Sustained Entry of Ca\textsuperscript{2+} Is Required to Activate Ca\textsuperscript{2+}-Calmodulin-dependent Phosphodiesterase 1A\textsuperscript{*}

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Regulation of adenyl cyclases (ACs) by Ca\textsuperscript{2+} requires capacitative Ca\textsuperscript{2+} entry (CCE) (Cooper, D. M. F. (2003) Biochem. J. 375, 517–529), but whether Ca\textsuperscript{2+}-sensitive phosphodiesterases (PDEs) are similarly discriminating has never been addressed. In the present study, a variety of conditions were devised to manipulate [Ca\textsuperscript{2+}]\textsubscript{i}, so that we could ask whether PDE1 selectively responds to different modes of elevating [Ca\textsuperscript{2+}]\textsubscript{i}, viz. Ca\textsuperscript{2+} released from intracellular stores and various modes of Ca\textsuperscript{2+} entry. In 1321N1 human astrocytoma cells, the endogenous PDE1 (identified as PDE1A by reverse transcriptase-PCR) was largely insensitive to Ca\textsuperscript{2+} released from carbachol-sensitive stores but was robustly stimulated by a similar rise in [Ca\textsuperscript{2+}]\textsubscript{i}, due to carbachol-induced Ca\textsuperscript{2+} influx. Gd\textsuperscript{3+}, which effectively blocked thapsigargin-induced CCE and its effect on PDE1A, also inhibited the activation of PDE1A by carbachol-induced Ca\textsuperscript{2+} entry. However, non-selective ionomycin-mediated Ca\textsuperscript{2+} entry also activated PDE1A, so that, unlike Ca\textsuperscript{2+}-sensitive ACs, PDE1A cannot discriminate between the different sources of Ca\textsuperscript{2+} entry. Fractionation of the cells revealed that the Ca\textsuperscript{2+}-calmodulin-stimulated PDE activity was not present at the plasma membrane but was associated with the cytosol and the organelar compartments of the cell. Therefore, the apparent disparity between PDE1A and ACs is likely to be the consequence of their differential subcellular localization. Nevertheless, in a physiological context, where artificial modes of elevating [Ca\textsuperscript{2+}]\textsubscript{i} are not available, as with ACs, a dependence on CCE would be evident, and it would be the duration of this influx of Ca\textsuperscript{2+} that would determine how long PDE1A was activated.

A rise in [Ca\textsuperscript{2+}]\textsubscript{i}, leads to an inhibition of cAMP accumulation in a variety of cell types (1–8). In some cases, the inhibition of cAMP accumulation is exerted on Ca\textsuperscript{2+}-inhibitable adenyl cyclases (ACs) (1–3, 5–7), whereas in others the effect may be due to inhibition of Ca\textsuperscript{2+}-sensitized ACs (1–5, 7–10). Inhibitors of cAMP accumulation are exerted on Ca\textsuperscript{2+} entry in a variety of cell types (1–8). In some cases, the inhibition of Ca\textsuperscript{2+}-sensitized ACs (1–5, 7–10) has been shown to be mediated by Ca\textsuperscript{2+}-calmodulin-dependent phosphodiesterases (PDE1) (4, 8). ACs are extremely discriminating in terms of the source of the Ca\textsuperscript{2+} to which they respond (9). In non-excitable cells, Ca\textsuperscript{2+}-sensitive ACs respond only to CCE (9), whereas other modes of elevating [Ca\textsuperscript{2+}]\textsubscript{i}, including release from intracellular stores (6, 7, 10) and ionophore- (6, 7, 10) or arachidonic acid-mediated Ca\textsuperscript{2+} entry (11), are ineffective. This dependence, along with other evidence, suggests that Ca\textsuperscript{2+}-sensitive ACs and CCE channels must be functionally co-localized and that cellular strategies are in place to ensure their association (12, 13). By contrast, although type I PDEs (PDE1) are known to be markedly stimulated by Ca\textsuperscript{2+} acting via calmodulin in vitro, little if anything is known about the mode of [Ca\textsuperscript{2+}]\textsubscript{i}, rise to which they will respond in the intact cell.

In the human astrocytoma cell line 1321N1, the activation of receptors that stimulate the formation of inositol 1,4,5-trisphosphate (InsP\textsubscript{3}) substantially inhibits cAMP accumulation (8, 14, 15). Complete reversal of the inhibition by PDE1-specific inhibitors is consonant with the agonist-evoked rise in [Ca\textsuperscript{2+}]\textsubscript{i},-activating PDE1 and hence increasing the rate of CAMP hydrolysis (16). Indeed, these early studies established that PDE1 activity is markedly increased when Ca\textsuperscript{2+} is introduced into the extracellular medium (14, 15). Therefore, PDE1 does seem to be regulated by [Ca\textsuperscript{2+}]\textsubscript{i}, but the source of the Ca\textsuperscript{2+} to which PDE1 responds and whether PDE1 is as discriminating as Ca\textsuperscript{2+}-sensitive ACs has never been addressed.

