, 1H, H8), 12.30 (broad s, 1H, N^1H); ^{31}P NMR ($CDCl_3$, 121.5 MHz) δ −5.5 and −8.5 ppm; MS *m/z* $(M+H)^+$ (calculated, 558.1601; observed, 558.1611).

Stability of Phosphate Acetoxymethyl Esters

Stability measurements of phenylphosphonate bis(acetoxymethyl)ester (**3**) and N^6,2′-*O*-dibutyryl cAMP acetoxymethyl ester (Bt_2cAMP/AM, **4a/4b**) in Hanks' balanced salt solution were carried out on a capillary electrophoresis system (Dionex CES, Sunnyvale, CA). The capillary was 67 cm long with internal diameter 75 μm, the buffer contained 10 mM sodium borate, 50 mM boric acid, pH 8.5, absorbance was measured at 254 nm, the applied voltage was 20 kV, injection-mode was gravity, and injection period was 10 s. Samples were dissolved to a final concentration of 1 mM in 50 μl of Hanks' balanced salt solution with 20 mM HEPES at pH 7.4.

Observation of FlCRhR in Response to Bt_2cAMP/AM in REF-52 Fibroblasts

The labeled cAMP-dependent kinase type II FlCRhR (type II catalytic subunit labeled with fluorescein, type II regulatory subunit labeled with tetramethyl rhodamine) was microinjected into REF-52 fibroblasts and imaged as previously described (27). Cells injected with FlCRhR were illuminated at 495 nm to excite fluorescein. The emissions of fluorescein and rhodamine were monitored with a low light level video camera at 500–530 nm and >570 nm (bandpass and long pass filters, respectively). Alternate positioning of the interference filters yielded pairs of fluorescent images for ratioing. Each ratio image was corrected by an analogous image of a shading sample of spatially uniform, equimolar fluorescein and rhodamine to cancel out geometric variations of the camera and optics as much as possible.

In Vitro Assay for Dissociation of Purified cAMP-dependent Kinase

cAMP or its derivatives was added to 50 nM labeled cAMP-dependent kinase type I FlCRhR in 135 mM KCl, 5 mM $MgCl_2$, 10 mM K-MOPS, pH 7.3, and 3 mM ATP. Kinase activation was measured by loss of intersubunit fluorescence energy transfer (27), which was monitored by ratioing the emissions at 518–586 nm while exciting at 495 nm. Ratios were converted to percent activation assuming that 0 and 100% activation result from no added or 200 μM cAMP, respectively.

Short Circuit Current Measurements of Chloride Secretion of T_{84} Cells in Response to Bt_2cAMP/AM and (Bt_2cAMP)

Cells of the human colonic epithelial cell line, T_{84}, were cultured and grown to confluence as previously described on polycarbonate membrane filters (5-μm pore size, Nuclepore, Pleasanton, CA) coated with rat tail collagen, glued to Lexan rings with an internal area of 1.98 cm^2 (28, 29). The rings were mounted into modified Ussing chambers (30) to measure cAMP-mediated chloride secretion, and bathed on each side with warmed Ringer's solution, gassed continuously with 95% O_2, 5% CO_2. The spontaneous potential difference across the monolayer was continuously short circuited via an automatic voltage clamp (W. P. Instruments, New Haven, CT) and Ag/AgCl electrodes, except for brief periods (2–5 s) at each time point when the open circuit potential difference was measured via calomel electrodes. Previous studies (31) using the T_{84} cell line have shown that the magnitude of the short circuit current (I_{SC}) is wholly reflective of the amount of transepithelial chloride secretion. The cells were allowed to equilibrate for approximately 20 min after mounting, then Bt_2cAMP/AM, Bt_2cAMP, 8-Br-cAMP, or 8-pCPT-cAMP were added, and I_{SC} was recorded for a further 60 min. For dose-response experiments, the response to a given stimulus was expressed as the change in I_{SC} recorded 12 min after addition of the cAMP derivatives.

