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COMPARISON OF THE EFFECT OF ISOBUTYLMETHYLXANTHINE AND PHOSPHODIESTERASE-SELECTIVE INHIBITORS ON cAMP LEVELS IN SH-SY5Y NEUROBLASTOMA CELLS

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Abstract—A comparison of the effects of various phosphodiesterase (PDE) inhibitors upon cellular cAMP levels was undertaken in human neuroblastoma SH-SY5Y cells. When inhibitors such as rolipram and Ro 20 1724 (selective for the low K_m cAMP-specific PDE) were used, cAMP levels were seen to rise dramatically under basal (≤60 fold) or forskolin-stimulated (≤200 fold) conditions. However, the non-selective PDE inhibitor isobutylmethylxanthine (IBMX) was 7–18% as effective as these other agents even at 1 mM. The poor efficacy of IBMX was not attributable to concomitant increases in cGMP, to alterations in cAMP egress or to a lack of sensitivity of the cellular PDEs to IBMX inhibition. In additivity experiments, IBMX potently and rapidly reduced cAMP that had accumulated after rolipram treatment. The fact that the agonist 2-chloroadenosine can enhance cAMP accumulation in these cells, and that cAMP elevated by rolipram or forskolin can be reduced by adenosine deaminase and theophylline suggest that cell-derived adenosine enhances cAMP in these cells in an autocrine fashion. Since IBMX is an adenosine receptor antagonist, it is suggested that its blockade of endogenous adenosine effects is at least partly responsible for its poor response when compared to other PDE inhibitors which are weaker adenosine receptor antagonists. These results forewarn against assuming that similar levels of cAMP accumulate after application of PDE inhibitors in these cells.

Since phosphodiesterase (PDE§) activity represents the major route of elimination of the cAMP signal [1–3], application of PDE inhibitors can provide a way of elevating cAMP levels which circumvents receptor activation. Although there are now a number of agents which are selective for some PDE isoenzymic forms [1, 2], the inability to inhibit selectively some PDE families (most notably PDE II, the cGMP-stimulated PDE) necessitates using non-selective inhibitors such as 1-methyl-3-isobutylxanthine (IBMX) to inhibit total PDE activity. However, IBMX and other xanthine-derived "PDE inhibitors" are notorious for their ability to act at other loci [4–6], including antagonizing adenosine receptors [7–9], and this illustrates the potentially composite effect of these compounds upon cAMP metabolism. Adenosine receptors are currently divided into A_1- and A_2-subtypes which are capable of coupling negatively and positively to adenylyl cyclase, respectively [10]. The human neuroblastoma cell line SH-SY5Y and the parent line SK-N-SH are widely used as they are considered to be a neuronally relevant cell model for the investigation of Ca^{2+} homeostasis by phosphoinositide hydrolysis [11–14], cross-talk at the level of second messenger generation [15–17] and stimulus secretion coupling [18, 19]. That neuroblastoma cells express adenosine receptors has been observed previously [20, 21], and SH-SY5Y cells, in particular, may well express A_2-type receptors coupled positively to cAMP production [22]. In such cells, we show that application of a variety of PDE IV-selective inhibitors results in dramatic rises in cAMP whereas IBMX is significantly less able to cause such increases in second messenger concentration. We suggest that this is partly due to IBMX antagonizing the adenylyl cyclase-stimulating effect of endogenous adenosine. The other PDE inhibitors cannot block this stimulation, and therefore act in synergy with endogenous nucleoside to give the observed larger responses.

MATERIALS AND METHODS

Cell culture. SH-SY5Y neuroblastoma cells were obtained from Dr J. Beidler (Sloane-Kettering Institute, NY, U.S.A.) and were cultured in monolayer according to Ref. 23. Cells (passage 70–95) were grown in 175 cm^2 flasks in minimum essential medium supplemented with 10% newborn calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone and 2 mM glutamine. Cells were harvested in 10 mM HEPES/0.9% NaCl/0.02% EDTA (w/v) pH 7.4, centrifuged at 350 *g* and the pellet washed twice in modified Krebs–Ringer bicarbonate (KRB) (118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM K_2HPO_4, 1.2 mM $MgSO_4$, 4.7 mM KCl,

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§ Abbreviations: IBMX, 1-methyl-3-isobutylxanthine; PDE, phosphodiesterase; KRB, Krebs–Ringer bicarbonate; ADA, adenosine deaminase; IC_{50}, concentration giving half-maximal inhibition; DMSO, dimethyl sulphoxide.