In the present study, we first characterized the various modes by which [Ca\textsuperscript{2+}]\textsubscript{i}, could be elevated in 1321N1 cells. We then established a variety of conditions to manipulate [Ca\textsuperscript{2+}]\textsubscript{i}, so that we could ask whether PDE1 discriminates between Ca\textsuperscript{2+}-sensitive signaling pathways, viz. Ca\textsuperscript{2+} released from carbachol-sensitive stores, and the different modes of Ca\textsuperscript{2+} entry. We also established that RT-PCR that PDE1A was the only PDE isoform expressed in these cells. Furthermore, we compared the effect of Ca\textsuperscript{2+} entry following stimulation with the muscarinic agonist carbachol (CCh) or non-selective entry mediated by the ionophore, ionomycin, and the triggering of CCE by the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase inhibitor, thapsigargin, on isoproterenol-evoked CAMP accumulation. Our findings establish that Ca\textsuperscript{2+} entry is the major stimulus for PDE1A in 1321N1 cells, but unlike Ca\textsuperscript{2+}-sensitive ACs, PDE1A does not discriminate between different modes of Ca\textsuperscript{2+} entry. We wondered whether the lack of selectivity for the source of Ca\textsuperscript{2+} entry was related to the subcellular distribution of PDE1A. Following fractionation of the cells, we found that Ca\textsuperscript{2+}-calmodulin-stimulated PDE activity was detected in the cytosol and the non-plasma membrane organelar compartments of the cell. Therefore, it would appear that the more diffuse subcellular organization of PDE1A renders it susceptible to non-selective Ca\textsuperscript{2+} entry. Nevertheless, under physiological conditions, CCE is the dominant Ca\textsuperscript{2+} entry pathway in these cells and the dependence of PDE1A activation on CCE would be evident.

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\textsuperscript{1} The abbreviations used are: [Ca\textsuperscript{2+}]\textsubscript{i}, intracellular free Ca\textsuperscript{2+} concentration; AC, adenyl cyclase; 2-APB, 2-aminoethoxydiphenyl borate; CCE, capacitative Ca\textsuperscript{2+} entry; CCh, carbachol; IBMX, 3-isobutyl-1-methylxanthine; MMX, 8-methoxymethyl 3-isobutyl-1-methylxanthine; NCCE, non-capacitative Ca\textsuperscript{2+} entry; PDE, cyclic nucleotide phosphodiesterase; RT, reverse transcriptase; Tg, thapsigargin; InsP\textsubscript{3}, inositol 1,4,5-trisphosphate; BSA, bovine serum albumin; KBS, Krebs-buffered saline; PBS, phosphate-buffered saline.
**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin (BSA; fraction V), calmodulin (bovine brain), cAMP, carbachol (CCh), forskolin, gadoxolinium (III) chloride, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), tetraethylammonium (TEA) chloride, isoproterenol, 8-methoxy-3-isobutyl-1-methylxanthine (IBMX), 3H-methyl, 3H-adenosine, [2,8-3H]cAMP, [2-3H]adenine, RNA Isolation kit was from Promega (Southampton, UK) and SuperScript II enzyme from Invitrogen.

**Cell Culture**—1321N1 human astrocytoma cells (European Collection of Cell Cultures, Porton Down, UK) were grown in Dulbecco’s modified Eagle’s medium supplemented with fetal bovine serum (10%) and 1-glutamine (2 mm) and maintained at 37 °C in a humidified atmosphere (95% air: 5% CO2). Because the efficacy of muracetic agonists to inhibit isoproterenol-evoked cAMP accumulation in 1321N1 cells is considerably less in 1- to 3-day cultures compared with that in cells cultured for 4-12 days (15), all experiments were carried out using cells that had been subcultured for 5-7 days prior to use. In 1321N1 cells that were grown for 5 days in 75-cm2 flasks were used. 1321N1 cells were grown for 5 days in 150-mm diameter dishes and then detached with PBS containing 0.03% w/v EDTA and centrifuged at 19500 g for 5 min. The supernatant was removed and the pellet was resuspended in 1 ml of assay buffer (50 mm Tris-HCl, pH 7.4) supplemented with 1 mm phenylmethylsulfonyl fluoride, 1 mm benzamidine, 2 mm 4-2-aminoethylbenzenesulfonyl fluoride, 1 mm EDTA, 130 μM bestatin, 14 μM E-64, 1 μM leupeptin, 0.3 μM aprotinin, and 1 μM of DNase I. The cells were then lysed by repeatedly passing the cell suspension through a 21-gauge needle. Unbroken cells and nuclei were removed from the lysate by low speed centrifugation (195 × g, 5 min, 4 °C). The supernatant was removed and centrifuged (17,257 × g, 15 min, 4 °C) to pellet the crude membrane fraction (containing plasma membranes) (19), and the resultant supernatant (“crude cytosolic” fraction) was either used immediately or fractionated further before use. To separate the cytosol from the non-plasma membrane organellar components of the cell, the “crude cytosolic” fraction was centrifuged at high speed (105,000 × g, 60 min, 4 °C) (20). The final supernatant was designated as the cytosolic fraction and the pellet as the “organellar fraction.” Both fractions were resuspended in an equal total volume of 50 mm Tris-HCl supplemented with 0.2% w/v BSA and 4 mm dithiothreitol (pH 7.4) and used immediately. The protein content was determined by using the bicinchoninic acid method (22).