Aggregation/Dispersion of Angelfish Melanophores on Scales in Response to Membrane-permeant cAMP Derivatives

Melanophores were isolated on scales of wild type fish (*Pterophyllum scalare*) as previously described (32). Scales in fish Ringer's (103 mM NaCl, 1.8 mM KCl, 2 mM $CaCl_2$, 0.8 mM $NaHCO_3$, 5 mM Tris) were digested with 1 mg/ml collagenase type 1A (activity >500 unit/mg) for 20 min to remove the overlaying epidermal layer. Each scale, carrying 60–100 melanophores, was placed in a dish and was rinsed three times with Ringer's. The mostly dispersed melanophores were treated with 100 nM epinephrine acting as an α_2-adrenergic agonist to lower endogenous cAMP and cause aggregation of their pigment granules. After counting the newly aggregated cells, the various cAMP derivatives were added in the continued presence of the α_2 agonist. The average degree of dispersion was quantified by multiplying the numbers of cells with aggregated, partially dispersed, and completely dispersed pigment granules by 0, 50, and 100, respectively. The sum of the products was divided by the total number of cells to give values between 0 (complete aggregation) and 100 (complete dispersion), referred to in Fig. 3, *A* and *B*, as the average dispersion score. At the end of some experiments, the cAMP derivatives were washed out, and epinephrine (100 nM) was readded to check reversibility and cell viability.

RESULTS

Synthesis

The most general and economical synthetic route to acetoxymethyl phosphate esters seemed to be alkylation of the parent phosphate anions by acetoxymethyl halides. The instability of acetoxymethanol precludes its phosphorylation. Preliminary synthetic attempts, similar to the experiments of Srivastva and Farquhar (15), were performed on 4-methylumbelliferyl phosphate and phenyl phosphonate as readily available model compounds detectable by UV absorption and bearing no competing nucleophilic centers. 4-Methylumbelliferyl phosphate bis(acetoxymethyl)ester (**1**) was successfully prepared in 73% yield by suspending the disilver salt of 4-methylumbelliferyl phosphate in dry acetonitrile, adding AM-Br (26) and sonicating the heterogeneous mixture at frequent intervals for 24 h. The ^1H NMR of the supernatant showed an AB doublet at 5.7 ppm for the methylene group of the acetoxymethyl ester, a typical pattern for all phosphate acetoxymethyl esters reported here. The synthesis of phosphate tris(acetoxymethyl)ester (**2**) offered a possibility to directly monitor the progress of the reaction. Yellow Ag_3PO_4 was reacted with AM-Br as described above. Disappearance of the color after 36 h indicated completion of the reaction. The product was the only compound in the organic phase (98% yield).

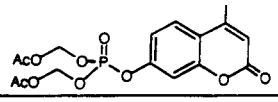
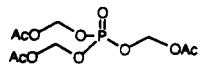
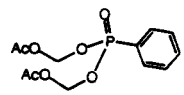
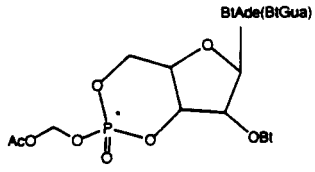
An alternative to silver salts was desirable for polyphosphates or molecules bearing oxidizable functionalities. Direct treatment of phenylphosphonic acid with an excess of the hindered base DIEA and AM-Br eventually gave an 86% yield of the phenylphosphonate bis(acetoxymethyl)ester (**3**). Analogous reactions worked albeit in lower yield (Table I) for the biologically more interesting Bt_2cAMP/AM, **4a/4b**, and Bt_2cGMP/AM, **5a/5b**. The commercially available sodium salts of Bt_2cAMP and Bt_2cGMP were converted into the free acids on Dowex 50W-X8 and then into DIEA salts. Reaction took place in dry CH_3CN with an excess of DIEA and AM-Br for 5 days at room temperature. Both nucleotide AM esters were purified on Silica Gel 60 (CH_3CN/hexane 19:1, v/v) after evaporation of the solvent. The two diastereomers of Bt_2cAMP/AM (**4a/4b**) were isolated in yields of 37 and 21% for the fast and slow-eluting isomers, the latter coeluting with residual DIEA. ^{31}P NMR resonances were −5.0 and −8.0 ppm, respectively, but absolute configurations were not determined. The analogous two diastereomers of Bt_2cGMP/AM (**5a/5b**) could not be separated under the described conditions but were free of DIEA. Bt_2cAMP/AM was also prepared by alkylating the silver salt of Bt_2cAMP with AM-Br in CH_3CN with frequent sonication for 24 h. Interestingly, these heterogeneous conditions reversed the enantiomeric preference, giving the fast and slow-migrating isomers in 10 and 35% yields.

