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SPECIAL REPORT

Enhanced inhibition of the EDHF phenomenon by a phenyl methoxyalaninyl phosphoramidate derivative of dideoxyadenosine

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In rabbit arteries endogenous production of cAMP facilitates electrotonic signalling *via* gap junctions, thus explaining the ability of P-site inhibitors of adenylyl cyclase to attenuate EDHF-type responses. In the present study, we show that a lipophilic phosphoramidate pronucleotide derivative of dideoxyadenosine, 2′,3′-ddA-PMAPh, exhibits enhanced activity as an inhibitor of EDHF-type smooth muscle hyperpolarizations induced by acetylcholine (ACh) compared to the parent nucleoside 2′,3′-ddA, and that the effects of both compounds can be reversed by the cAMP phosphodiesterase inhibitor IBMX. Neither 2′,3′-ddA nor 2′,3′-ddA-PMAPh depress ACh-evoked endothelial hyperpolarization directly. Modifications in the lipophilicity of dideoxyadenosine and its direct intracellular delivery as a mononucleotide may thus enhance the ability to inhibit adenylyl cyclase and depress electrotonic signalling *via* myoendothelial gap junctions.
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Abbreviations: ACh, acetylcholine; Ado, adenosine; cAMP, cyclic adenosine 3′,5′-monophosphate; ddA, dideoxyadenosine; DMSO, dimethylsulphoxide; EDHF, endothelium-derived hyperpolarizing factor; IBMX, 3-isobutyl-1-methylxanthine; PMAPh, phenyl methoxyalaninyl phosphoramidate

Introduction In rabbit arteries, agonists such as acetylcholine (ACh) stimulate an endothelial hyperpolarization that is transmitted through the vascular wall *via* myoendothelial and homocellular smooth muscle gap junctions to promote mechanical relaxation (Chaytor *et al*., 1998; 2003; Griffith *et al*., 2002). Electrotonic signalling may therefore underpin the NO- and prostanoid-independent relaxations that are often attributed to a freely transferable endothelium-derived hyperpolarizing factor or EDHF. ACh also stimulates prostanoid-independent synthesis of cAMP by the endothelium, thereby facilitating the spread of endothelial hyperpolarization through an action that involves phosphorylation of the connexin proteins that form gap junction channels and/or their rapid recruitment to the cell membrane (Paulson *et al*., 2000; Van Rijen *et al*., 2000; Taylor *et al*., 2001; Griffith *et al*., 2002). P-site inhibitors of adenylyl cyclase, such as the dideoxyadenosine nucleosides 2′,3′-ddA and 2′,5′-ddA, have thus been shown to attenuate EDHF-type smooth muscle hyperpolarizations and relaxations of rabbit arteries and veins when applied at concentrations in the range 30–200 μM (Griffith & Taylor, 1999; Taylor *et al*., 2001; Griffith *et al*., 2002; Chaytor *et al*., 2002; 2003). In cultured bovine endothelial cells, such P-site ligands also attenuate forskolin and isoproterenol-evoked increases in cAMP content with IC_{50} values in the range 300–500 μM (Legrand *et al*., 1990), whereas in experiments with cell-free fractions or recombinant adenylyl cyclase IC_{50} values are in the low micromolar range (Johnson *et al*., 1997; Desaubry & Johnson, 1998; Shoshani *et al*., 1999). Such apparently discrepant observations are likely to reflect the relatively poor ability of nucleosides to cross cell membranes and access the intracellular compartment, as well as their metabolism to dideoxyinosine by adenosine deaminase, followed by subsequent degradation to hypoxanthine in intact cell systems (Cooney *et al*., 1987).

A variety of purine nucleosides, including 2′,3′-ddA, have also been shown to become potent inhibitors of viral reverse transcriptases following intracellular conversion to their corresponding triphosphates by kinases (Cooney *et al*., 1987). The antiviral activity of such nucleosides can therefore be enhanced by the addition of a phosphate moiety shielded by hydrophobic groups, thereby generating a lipophilic prodrug that readily enters the cell, where it is converted to the free mononucleotide (Siddiqui *et al*., 1999; Cahard *et al*., 2004). Since the addition of phosphates to ddA nucleosides is also known to increase their inhibitory activity against adenylyl cyclase (Johnson *et al*., 1997; Desaubry & Johnson, 1998; Shoshani *et al*., 1999), in the present study, we have investigated whether a 'masked phosphate' phenyl methoxyalaninyl phosphoramidate derivative of 2′,3′-ddA (2′,3′-ddA-PMAPh) is a more potent inhibitor of the EDHF phenomenon than the parent nucleoside itself.