**Measurement of Cyclic Nucleotide Phosphodiesterase Activity—**Phosphodiesterase activity was measured as described by Shahid and Ni- tovis (10) with some modifications. Briefly, both cytosolic and organellar fractions were incubated (15 min, 30 °C) with 5 μCi of [2,8-3H]cAMP, 3 μM MgCl2, 1 mm 5′,0.24 μM calmodulin, 200 μM EGTA, and various Ca2+ concentrations (see under “Determination of Free Ca2+ Concentrations” below) in 50 mm Tris-HCl (pH 7.4) in a final volume of 200 μL. Reactions were terminated by harvesting the assay mixture for 3 min by mixing 100 μL of mixture (-3000 cpm) was added to each sample to measure the recovery of [2,8-3H]cAMP from aluminina columns (23). The amount of [2,8-3H]cAMP that was hydrolyzed was quantified by expressing the corresponding [2,8-3H]cAMP counts following recovery from the columns as a function of the total [2,8-3H]cAMP added to each assay tube. Blank values were obtained by using previously boiled preparations. There was no significant difference between the decrease in the amount of cAMP detected with boiled enzymes and when no protein was added. The amount of cAMP that was hydrolyzed was expressed as either picomoles of cAMP/min/mg of protein or picomoles of cAMP/min/μl of fraction.

**Determination of Free Ca2+ Concentrations—**Free Ca2+ concentrations were calculated as previously described (24). Briefly, this involved an iterative computing program that solved equations that described the mixture formed with free and bound Ca2+ buffer components (see under “Measurement of Adenylyl Cyclase Activity” and “Measurement of Phosphodiesterase Activity” above). Final assay mixture concentrations of free Ca2+ (in the presence of 200 μM EGTA) are shown.

**Reverse Transcription-PCR—**Total RNA was extracted from 1.5 × 108 1321N1 cells using the SV total RNA isolation kit (Promega). RNA (1 μg) was reverse-transcribed by Superscript II enzyme with 0.5 μg of oligo(dT)16. The reaction mixture was incubated at 42 °C for 50 min, followed by a further incubation at 70 °C for 15 min. PCR was performed using 10 ng of cDNA, 10 pmol of each of the two specific primers, 1.5 μM MgCl2, 0.2 μM dNTPs, and 2.5 units of TaqDNA polymerase. The specific primers were CCATTGTCCCATATTCATGAGC, a N-terminal primer common to all PDE isoforms; TCTAACAATTGTTTGATC, a C-terminal primer specific for PDE1A; GTGCAAAGTAGCATGTTTGC, a C-terminal primer specific for PDE1B; and TTCTCTTCTTTGGTACCTTGGC, a C-terminal primer specific for PDE1C. The amplification profile consisted of heating the mixture at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 cycles (an initial heating to 94 °C for 5 min was performed). To ensure the fidelity of mRNA extraction and reverse transcription, the integrity of RNA was subjected to PCR amplification with oligonucleotide primers specific for the constitutively expressed gene β-actin.

**Statistical Analysis—**Results are shown as the means ± S.E. of at least three individual experiments. Statistical significance was assessed by using paired Student’s t test, where p ≤ 0.05 was considered significant.
RESULTS

CCh Mediates a Ca\(^{2+}\)-dependent Decrease in Isoproterenol-evoked cAMP Accumulation—Stimulation of endogenous β-adrenoceptors in 1321N1 cells with isoproterenol evoked a concentration-dependent increase in cAMP accumulation (EC\(_{50}\) = 8.7 ± 0.1 nM, n = 3) (Fig. 1A, inset). The time course for cAMP accumulation after stimulation with a maximal concentration of isoproterenol (10 μM) in the presence and absence of CCh (1 mM) was examined (Fig. 1A). In Ca\(^{2+}\)-free conditions, isoproterenol stimulated a time-dependent increase in cAMP accumulation that reached a maximum level after 10 min. A similar profile was observed in cells that were co-stimulated with isoproterenol and CCh in Ca\(^{2+}\)-free conditions or isoproterenol alone in the presence of 3 mM CCh. However, co-stimulation with isoproterenol (10 μM) and CCh (1 mM) in Ca\(^{2+}\)-containing medium produced two notable effects. First, there was a significant decrease in the amount of cAMP detected after each time interval compared with that evoked by isoproterenol alone, and second, the time required to achieve a maximal cAMP response was reduced from 10 to 2 min (Fig. 1A).