Stability of Acetoxymethyl Esters against Hydrolysis

In order to determine the lifetime of acetoxymethyl esters in incubation media, we investigated the hydrolysis of phenylphosphonate bis(acetoxymethyl)ester (**3**) and Bt_2cAMP/AM (**4a/4b**) in Hanks' balanced salts solution with 20 mM HEPES at pH 7.4, a typical extracellular medium for mammalian cells. The esters and their hydrolysis products were

TABLE 1

Structures of acetoxymethyl esters of various organic phosphates

comp.	structure	counter[a] ion M^+	yield[b]	^{31}P-NMR [ppm]
1		Ag^+	72%	-9.1
2		Ag^+	98%	-2.25
3		$HDIEA^+$	86%	18.70
4a/4b		$HDIEA^+$ Ag^+	59% 52%	-8.0/[c] -5.0
5a/5b		$HDIEA^+$	40%	-5.5/[c] -8.5

[a] M^+ specifies the counter ion for the phosphate-containing starting material; $HDIEA^+$ = diisopropylethylammonium.
[b] Yield by weight unless otherwise noted.
[c] Shift values for both diastereomers.

analyzed by capillary electrophoresis (Dionex CES, Sunnyvale, CA) in sodium borate, pH 8.5. Frequent monitoring of the hydrolyses gave half-lives of 23 days and 36 h for **3** and **4a/4b**, respectively (data not shown). The difference in the reaction time can be explained by the generally higher stability of phosphonate diesters compared to phosphate triesters (33).

Biological Tests

Activation of Intracellular Protein Kinase A—The premier target of cAMP in most cells is the cAMP-dependent protein kinase (34). To show that this important enzyme can be activated by extracellular application of Bt_2cAMP/AM, we used a recently developed assay for protein kinase A activation in single cells (27). When protein kinase A is doubly labeled with fluorescein on its catalytic subunits and rhodamine on its regulatory subunits to produce FlCRhR, fluorescence energy transfer from the fluorescein to rhodamine occurs in the holoenzyme complex but is disrupted upon activation and dissociation of the subunits. The change in the ratio of fluorescein to rhodamine emissions parallels the increase in kinase activity and can be nondestructively imaged in single cells. REF-52 fibroblasts were microinjected with FlCRhR and emission ratio images recorded at room temperature as previously described (27). 30 min after injection, 0.1, 1, or 10 μM Bt_2cAMP/AM were added extracellularly (Fig. 1*A*). The highest dose yielded a maximal change in fluorescence ratio within 15 min. The intermediate dose gave a shallower slope and a lower plateau to approximately 80% of the maximal change. Interestingly, the onset of the separation of regulatory and catalytic subunit of FlCRhR occurred roughly 2 min after the addition of the cAMP derivative. Much the same delay and overall time course occurred with nonesterified Bt_2cAMP, although much higher concentrations, 1 mM, were required (Fig. 1*B*). Other widely used, supposedly lipophilic cAMP