Methods Iliac arteries were obtained from male NZW rabbits (2–2.5 kg) killed by sodium pentobarbitone (120 mg kg^{-1}; i.v.) and transferred to oxygenated (95% O_2, 5% CO_2) Holmans buffer (composition in mM: 120 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.3 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose and 10 sucrose) at room

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temperature. Arterial strips were prepared and held adventitia down in an organ chamber superfused (2 ml min^{-1} at 37°C) with oxygenated Holmans solution containing the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) and the cyclooxygenase inhibitor indomethacin (10 μM). Endothelial and subintimal smooth muscle membrane potentials were recorded by conventional whole-cell patch-clamp and sharp electrode intracellular techniques, respectively, using glass capillary microelectrodes (tip resistance 80–180 MΩ) filled with 3 M KCl. ACh was administered at a concentration (3 μM) that evokes maximal hyperpolarization in the rabbit iliac artery (Griffith *et al*., 2002; Chaytor *et al*., 2003). In some experiments, 2′,3′-ddA, 2′,3′-ddA-PMAPh or the corresponding derivative of adenosine (Ado-PMAPh), synthesized as previously described (Siddiqui *et al*., 1999), were included in the buffer 30 min prior to the addition of ACh. Stock solutions of these agents were prepared in DMSO, which had no effect in control experiments.

Statistics Hyperpolarizations evoked by ACh under the different experimental conditions were compared by ANOVA, followed by Dunnett's multiple comparison test. IC_{50} values for 2′,3′-ddA and 2′,3′-ddA-PMAPh were derived as means and 95% confidence intervals (GraphPad Prism 3.03). Results are otherwise given as mean±s.e.m., where n denotes the number of animals studied for each data point. $P<0.05$ was considered significant.

Results The resting membrane potential of subintimal smooth muscle cells was −45.2±2.4 mV ($n=20$) and not affected by incubation with 2′,3′-ddA (50–500 μM), 2′,3′-ddA-PMAPh (1–200 μM), Ado-PMAPh (10–300 μM) or the combination of IBMX (30 μM) with 2′,3′-ddA (200–500 μM) or 2′,3′-ddA-PMAPh (50–200 μM). Addition of 3 μM ACh evoked subintimal smooth muscle hyperpolarizations of 18.0±2.6 mV that were unaffected by preincubation with 50 μM 2′,3′-ddA (Figure 1; $n=12$ and 4, respectively). However, increasing the concentration of 2′,3′-ddA to 200 and 500 μM caused a reduction in the ACh-evoked hyperpolarization to 9.3±2.3 and 10.0±1.2 mV, respectively (Figure 1; $n=3$ and 4, $P<0.05$ for both). The combination of IBMX (30 μM) and 2′,3′-ddA (200 μM) did not significantly alter ACh-evoked hyperpolarization compared to control (Figure 1; $n=4$). Incubation with 2′,3′-ddA-PMAPh caused a concentration-dependent inhibition of ACh-evoked responses, with smooth muscle hyperpolarization reduced from 22.5±1.8 to 13.8±2.5, 8.8±1.5 and 4.0±1.7 mV in the presence of 10, 50 and 200 μM 2′,3′-ddA-PMAPh, respectively (Figure 1; $n=4$–8, $P<0.05$, 0.005 and 0.001, respectively). ACh-evoked hyperpolarizations observed in the presence of both IBMX (30 μM) and 2′,3′-ddA-PMAP (50 or 200 μM) did not significantly differ from control (Figure 1; $n=3$ and 4, respectively). IC_{50} values for 2′,3′-ddA and 2′,3′-ddA-PMAP were estimated as 86.2 (72.1–102.6) μM and 12.6 (8.2–18.6) μM, respectively. Incubation with Ado-PMAPh at concentrations up to 300 μM did not significantly affect smooth muscle hyperpolarizations evoked by ACh (Figure 2).