To confirm that the effect of CCh on isoproterenol-evoked cAMP accumulation was indeed Ca\(^{2+}\)-dependent, cells were depleted of Ca\(^{2+}\) by pre-treatment with ionomycin in the absence of extracellular Ca\(^{2+}\). This has the effect of causing extrusion of Ca\(^{2+}\) from intracellular stores out of the cell. Under such circumstances, it was possible to isolate any potential action of CCh on isoproterenol-evoked cAMP accumulation that was independent of Ca\(^{2+}\) mobilization from Ca\(^{2+}\)-dependent effects. Because the removal of both extracellular and intracellular sources of Ca\(^{2+}\) eliminated the inhibitory effect of CCh on isoproterenol-evoked cAMP accumulation (Fig. 1B), it is possible to conclude that the observed inhibition of CCh accumulation by CCh is a consequence of a rise in [Ca\(^{2+}\)].
The relative effects of what was assumed to be Ca\(^{2+}\) release from InsP\(_3\)-sensitive stores and Ca\(^{2+}\) entry on isoproterenol-evoked cAMP accumulation were compared as a function of the total inhibition evoked by CCh, i.e. that attributable to the increase in [Ca\(^{2+}\)], due to release plus entry in the presence of extracellular Ca\(^{2+}\). Of the total inhibition of the cAMP response by CCh, 22 ± 6% (n = 25) (viz. in the absence of extracellular Ca\(^{2+}\)) was dependent on Ca\(^{2+}\) released from intracellular stores and 78 ± 6% (n = 25) on Ca\(^{2+}\) influx.

The extrusion of cAMP from the cell may provide a theoretical means (in addition to phosphodiesterases) whereby an intracellular cAMP signal may be rapidly diminished (25). Although the identity of the cAMP transporters remains unclear (25), we considered the possibility that the rise in [Ca\(^{2+}\)] evoked by CCh could enhance the extrusion of cAMP from the cell. It turned out that, in the presence of 3 mM Ca\(^{2+}\), the amount of cAMP detected in the extracellular medium following exposure to isoproterenol (10 μM) and CCh (1 mM) was 11.88 ± 3.27% (n = 4) of the total cAMP level compared with 7.96 ± 1.10% (n = 4) with isoproterenol alone. Obviously, changes in this small fraction of the total cAMP cannot account for the present effects of CCh on isoproterenol-evoked cAMP accumulation.

Characterization of Adenylyl Cyclase in 1321N1 Cells—The regulation of adenylyl cyclases (ACs) by elevated [Ca\(^{2+}\)], provides a means whereby Ca\(^{2+}\) can modulate the cAMP pathway at the earliest opportunity. Of the 10 known isoforms of AC, five are either stimulated or inhibited by physiological increases in [Ca\(^{2+}\)] (9). AC1, AC3, and AC8 are activated by Ca\(^{2+}\) in a calmodulin-dependent manner, whereas AC5 and AC6 are Ca\(^{2+}\)-inhibitable, but calmodulin-independent (9). Therefore, a possible explanation for the inhibitory effect of CCh on isoproterenol-evoked cAMP accumulation (Fig. 1A) could be the inhibition of AC activity by Ca\(^{2+}\). To address this issue, the sensitivity of ACs to Ca\(^{2+}\) was measured using membranes prepared from 1321N1 cells. AC activity in 1321N1 membranes was inhibited by Ca\(^{2+}\) in a concentration-dependent manner (IC\(_{50}\) = 273 ± 25 μM, n = 3), which was unaffected by calmodulin (IC\(_{50}\) = 279 ± 80 μM, n = 3) (Fig. 2). Thus 1321N1 cell membranes express either AC5 or AC6 adenylyl cyclase. However, inhibition of adenylyl cyclase cannot play any role in the inhibition of cAMP accumulation observed in the present studies, because the inhibition is eliminated in the presence of PDE inhibitors (see below); a situation that does not arise when direct inhibition of adenylyl cyclase occurs in intact cells (2, 7).

The Activation of PDE1A by Ca\(^{2+}\) Entry Underlies the Mechanism for the Inhibition of Isoproterenol-evoked cAMP Accumulation—Of the 11 known members of the PDE family (26), only PDE1 (27) and PDE4 (28) are directly regulated by a rise in [Ca\(^{2+}\)]. However, the mechanisms whereby Ca\(^{2+}\) regulates these enzymes are different: PDE1 is directly activated following the binding of Ca\(^{2+}\)-calmodulin (29), whereas intracellular Ca\(^{2+}\) facilitates the association of PDE4A1 with the Golgi membrane (28) without affecting its activity (30). Indirect effects of Ca\(^{2+}\) (e.g. protein kinase C) on other Ca\(^{2+}\)-insensitive PDEs may also account for the enhanced cAMP hydrolysis in the presence of CCh.