8 mM HEPES, 1.3 mM $CaCl_2$, 10 mM glucose). Cells were finally resuspended in KRB to give a protein concentration of 4–5 mg/mL as determined by the method of Lowry *et al.* [24] using bovine serum albumin as standard.

Incubation and determination of cAMP. Incubations of cells with agents were performed in polypropylene insert vials in a shaking water bath at 37°. Aliquots of the cell suspension (~200 μg protein) were incubated in a final volume of 300 μL of KRB previously equilibrated with 95% O_2/5% CO_2. The vials were gassed and capped and incubations with various PDE inhibitors or dimethyl sulphoxide (DMSO) vehicle [0.8% (v/v)] were conducted for 20 min. Forskolin (1 μM) was added for a further 10 min, the reactions were stopped by the addition of conc. HCl and the samples were placed on ice for 15 min. After neutralization with NaOH and subsequent centrifugation, samples were assayed for cAMP using the binding protein assay of Brown *et al.* [25]. At the end of experiments, cell viability was assessed by a dye exclusion method using Azur A [26] [incubation with 0.04% (w/v) in glucose-supplemented phosphate-buffered saline for 10–15 min at room temperature] and was routinely found to be ≥85%.

Determination of cGMP. Cells were treated identically as above, except that neutralized cell extracts were examined for cGMP according to Chilvers *et al.* [27].

Determination of SH-SY5Y PDE activity. Examination of cell PDE activity was performed by homogenizing cells in 50 mM Tris–HCl/4 mM EDTA (pH 7.5) at 4° with a polytron at setting 5 for 30 sec. A low speed centrifugation (350 *g*, 2 min) isolated unbroken cells and nuclei, and the resultant supernatant was used in aliquots (~50 μg protein) as the source of "total PDE". The assay of PDE activity was performed according to Arch and Newsholme [28] using 1 μM cAMP as substrate, and a 15 min incubation protocol at 37° (the reaction was linear for at least 20 min, results not shown). All inhibitors were dissolved in DMSO and diluted into assay buffer. The results are expressed as a percentage of vehicle controls.

Data analysis. Data were examined using Student's *t*-test (unpaired), and considered to be significant when $P \leq 0.05$. Given experimental variation in absolute changes in second messenger concentration, some data are expressed as a percentage of the cAMP accumulation evoked by a combination of 1 μM forskolin and 1 mM IBMX.

Materials. Rolipram was a gift from Schering Health Care Ltd (Burgess Hill, West Sussex, U.K.); Ro 20-1724 was a gift from Roche Products Ltd (Welwyn, U.K.). Forskolin, IBMX, 2-chloroadenosine, 5′-nucleotidase (*Crotalus* venom), Azur A, cGMP (sodium salt) and Dowex 1-X8 (200–400 mesh, chloride form) were purchased from the Sigma Chemical Co. (Poole, U.K.). Adenosine deaminase (in glycerol) and cAMP (sodium salt) were from Boehringer Mannheim (Germany). [2,8-^{3}H]cAMP (33.5 Ci/mmol) was obtained from NEN Products, DuPont Ltd (Stevenage, U.K.). Anti-cyclic GMP antibody, cGMP 2′-*O*-succinyl 3-[^{125}I]iodo tyrosine methyl ester (~2000 Ci/mmol) and [8-^{3}H]cAMP (41 Ci/mmol) were from Amersham International (Amersham, U.K.). Theophylline and other reagents were obtained from Fisons (Loughborough, U.K.).