In patch-clamp experiments, the resting membrane potential of endothelial cells was −45.0±3.2 mV ($n=6$) and not significantly different from the corresponding subintimal smooth muscle membrane potential. Neither 200 μM 2′,3′-ddA nor 200 μM 2′,3′-ddA-PMAPh affected the resting

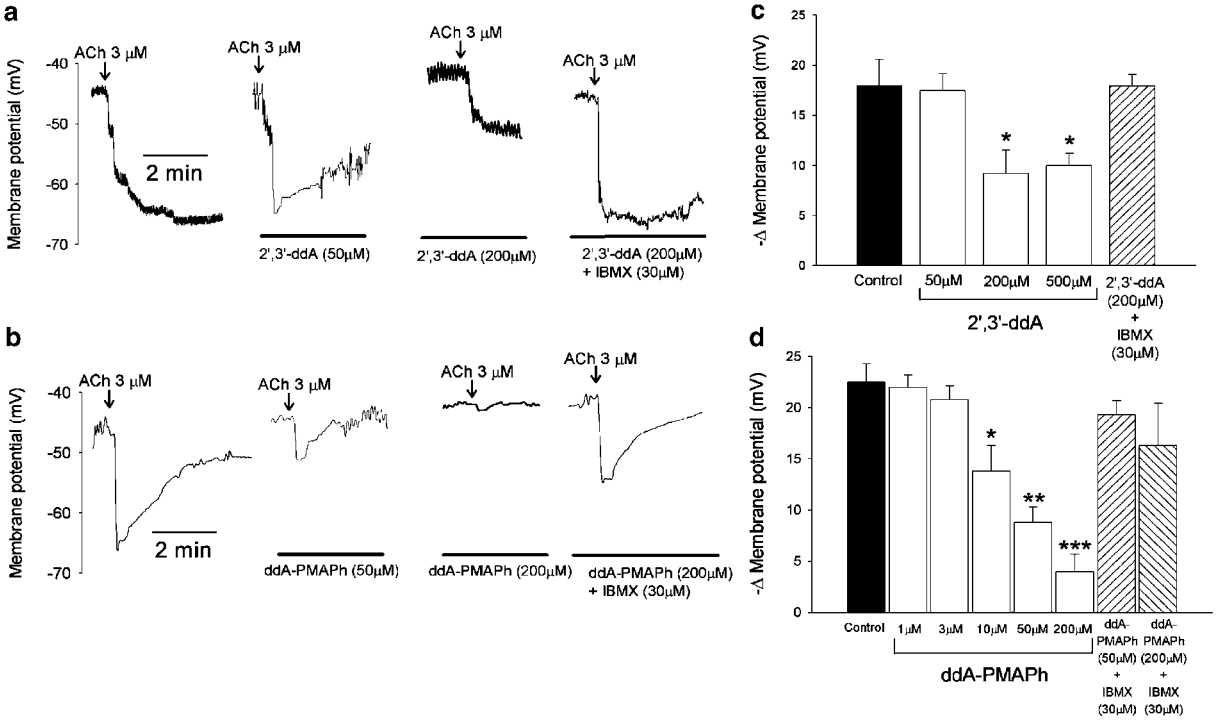

Figure 1 Concentration-dependent inhibition of subintimal smooth muscle hyperpolarizations evoked by 3 μM ACh by 2′,3′-ddA and its phenyl methoxyalaninyl phosphoramidate derivative 2′,3′-ddA-PMAPh in the rabbit iliac artery. (a, b) Representative traces showing that the cAMP phosphodiesterase inhibitor IBMX prevented the inhibitory effects of both compounds. (c, d) Histograms giving peak changes in membrane potential as mean±s.e.m. ($n=3$–12). *, ** and *** denote $P<0.05$, $P<0.005$ and $P<0.001$, respectively.

endothelial membrane potential or hyperpolarizations stimulated by 3 μM ACh (Figure 3).

Discussion We have shown that a lipophilic phosphoramidate pronucleotide derivative of dideoxyadenosine, 2′,3′-ddA-PMAPh, possesses enhanced activity as an inhibitor of EDHF-type subintimal smooth muscle hyperpolarizations induced by ACh compared to its parent compound 2′,3′-ddA in the rabbit iliac artery. At a concentration of 200 μM, 2′,3′-ddA-PMAPh almost abolished the smooth muscle hyperpolarizing response to ACh, whereas the maximal reduction obtained with 2′,3′-ddA was only ~50% at concentrations of 200–500 μM. The IC_{50} value for 2′,3′-ddA-PMAPh was correspondingly decreased some seven-fold in magnitude compared to that for 2′,3′-ddA. Whole-cell patch clamping confirmed that neither compound affected the initiating endothelial hyperpolarizing response evoked by ACh. These data are consistent with the hypothesis that 2′,3′-ddA and 2′,3′-ddA-PMAPh modulate the EDHF phenomenon by inhibiting electrotonic signalling *via* myoendothelial gap junctions, rather than the mechanisms that underpin endothelial hyperpolarization. Their action was confirmed to be linked to inhibition of adenylyl cyclase, since the ability of both compounds to attenuate the smooth muscle hyperpolarizing response to ACh was prevented by IBMX, a cAMP phosphodiesterase inhibitor that does not enhance endothelium-dependent subintimal smooth muscle hyperpolarizations evoked by ACh in the rabbit iliac artery when administered alone (Griffith *et al*., 2002). Although IBMX also inhibits cGMP phosphodiesterases, we have previously shown that ACh fails to promote cGMP accumulation in the rabbit iliac artery in the presence of 300 μM L-NAME, so that activation of smooth muscle K^+ channels *via* cGMP-dependent phosphorylation does not complicate interpretation of the findings (Taylor *et al*., 2001; Chaytor *et al*., 2002).