Three forms of PDE have been identified in 1321N1 cells: PDE1, a Ro20-1724-sensitive PDE (presumably PDE4) and a putative cGMP-stimulated PDE (16). Therefore, we employed selective PDE inhibitors to identify the PDE species that might mediate the inhibitory effect of CCh on isoproterenol-evoked cAMP accumulation (Fig. 3). The PDE1-specific inhibitor MMX (31, 32) reversed the inhibitory effects of CCh on isoproterenol-evoked cAMP accumulation in a concentration-dependent man-

![FIG. 2. Adenylyl cyclase activity in 1321N1 cells is inhibited by Ca\(^{2+}\). Membranes prepared from 1321N1 cells were assayed for adenylyl cyclase activity in the presence of the concentrations of Ca\(^{2+}\) shown. Membranes were assayed with 10 μM forskolin, 10 μM isoproterenol, and 100 μM ATP in either the absence (filled circles) or presence (open circles) of 1 μM calmodulin. The results show the means ± S.E. of three independent experiments.](http://www.jbc.org/)
Regulation of PDE1A by Ca\(^2+\)

**A.**

![Graph showing reversal of inhibition (%)](image)

**Figure 3.** PDE1 mediates the effect of CCh on isoproterenol-evoked cAMP accumulation. A, concentration-dependent reversal of the effect of CCh (1 mM) on isoproterenol (10 \(\mu M\), 5 min)-evoked cAMP accumulation (mean ± S.E., \(n = 6\)) by MMX. The results show the increase in cAMP accumulation after subtraction of the responses to isoproterenol alone (10 \(\mu M\), 5 min; solid bars) or in the presence of CCh (1 mM; open bars) is shown after pre-treatment with IBMX (50 \(\mu M\), 10 min), MMX (50 \(\mu M\), 10 min), rolipram (Roli; 10 \(\mu M\), 10 min), and Ro20-1724 (Ro20; 10 \(\mu M\), 10 min). A and B, cells were stimulated in nominally Ca\(^2+\)-free KBS supplemented with 3 m\(M\) CaCl\(_2\). The results show the means ± S.E. of four independent experiments. Asterisks denote a statistical difference between responses to isoproterenol alone and isoproterenol in the presence of CCh \((p < 0.05)\).
though arachidonic acid-dependent NCCE has only been demonstrated in a few cell types (41, 46), it seemed reasonable to entertain the possibility that CCh might activate both NCCE and CCE in 1321N1 cells.

In nominally Ca\(^{2+}\)-free medium, CCh (1 mM) evoked a transient increase in \([\text{Ca}^{2+}]_i\) (1143 ± 152 nm, n = 5), which rapidly returned to its basal level (t\(_{1/2}\) = 24 ± 2 s, n = 5) (Fig. 6A). The subsequent addition of Ca\(^{2+}\) to the medium, triggered a second Ca\(^{2+}\) signal that represented Ca\(^{2+}\) entry. The peak increase in \([\text{Ca}^{2+}]_i\), that followed Ca\(^{2+}\) entry was 712 ± 109 nm (n = 5). The Ca\(^{2+}\) signal attributable to Ca\(^{2+}\) entry did not return to the basal level, but achieved a steady state at an elevated \([\text{Ca}^{2+}]_i\). The rate at which the Ca\(^{2+}\) signal reached the steady-state was significantly longer for the Ca\(^{2+}\) entry phase than that of the release phase (half-time, t\(_{1/2}\) = 93 ± 29 s, n = 5).

The effects of the CCE blocker, Gd\(^{3+}\), on Ca\(^{2+}\) entry induced by 1 mM CCh is shown in Fig. 6A. Preincubating cells (5 min) with 10 \(\mu\)M Gd\(^{3+}\) inhibited CCh-induced Ca\(^{2+}\) entry by 86 ± 2% (n = 5) (cf., Fig. 5B). Therefore, the inhibition of isoproterenol-evoked cAMP accumulation by CCh shown in Fig. 2 can be interpreted to be the consequence of CCh-induced CCE activating PDE1A. This, however, does not exclude the possibility that, at lower concentrations of CCh, NCCE could regulate both NCCE and CCE in 1321N1 cells.