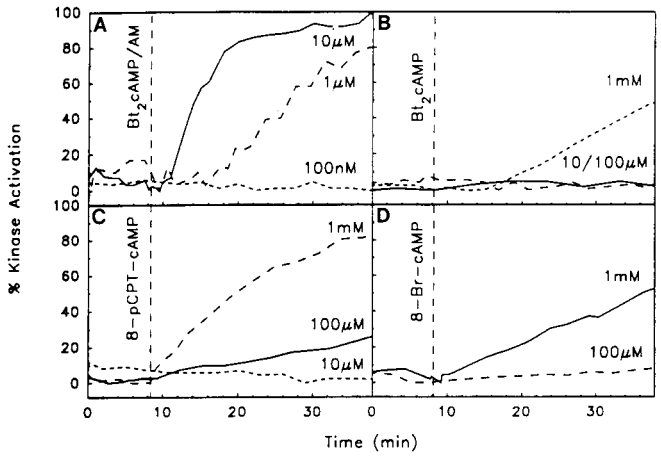


FIG. 1. **Dissociation of intracellular cAMP-dependent protein kinase by extracellularly applied cAMP derivatives.** The kinase had been labeled *in vitro* with fluorescein on its catalytic subunits and rhodamine on its regulatory subunits, recombined into holoenzyme (FlCRhR), and microinjected into single REF-52 fibroblasts about 30 min before the start of these records. Various parallel runs with different doses of Bt_2cAMP/AM, as well as other cAMP derivatives, have been superimposed (*dashed* and *solid lines*, respectively) with additions at the vertical time marker. Each trace is the mean of 3–10 single-cell experiments. An increase in fluorescence ratio of fluorescein (500–530 nm) to rhodamine (>570 nm) emission indicates dissociation of FlCRhR and loss of energy transfer. Ratio values were normalized in each experiment to give percent kinase activation. Minimum values were obtained by the lowest fluorescence ratio, usually shortly before the addition of a cAMP derivative. Maximal values shown here were obtained by applying a high dose of forskolin (50 μM) to elevate cAMP in the cells maximally at the end of the experiment (not shown).

derivatives showed no delay in beginning to activate protein kinase A, but millimolar concentrations were still required (Fig. 1, *C* and *D*).

To prove that intracellular enzymatic hydrolysis of the ester groups is required, we examined the binding properties of Bt_2cAMP/AM and Bt_2cAMP to FlCRhR *in vitro*. The highest concentration of Bt_2cAMP/AM used in the other assays (10 μM) gave no separation of the subunits, while Bt_2cAMP was roughly 1/100 as potent as cAMP probably due to contamination by 1% monobutyryl-cAMP as specified by the supplier (Sigma) (Table II).

Activation of Intestinal Cl^- Transport—The above fluorescence assay for dissociation of protein kinase A focuses on the immediate molecular target of cAMP action but involves a relatively novel readout. We felt that comparison with more traditional measures of physiological activation at the whole tissue level would also be valuable, especially where a direct comparison could again be made to Bt_2cAMP and other cAMP derivatives lacking the AM ester. One of the many well known cell functions controlled by cAMP is intestinal transepithelial Cl^- secretion (35). A convenient test system is the intestinal cell line T_{84}, in which chloride secretion can be continuously monitored by mounting confluent monolayers of cells in Ussing chambers (30). Fig. 2*A* shows the Cl^- secretion measured as short circuit current (I_{SC}) across the cells. The addition of Bt_2cAMP/AM at a concentration of 3 μM to the serosal bathing solution caused an increase in I_{SC} with a maximum after 20 min. Higher concentrations of the derivative caused faster but not significantly greater responses, whereas lower concentrations reached lower maximum I_{SC} values. The I_{SC} values obtained at an arbitrary intermediate time, 12 min after addition of various cAMP derivatives, were used to determine the dose dependence (Fig. 2*B*). The dose-response curves were parallel, with EC_{50} values of 2 and 400 μM for Bt_2cAMP/AM and Bt_2cAMP, respectively. Therefore, the introduction of the acetoxymethyl group on the phosphate increased the potency by 200-fold in this assay, presumably by circumventing the permeability barrier. Furthermore, the acetoxymethyl ester seems to be cleaved inside T_{84} cells without significant delay, since the two agents gave essentially indistinguishable kinetics of activation. Experiments with the cAMP derivatives 8-Br-cAMP and 8-pCPT-cAMP showed activation of Cl^- secretion with EC_{50} values of 1.5 mM and 100 μM, respectively. (The EC_{50} values of the derivatives without AM ester suggest that increasing lipophilicity results in an enhanced potency to induce Cl^- secretion.)

Intracellular Motility in Angelfish Melanophores—The above intestinal cell system is one in which classical cAMP derivatives are active extracellularly, albeit at high concentrations, so that phosphate esterification is helpful but not essential. A greater challenge would be a cAMP-controlled preparation in which nonesterified Bt_2cAMP is ineffective

TABLE II

In vitro assay for dissociation of purified cAMP-dependent kinase

	cAMP			Bt_2-cAMP[a]		Bt_2cAMP/AM
Conc. μM	1	10	200[b]	10	100	10
% kinase activation[c]	65	90	≡100	15	59	0

[a] The slight residual activity of Bt_2cAMP is probably due to an impurity of N^6-monobutyryl cAMP (1%) as specified by the supplier.

[b] 200 μM cAMP was considered the maximal dose necessary to fully dissociate FlCRhR.

[c] Labeled cAMP-dependent kinase type I (FlCRhR). This labeled isoform is more stable against subunit dissociation in the absence of cAMP at the low enzyme concentrations used in this assay than labeled type II.