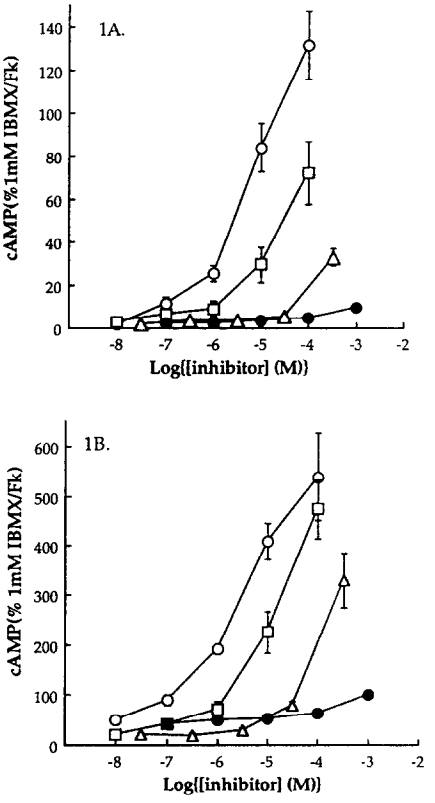

Fig. 1. Effect of selective and non-selective PDE inhibition upon cAMP levels in basal and forskolin (Fk)-treated cells. Various PDE inhibitors or their DMSO vehicle (0.8% v/v) were preincubated with cells for 20 min (A). Fk (1 μM) was then added to half the samples (B) for a further 10 min and acid arrested. Cyclic AMP was determined by the mass assay of Brown *et al.* [25] (Materials and Methods). Papaverine (△); rolipram (○); Ro 20 1724 (□); IBMX (●). Basal cAMP = 11.59 ± 2.34; 1 μM Fk = 255.51 ± 67.96; 1 mM IBMX = 52.85 ± 19.99; IBMX/Fk = 531.93 ± 194.04 pmol/mg protein. Results are means ± SEM for 3–5 experiments performed in triplicate.

RESULTS

The effect of PDE inhibition upon cAMP levels in these cells was examined. Figure 1 shows the effect of increasing concentrations of phosphodiesterase inhibitors on cAMP accumulation in SH-SY5Y cells in the absence and the presence of 1 μM forskolin. This concentration of the adenylyl cyclase activator was chosen since it gave a robust, ~10 fold increase in cAMP level, a level which was ~50% that elicited by 10 μM (N = 3; not shown). The most striking observation was the difference in effectiveness of the "non-selective" PDE inhibitor IBMX and the other agents, whether non-selective (papaverine) or selective for the low K_m cAMP PDE (Ro 20 1724, rolipram) [1–3]: whereas 1 mM IBMX raised basal

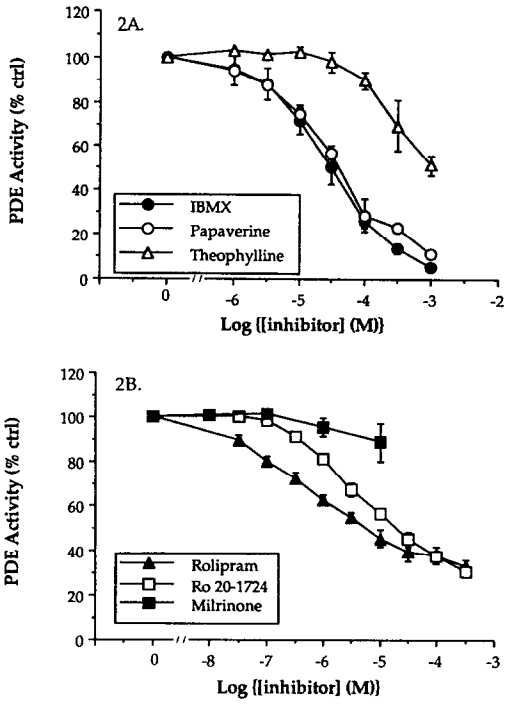

Fig. 2. Inhibition of PDE activity in a homogenate of SH-SY5Y cells by a variety of selective and non-selective inhibitors. Homogenate was prepared as in Materials and Methods and represents total cellular PDE activity (soluble plus particulate). Assay was conducted with 1 μM cAMP as substrate for 15 min at 37° in the presence and absence of varying inhibitor concentrations. All data are means ± SEM of three experiments performed in triplicate except for the theophylline data set which was mean ± range of two experiments performed in triplicate. Control values were 1412 ± 180 pmol adenosine produced/15 min/mg protein (N = 15 observations in triplicate).