A Generalized Rise in \([\text{Ca}^{2+}]_i\], Can Activate PDE1A—To determine whether a similarly intimate relationship existed between PDE1A and CCE in 1321N1 cells, we compared the effects of Tg-induced CCE with a generalized ionophore-mediated Ca\(^{2+}\) entry on PDE1 activity. At submicromolar concentrations, ionomycin...
selectively permeabilizes intracellular membranes (47) facilitating the flux of Ca\(^{2+}\) from organelles into the cytosol to trigger CCE. However, at higher concentrations, ionomycin also permeabilizes the plasma membrane to divalent cations (48) to cause a generalized increase in \([\text{Ca}^{2+}]_i\) that is independent of Ca\(^{2+}\) entry channels and which cannot be blocked by CCE channel blockers.

Ionomycin (10 \(\mu\)M, 15 min) evoked a dramatic increase in \([\text{Ca}^{2+}]_i\) that corresponded to the emptying of intracellular Ca\(^{2+}\) stores (Fig. 7A). The subsequent addition of Ca\(^{2+}\) to the extracellular medium caused a concentration-dependent increase in \([\text{Ca}^{2+}]_i\). The effect of pre-treatment with 10 \(\mu\)M Gd\(^{3+}\) on the inhibition of isoproterenol-evoked cAMP accumulation by CCh-induced Ca\(^{2+}\) entry is also shown. Results show the means \(\pm\) S.E. of eight independent experiments. The asterisk denotes a significant difference from the response to isoproterenol alone (\(p \leq 0.05\)).

Under identical conditions to those used in the Ca\(^{2+}\) measurements, ionomycin alone did not significantly affect isoproterenol-evoked cAMP accumulation (Fig. 7C). However, the addition of 30 \(\mu\)M Ca\(^{2+}\) to the extracellular medium produced a substantial inhibition of isoproterenol-evoked cAMP accumulation in ionomycin-pre-treated cells (41 \(\pm\) 8\%, \(n = 4\)). The effect was completely reversed by IBMX (50 \(\mu\)M) and MMX (50 \(\mu\)M) (Fig. 7C). This result establishes that not only CCE, but also a nonspecific, generalized rise in \([\text{Ca}^{2+}]_i\), can activate PDE1 in 1321N1 cells.

**PDE1A Is Distributed within the Cytosol and Non-plasma Membrane Organellar Compartments of 1321N1 Cells**—The functional co-localization of Ca\(^{2+}\)-sensitive ACs with CCE channels is reinforced by their subcellular distribution (12, 13). The disparity between the selectivity of ACs and the non-selectivity of PDE1 for particular sources of Ca\(^{2+}\) entry pathways might therefore be explained by the spatial distribution of
the enzymes within the cell. To examine this possibility, we initially measured Ca^{2+}-calmodulin-dependent PDE activity in crude membrane and “crude cytosolic” fractions prepared from 1321N1 cells (Fig. 8). In the presence of calmodulin, Ca^{2+} evoked a concentration-dependent increase in PDE activity in the cytosolic fraction (Fig. 8A), which was completely inhibited by the PDE1-specific inhibitor MMX (50 μM) (Fig. 8B). By contrast, Ca^{2+}-calmodulin-dependent PDE activity was not detected in the membrane fraction (Fig. 8C).

We considered the possibility that the crude cytosol described above could include non-plasma membrane organelles with which PDE1A would be associated. Therefore, we fractionated the crude cytosolic fraction into cytosol and organelar components and measured the Ca^{2+}-calmodulin-dependent PDE activity. AC activity was also measured to show the subcellular distribution of PDE1A relative to plasma membrane-associated ACs. Ca^{2+}-calmodulin-dependent PDE activity occurred in the cytosol and, to a slightly lesser extent, in the organellar components of the cells (Fig. 9A, panels i and ii), but was absent from the plasma membrane (Fig. 9A, panel iii). In all cases, the Ca^{2+}-calmodulin-dependent increase in PDE activity was abolished by the PDE1-specific inhibitor MMX (50 μM). In contrast, all AC activity was localized to the crude plasma membrane fraction (Fig. 9B) (19).

The experiments described above examined the subcellular distribution of PDE1A at resting [Ca^{2+}]_{i}. Because the effect of elevated [Ca^{2+}]_{i} on the subcellular localization of PDE1A has never been explored, we entertained the further possibility that the intracellular targeting of PDE1A may be a function of the rise in [Ca^{2+}]_{i} that follows agonist stimulation in a manner analogous to PDE4A1 (28). 1321N1 cells were treated with ionomycin (10 μM) and CaCl_{2} (30 μM) as shown in Fig. 7 prior to lysis, and the PDE activity in the different cellular fractions was measured (data not shown). Increasing [Ca^{2+}]_{i}, prior to