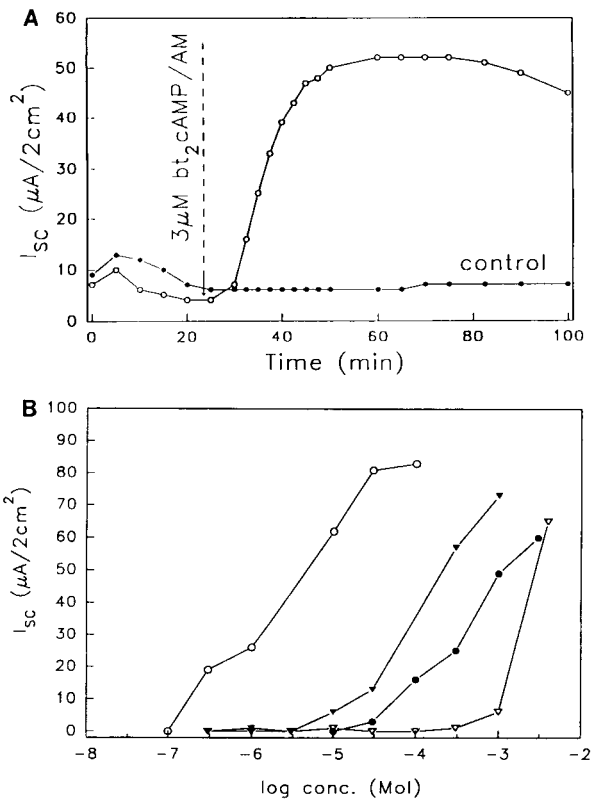


FIG. 2. **Chloride secretion in response to cell permeant cAMP-derivatives.** *A*, time course of the Bt_2cAMP/AM effect on short circuit current (I_{SC}) across T_{84} cell monolayers in modified Ussing chambers (30). 3 μM Bt_2cAMP/AM was added to the basolateral reservoir 24 min after mounting (○). In the control experiment (●), 3 μM Bt_2cGMP/AM was added instead. Its lack of effect probably reflects the known inability of N^2-Bt-cGMP to activate cGMP-dependent protein kinase (6), whereas N^6-Bt-cAMP does activate cAMP-dependent protein kinase. *B*, dose-response relations for Bt_2cAMP/AM (○) *versus* 8-pCPT-cAMP (▼), Bt_2cAMP (●), and 8-Br-cAMP (▽). I_{SC} values were taken 12 min after adding each compound. Only one concentration of one cAMP derivative was applied per monolayer.

even at millimolar concentrations, the highest practical dose range. Fish dermal chromatophores exhibit a tightly regulated movement of pigment granules either inward into a highly aggregated central mass, or outward, dispersing the pigment throughout the cell. In angelfish (*Pterophyllum scalare*) melanophores this movement is microtubule based and cAMP regulated (32, 36) but relatively refractory to external cAMP analogues. Melanophores permit a visual single-cell assay for the ability of cAMP analogues to enter cells and mimic cAMP actions. The melanophores were isolated on angelfish scales and the epidermis was stripped off (see "Experimental Procedures"). The 60–100 melanophores/scale were pretreated with an α_2-adrenergic agonist to reduce endogenous cAMP and start with full aggregation. Extracellular Bt_2cAMP/AM then caused dispersion of the pigment in a dose-dependent manner (Fig. 3*A*). A concentration of 100 μM Bt_2cAMP/AM was enough to cause essentially complete dispersal. However, 1 mM gave a slightly faster onset of action and could not be readily reversed by removal of the extracellular Bt_2cAMP/AM and administration of epinephrine, whereas the effect of 100 μM was easily reversed (Fig. 3*B*). Dispersion was just detectable at 1 μM and half-maximal near 10 μM (Fig. 3*A*). By comparison, 1 mM Bt_2cAMP was unable to cause any detectable dispersion. Hence the AM ester group increased the potency by more than 1000 in this assay. The effectiveness