The first step in the intracellular metabolism of 2′,3′-ddA-PMAPh is a carboxylesterase-mediated hydrolysis of the carboxylic ester function of the amino-acid moiety, followed by spontaneous elimination of phenol (Figure 4, *step 1*). Subsequent enzymatic cleavage of the phosphorus–nitrogen bond (Figure 4, *step 2*) delivers the nucleotide monophosphate 2′,3′-dd-5′-AMP directly, thereby bypassing conversion of 2′,3′-ddA to 2′,3′-dd-5′-AMP by adenosine kinase or *via* dideoxyinosine (2′,3′-ddI) (Siddiqui *et al*., 1999). Since the ability of 2′,3′-ddA and 2′,5′-ddA nucleosides to inhibit adenylyl cyclase is markedly increased by addition of successive phosphate groups at the 5′ or 3′ locations, respectively, inhibition of EDHF-type hyperpolarizations by 2′,3′-ddA-PMAPh may not simply involve direct intracellular delivery of 2′,3′-dd-5′-AMP, but also its subsequent conversion by nucleotide kinases to 2′,3′-dd-5′-ATP (Figure 4, *step 3*), a potent inhibitor of rat brain adenylyl cyclase with an IC_{50} value of 0.76 μM (Johnson *et al*., 1997; Desaubry & Johnson, 1998; Shoshani *et al*., 1999). It is unlikely that non-nucleotide breakdown products of the prodrug (methanol, phenol and alanine) themselves impair gap junctional communication, as the PMAPh derivative of adenosine, which is expected to release 5′-AMP and identical breakdown products (Cahard *et al*., 2004), did not affect ACh-evoked

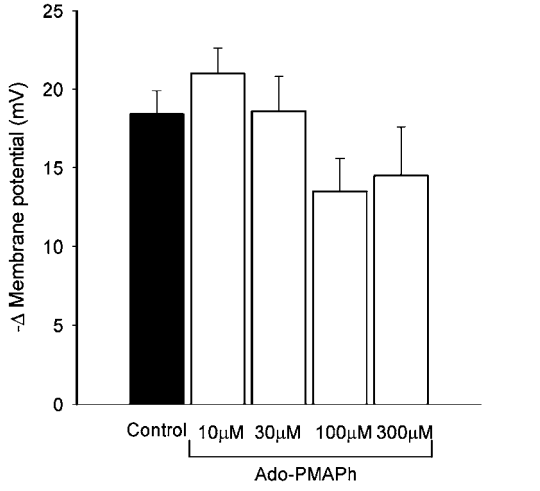

Figure 2 Histograms showing that the PMAPh derivative of adenosine did not affect subintimal smooth muscle hyperpolarizations evoked by 3 μM ACh in the rabbit iliac artery ($n = 5$–8).

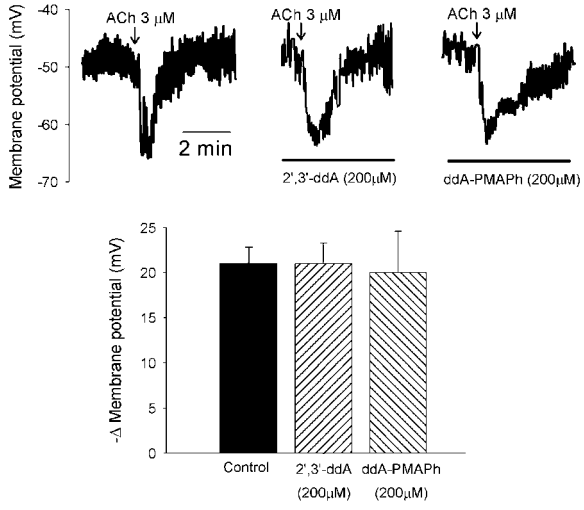

Figure 3 Representative traces and histograms showing that 2′,3′-ddA and 2′,3′-ddA-PMAPh did not attenuate endothelial hyperpolarizations evoked by 3 μM ACh in rabbit iliac arteries in whole-cell patch-clamp experiments ($n = 3$–6).