**Fig. 7.** PDE1 is activated by a generalized increase in [Ca^{2+}]_{i}. After 1 min in nominally Ca^{2+}-free KBS, cells were incubated with ionomycin (10 μM, 15 min) prior to the addition of CaCl_{2} into the extracellular medium. A, Ca^{2+} entry in ionomycin-pre-treated cells after the addition of 70 μM (a), 30 μM (b), 10 μM (c), and 0 mM (d) CaCl_{2}. B, ionomycin-treated cells were incubated with the CCE blocker 2-APB (100 μM) (gray line) in nominally Ca^{2+}-free KBS for 5 min before the addition of 30 μM CaCl_{2}. The control response is shown by the black line. Traces (A and B) are representative of at least three independent experiments. C, under identical conditions to A and B, cells were incubated with ionomycin (10 μM, 15 min) in nominally Ca^{2+}-free medium before the addition of isoproterenol (10 μM, 5 min) in the presence of 30 μM extracellular Ca^{2+}. Where indicated, IBMX (50 μM) and MMX (50 μM) were present 10 min before and throughout the experiment. Results are the means ± S.E. of four experiments. The asterisk denotes a statistically significant difference from the response to isoproterenol alone (p ≤ 0.05).
ACs have been postulated to lie close to CCE channels in the plasma membrane. In contrast, very little is known about the regulation of Ca$^{2+}$-sensitive PDEs by Ca$^{2+}$. Although earlier work has established that activation of Ca$^{2+}$-calmodulin-dependent PDE1 requires extracellular Ca$^{2+}$ (15, 16) the nature of the Ca$^{2+}$ signal involved has never been explored. In the present study, we first characterized the various modes of [Ca$^{2+}$], elevation in the human astrocytoma cell line, 1321N1. By using selective PDE inhibitors, we established that 1321N1 cells express endogenous PDE1, and through RT-PCR identified PDE1A as the only PDE1 isoform expressed in these cells. We then manipulated [Ca$^{2+}$], by various means to ask (i) whether Ca$^{2+}$ release from intracellular stores regulates PDE1 activity, (ii) whether CCE activates PDE1 and, (iii) whether any other mode of elevating [Ca$^{2+}$], could regulate PDE1. Finally, we explored the subcellular distribution of PDE1A as a potential determinant in its selectivity toward Ca$^{2+}$ from a particular source.

In 1321N1 cells, CCh evokes an increase in [Ca$^{2+}$], through the formation of InsP$_3$ and the subsequent release of Ca$^{2+}$ from InsP$_3$-sensitive stores (49). Because removal of extracellular Ca$^{2+}$ almost eliminates the inhibitory effects of InsP$_3$-coupled agonists on cAMP accumulation in 1321N1 cells, we can infer that release of Ca$^{2+}$ from intracellular stores plays little role in the regulation of PDE1A. The importance of Ca$^{2+}$ entry versus Ca$^{2+}$ release for the regulation of PDE1A is emphasized when the substantial, but relatively ineffective rise in [Ca$^{2+}$], due to Ca$^{2+}$ release is compared with the robust effect of a similar rise in [Ca$^{2+}$], due to Ca$^{2+}$ influx (see Fig. 6).

In non-excitable cells, CCE plays a critical role in determining the amplitude of sustained elevations in [Ca$^{2+}$], and replenishing depleted intracellular Ca$^{2+}$ stores. Although the identity of store-operated Ca$^{2+}$ channels remains unclear, prime candidates for the role are mammalian homologues of the Drosophila transient receptor potential (trp) protein (50). The localization of these trp proteins and Ca$^{2+}$-sensitive ACs in cholesterol-rich domains of the plasma membrane (51) may provide the means whereby Ca$^{2+}$-sensitive ACs are exclusively regulated by CCE. Our findings show that CCE triggered independently of receptor activation (i.e. Tg) causes a profound increase in PDE1A activity (Fig. 5). Although CCE clearly stimulates PDE1A, the use of Tg to induce CCE may overlook modes of Ca$^{2+}$ entry that are stimulated by agonists, in addition to CCE. For example, when CCh increases InsP$_3$ formation in 1321N1 cells (49), it may be assumed that the depletion of CCh-sensitive Ca$^{2+}$ stores will trigger CCE. However, CCh may also activate NCCE through a diacylglycerol/arachidonic acid-driven pathway. It turned out that known CCE blockers abolished CCh-induced Ca$^{2+}$ entry and its effect on PDE1 (Fig. 6). Therefore, we can conclude that CCE is the primary route of Ca$^{2+}$ entry and the major stimulus for PDE1A activation in 1321N1 cells.