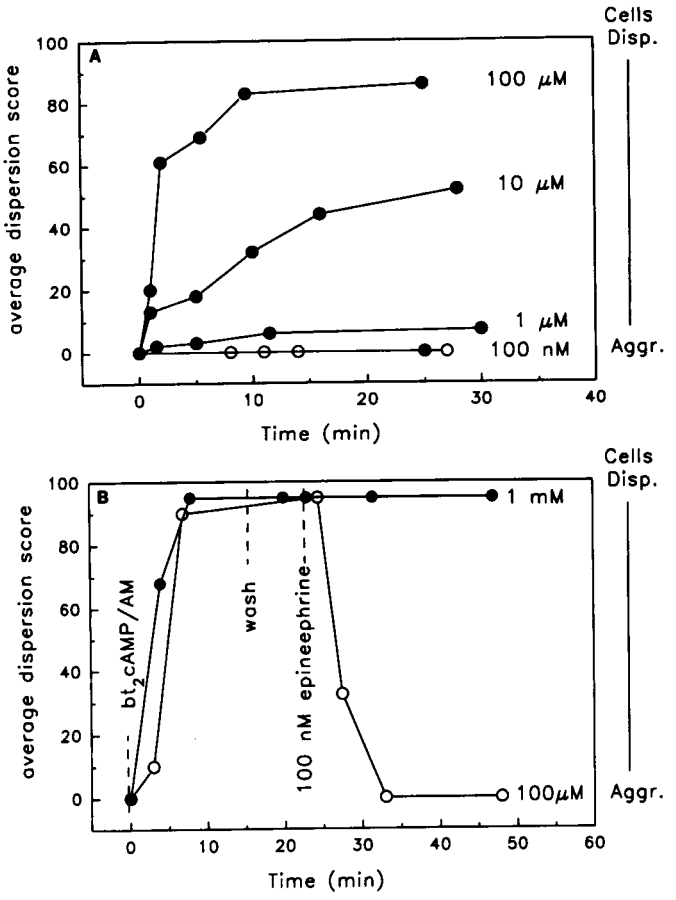


FIG. 3. **Dispersion of 60–100 angelfish melanophores on scales by Bt_2cAMP/AM or Bt_2cAMP.** Average dispersion score (see "Experimental Procedures") of 0 or 100 mean complete aggregation or dispersion respectively. *A*, dose-response of Bt_2cAMP/AM (●) compared to 1 mM Bt_2cAMP (○). Only one concentration of cAMP derivative was applied per scale. 100 nM epinephrine was present throughout to suppress endogenous production of cAMP. *B*, dispersion by high doses of Bt_2cAMP/AM followed by a wash and readdition of 100 nM of epinephrine to test for recovery.

of Bt_2cAMP/AM argues that the inertness of Bt_2cAMP in melanophores is due to poor permeability rather than susceptibility of Bt-cAMP to phosphodiesterases or selectivity of kinase binding sites for cAMP substitution (37).

DISCUSSION

Synthesis—Despite the low nucleophilicity and poor solubility of most phosphate ester polyanions in organic solvents, they proved susceptible to alkylation with acetoxymethyl bromide in fair to good yields. Silver salts were useful for compounds with single phosphates such as 4-methylumbelliferyl phosphate, cyclic nucleotides, and inorganic orthophosphate itself, and had the advantage of easy product isolation due to the insolubility of silver bromide. We also found that homogeneous reaction conditions with diisopropylethylamine instead of silver could work about as well, which is helpful because such conditions are more promising for reactions with polyphosphates such as derived from *myo*-inositol. The cyclic nucleotides had already been modified with butyryl groups to mask their free hydroxyl or amino groups to increase their solubility in organic solvents, and to enforce regioselectivity of alkylation. However, preliminary trials with unmodified cyclic AMP showed that the butyryl groups were not absolutely necessary, though synthetic yields declined considerably (data not shown). Butyryl groups, particularly amides, may sometimes be problematic if they are not hydrolyzable inside cells. Moreover, instances are known where butyric acid can have its own pharmacological effects at high concentrations (38–41). Fortunately, these are usually easily detectable by control experiments.

Stability and Biochemical Properties—The acetoxymethyl esters were surprisingly stable to hydrolysis in the absence of enzymes or strong base. They survived brief thin layer or column chromatography on silica. The loss of ester groups in buffer was moderate (5%/h) for Bt_2cAMP/AM at room temperature, measured by capillary electrophoresis. However, acetylesterase applied *in vitro* caused rapid hydrolysis as expected. Intracellular enzymatic hydrolysis is quite useful because it greatly increases the efficiency of utilization of the membrane-permeant derivative (12). Even though the intracellular volume is a tiny fraction of the total volume of a cell suspension, most of the added molecules will end up trapped inside the cells. By contrast, if the extra- and intracellular rates of removal of protecting groups were the same, most of the molecules would undergo wasteful hydrolysis in the much larger extracellular space. In the case of Bt_2cAMP/AM, extensive structure-activity relationships (6) show that both the phosphate charge and 2′-hydroxyl must be free for kinase activations, so that two enzymatic hydrolyses are necessary. The actual intracellular concentration of the active phosphate derivative will presumably reflect a balance between the rate of hydrolysis of the protective groups and the rate of catabolism of the intracellular messenger or analogue. If the breakdown rate is quite high, as expected for messengers involved in fast signal transduction, a relatively large concentration of the acetoxymethyl derivative may be required.