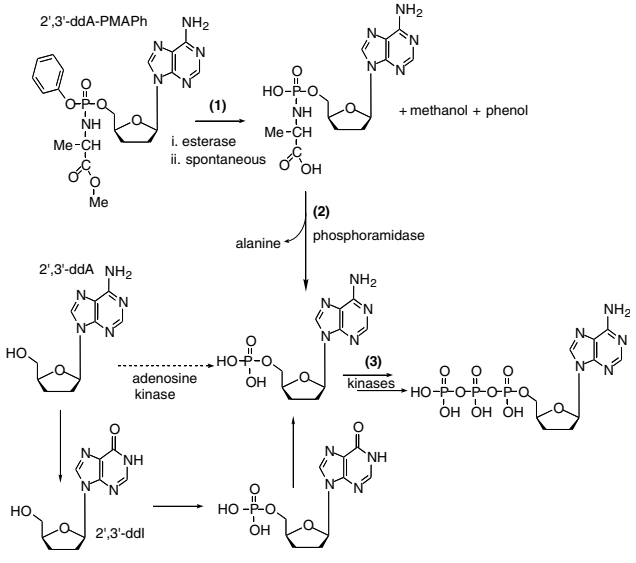

Figure 4 Schematic representation outlining the intracellular metabolism of 2′,3′-ddA-PMAPh and 2′,3′-ddA.

EDHF-type hyperpolarizations at concentrations up to 300 μM. Previous studies have shown that 5′-AMP itself is relatively inactive against adenylyl cyclase ($IC_{50} \sim 150\ \mu$M for the rat brain enzyme) and the corresponding triphosphate 5′-ATP is the natural substrate for the enzyme (Shoshani *et al*., 1999).

The mechanisms through which endothelium-dependent agonists promote prostanoid-independent cAMP synthesis remain unclear, but could involve stimulation of specific adenylyl cyclase isoforms by elevations in endothelial $[Ca^{2+}]_i$ or G protein-dependent activation of the enzyme by epoxyeicosatrienoic acid metabolites of arachidonic acid (Node *et al*., 2001; Taylor *et al*., 2001; Popp *et al*., 2002). In rabbit iliac and rat mesenteric arteries, EDHF-type responses are also accompanied by an endothelium-dependent increase in smooth muscle cAMP content (Taylor *et al*., 2001; Chaytor *et al*., 2002; Matsumoto *et al*., 2003). In rabbit arteries, this nucleotide response can be prevented by pharmacological blockade of gap junctions, although it remains uncertain if it is mediated by diffusion of endothelium-derived cAMP *via* myoendothelial gap junctions, modulation of smooth muscle adenylyl cyclase activity by electrotonically conducted changes in membrane potential, or intercellular transfer of an intermediate activator of adenylyl cyclase/inhibitor of cAMP phosphodiesterase (Taylor *et al*., 2001; Chaytor *et al*., 2002; Griffith *et al*., 2002). Such secondary elevations in smooth muscle cAMP levels may facilitate electrotonic signalling between smooth muscle cells and potentiate EDHF-type relaxations in rabbit and rat arteries (Taylor *et al*., 2001; Chaytor *et al*., 2002; Griffith *et al*., 2002; Matsumoto *et al*., 2003). It therefore remains to be determined to what extent the inhibitory action of ddA nucleosides against EDHF-type arterial relaxations involves inhibition of adenylyl cyclases present in smooth muscle cells.

It should be noted that the observed increase in the potency of 2′,3′-ddA-PMAPh against EDHF-type hyperpolarizations ($\sim$7-fold) is substantially less than the 200 times enhanced antiretroviral activity of 2′,3′-ddA-PMAPh compared to 2′,3′-ddA in CEM cell cultures (Siddiqui *et al*., 1999). The reasons for this variation in sensitivity are unclear, but could reflect the differences in prodrug hydrolysis by esterases and phophoramidases and subsequent nucleotide phosphorylation by kinases in different cell types. It also remains to be determined if the analogous phosphoramidate derivative of 2′,5′-ddA is a more effective inhibitor of the EDHF phenomenon than 2′,3′-ddA-PMAPh. Addition of phosphate groups at the 3′-site in particular enhances the ability of ddA nucleosides to inhibit adenylyl cyclase, with 2′,5′-dd-3′-ATP being a noncompetitive post-transition state inhibitor that is 10–20-fold more potent than either 2′,5′-dd-3′-AMP or 2′,3′-dd-5′-ATP (Johnson *et al*., 1997; Shoshani *et al*., 1999).

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