The dependence of PDE1 activity on Ca$^{2+}$ influx might reflect either the functional co-localization of the enzyme with Ca$^{2+}$ entry channels, localization of the enzyme near the plasma membrane, or simply a requirement for a sustained elevation of [Ca$^{2+}$], irrespective of its origin. To distinguish between these possibilities, we devised conditions where the effect on PDE1A activity of non-selective Ca$^{2+}$ influx could be compared with that of CCE. Unlike CCE, ionophore-mediated Ca$^{2+}$ entry is not restricted to discrete regions of the plasma membrane, but allows a nonspecific influx of Ca$^{2+}$ into the cell (6). The experimental conditions developed permitted ionomycin to cause a substantial increase in [Ca$^{2+}$], in the presence of a low concentration of Ca$^{2+}$ (30 μM) that evoked no CCE in Tg-treated cells (Figs. 5 and 7). The failure of CCE blockers to inhibit ionomycin-mediated Ca$^{2+}$ entry established that iono-

**DISCUSSION**

The regulation of ACs by Ca$^{2+}$ has been extensively characterized (9). In the intact cell, they show a remarkable dependence on CCE: neither release from intracellular stores (6, 7, 10) nor ionophore- (6, 7, 10) or arachidonic acid-mediated Ca$^{2+}$ entry (11) affect AC activity. As a consequence, Ca$^{2+}$-sensitive

**Fig. 8. Subcellular localization of PDE1A.** Crude cytosolic and membrane fractions prepared from 1321N1 cells were assayed for PDE activity in the presence of the concentrations of Ca$^{2+}$ shown. The fractions were assayed with 4 μM cAMP, 3 mM MgCl$_2$, 200 μM EGTA, and 0.24 μM calmodulin. A, the concentration-dependent stimulation of PDE activity in the crude cytosolic fraction by Ca$^{2+}$ (mean ± S.E., n = 5). B and C, the increase in PDE activity evoked by Ca$^{2+}$ in crude cytosolic (B) and crude membrane (C) fractions in the absence (solid bars) and presence (open bars) of 50 μM MMX. Results show the means ± S.E. of three independent experiments.
mecin stimulated nonspecific Ca\textsuperscript{2+} entry in 1321N1 cells. This ionomycin-mediated Ca\textsuperscript{2+} entry also activated PDE1A, and so we conclude that PDE1A requires sustained Ca\textsuperscript{2+} entry for activation, but unlike Ca\textsuperscript{2+}-sensitive ACs, it does not discriminate between the different routes of Ca\textsuperscript{2+} entry. However, the possibility should not be discounted that other PDE1 isoforms (viz. PDE1B or PDE1C) might be more selective for the source of Ca\textsuperscript{2+} to which they will respond. Future studies involving heterologous expression of the various isoforms might be insightful in this regard.

The disparity between the regulation of Ca\textsuperscript{2+}-sensitive ACs and PDE1A by Ca\textsuperscript{2+} is most likely to reflect the subcellular placement of these enzymes. A major contributing factor for the regulation of Ca\textsuperscript{2+}-sensitive ACs by CCE is their compartmentalization in cholesterol-rich domains of the plasma membrane (12, 13). Indeed, other proteins that are specifically regulated by CCE are also targeted to this domain, as are trp proteins (51, 52). Unlike ACs, which possess twelve transmembrane-spanning domains and are always associated with the plasma membrane, only some of the known PDEs have the potential to be associated with the plasma membrane, and this is dependent on lipid modifications to amino acids in their N-terminal regions (20, 26). The subcellular distribution of PDEs is largely determined by the formation of signaling complexes with scaffolding proteins (53). Although extremely little is known about the intracellular targeting of PDE1, at resting [Ca\textsuperscript{2+}], the enzyme is likely to be distributed within the cytosol (54) or associated with cytoskeletal components (55) (Fig. 9).

Although PDE1A does not discriminate between specific and nonspecific routes of Ca\textsuperscript{2+} entry it is highly selective for Ca\textsuperscript{2+} entering the cell versus Ca\textsuperscript{2+} released from intracellular stores. The dependence of PDE1A on Ca\textsuperscript{2+} entering the cell may be another manifestation of its subcellular distribution. Selective targeting of PDE1A to the sub-plasmalemmal space (possibly through association with cytoskeletal, accessory proteins, or organellar structures) could allow regulation of the enzyme by Ca\textsuperscript{2+} entry, while potentially shielding it from Ca\textsuperscript{2+} released from intracellular stores.

In conclusion, the present study has significantly refined our understanding of how PDE1 is regulated by [Ca\textsuperscript{2+}]. It is clear that Ca\textsuperscript{2+} released from intracellular stores plays little or no role in the regulation of PDE1A compared with robust activation by Ca\textsuperscript{2+} influx. However, PDE1A is unable to discriminate between the different sources of Ca\textsuperscript{2+} entry owing to its subcellular distribution. Nevertheless, it is worth noting that, in a physiological context, where artificial modes of elevating [Ca\textsuperscript{2+}] are not available, as with ACs, a dependence of PDE1A on CCE would be manifest, and it would be the duration of the influx of Ca\textsuperscript{2+} that would determine for how long PDE1A was activated.

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Sustained Entry of Ca\textsuperscript{2+} Is Required to Activate Ca\textsuperscript{2+}-Calmodulin-dependent Phosphodiesterase 1A

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