The hydrolysis of acetoxymethyl esters produces not only the protonated phosphoric acid derivative but also acetic acid and formaldehyde as byproducts. Of these, formaldehyde is obviously the most worrisome with regards to biological toxicity. However, extensive experience with acetoxymethyl esters of carboxylic acids applied to isolated cells and tissues has revealed surprisingly little toxicity at least in the short term (42, 43). In the current types of applications, micromolar concentrations of formaldehyde are gradually released over minutes to hours, whereas concentrations used for biological fixation are at least hundreds of millimolar. A few cell types such as retina, which is particularly sensitive to formaldehyde, do suffer when acetoxymethyl esters of carboxylates are applied alone. However, these toxic effects can be prevented by antidotes such as ascorbate and pyruvate (42, 43). These innocuous antidotes should be equally effective against formaldehyde produced from acetoxymethyl esters of phosphates. Another byproduct to be kept in mind is the proton load from the acids produced. Fortunately, most cells seem well supplied with pH-homeostatic mechanisms to cope with sizable endogenous production of acids during normal metabolism. Direct measurement with BCECF showed no significant pH drop during application of other phosphate AM esters at much higher doses than used here (44). In general, AM esters of chemically related but physiological inert phosphates provide ready controls for toxic effects of the delivery mechanism. Even the tris(acetoxymethyl) ester of inorganic orthophosphate is probably a good control for formaldehyde and proton side effects. Whether such toxicity would be more severe over longer time scales in intact organisms compared to acute applications to isolated cells remains to be determined. If so, other acyloxyalkyl esters, such as 1-acetoxyethyl to release acetaldehyde instead of formaldehyde, would be obvious candidates.

The butyryl groups in Bt_2cAMP and the substituents in 8-

Br-cAMP and 8-pCPT-cAMP are commonly thought of as rendering cAMP membrane-permeant. However, they do not mask the phosphate, whose negative charge is probably a greater impediment to membrane permeability than the hydrogen bonding capability of the N^6-amino and 2′-hydroxyl groups. The ability of the AM ester group on phosphate to improve the potency by over two orders of magnitude, despite the requirement for an additional hydrolytic step, shows that a major increase in permeability is possible and valuable. Thus, the unmasked single negative charge on a phosphodiester group hinders membrane permeability, by at least two orders of magnitude, a rough quantitative estimate for the barrier to loss of precious intracellular phosphodiesters or entry of potentially valuable phosphate-containing drugs. Intracellular cleavage of the AM ester group as well as at least one of the butyrates (on the 2′-OH) proved to be essential because both Bt_2cAMP and Bt_2cAMP/AM were not able to activate the cAMP-dependent kinase *in vitro*, as shown in Table II. Presumably, the relative resistance of N^6-monobutyryl cAMP, 8-Br-cAMP, and 8-pCPT-cAMP to phosphodiesterases is the major factor giving them some potency despite poor permeability (6, 45). Yet in some tissues like the fish melanophores, Bt_2cAMP cannot elicit intracellular cAMP actions at practical concentrations. AM esters of Bt_2cAMP or of other phosphodiesterase-resistant cAMP analogues should be helpful in such cases. Cyclic nucleotides caged with *o*-nitrobenzyl esters are also membrane permeant and offer faster kinetics of onset because they are gated by a photolytic flash (21, 24). However, AM esters are simpler to apply, because no UV is needed, and probably make more efficient use of any given number of molecules because enzymatic hydrolysis concentrates their release to the intracellular compartment whereas photolysis occurs everywhere. Success with cAMP esterification is the first step toward efficient transmembrane delivery of other messengers and modulators such as cGMP, GTP analogues, inositol polyphosphates, and cyclic ADP-ribose, which will be discussed in future reports.

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