levels of cAMP 4-fold, the type IV inhibitors at maximally or near maximally effective concentrations, elicited a much larger 20–55-fold increase (Fig. 1A). Papaverine may be too weak an agent to observe its maximum effect, but at 300 μM it increases cAMP some 15-fold. Forskolin itself enhanced accumulation of cAMP by ~20-fold, but nevertheless the disparity between IBMX and the other inhibitors was still marked in its presence (Fig. 1B).

The unexpected difference in the effect between IBMX and the other agents was examined further and found not to be due to a slower time course of action of IBMX: both IBMX and rolipram sustained their maximal effect after a 5–10 min preincubation (not shown). To confirm that the PDEs in these cells displayed their expected susceptibility to inhibition by all the agents, a cell homogenate was prepared, and the inhibition of PDE activity measured. Figure 2 indicates that IBMX and papaverine were capable of inhibiting ≥95% of total PDE activity as assessed with 1 μM cAMP as substrate. Whilst the PDE III-selective inhibitor, milrinone, had no real effect on cAMP hydrolysis at concentrations ≤10 μM, the

Table 1. Effect of PDE inhibitors upon cGMP mass levels in SH-SY5Y cells

[IBMX] (μM)	−Forskolin	+Forskolin
0	0.36 ± 0.03	0.46 ± 0.23
10	0.38 ± 0.07	0.45 ± 0.19
100	0.40 ± 0.13	0.49 ± 0.15
1000	0.55 ± 0.24	0.52 ± 0.22
Rolipram	0.44 ± 0.16	0.51 ± 0.12
Theophylline	0.43 ± 0.13	0.44 ± 0.16

Cells were preincubated with DMSO or inhibitors for 20 min prior to a 10 min stimulation with 1 μM forskolin. Rolipram = theophylline = 100 μM. Samples were assayed for cGMP as in Materials and Methods.

Results expressed as pmol/mg protein (mean ± SEM of three experiments performed in triplicate).

PDE IV-selective agents maximally inhibited the activity by ~70%. These data therefore suggest that the poor effect of IBMX could not be attributable to some anomalous behaviour of the PDE complement.

Unlike PDE IV-selective inhibitors, IBMX can also inhibit cGMP PDEs; this could lead to elevations of cGMP that might antagonize cAMP accumulation [29]. However, application of IBMX, theophylline or rolipram, in the presence or absence of forskolin, was insufficient to evoke a significant change in cGMP levels above control ($P > 0.40$) (Table 1), suggesting that cGMP could not be implicated in dampening cAMP accumulation elicited by IBMX.

Metazoan cells are also capable of reducing cAMP concentration by actively extruding the molecule from the cytoplasm, although the extent varies between cell types [30, 31]. In these neuroblastoma cells, the localization of cAMP after agent addition had not been established since the experiments were conducted in such a way that no attempt was made to discriminate between intra- and extracellular cyclic nucleotide accumulation. Given that methylxanthines can inhibit cAMP egress [5], it was imperative to ascertain that differences in cAMP distribution did not underly the poor effect of IBMX. Figure 3 shows that after treatment there was no significant difference between intracellular and total cAMP accumulation, whether after PDE inhibition, forskolin or a combination of both. Apparently in these cells, egress does not make any significant contribution to the reduction in intracellular cAMP, and importantly, the disparity between IBMX and rolipram, evident irrespective of whether "total" or "intracellular" cAMP was measured, cannot be attributed to their differential effects upon cAMP distribution. Interestingly, the co-incubation of IBMX and rolipram gave results similar to that with IBMX alone, and this observation was expanded upon in the next set of experiments.

That IBMX is acting at a locus (other than PDE inhibition) which leads to a reduction in cAMP synthesis was substantiated by the additivity experiments depicted in Fig. 4. Here, cAMP was significantly increased by rolipram (± forskolin) and the effect of increasing IBMX concentration on this

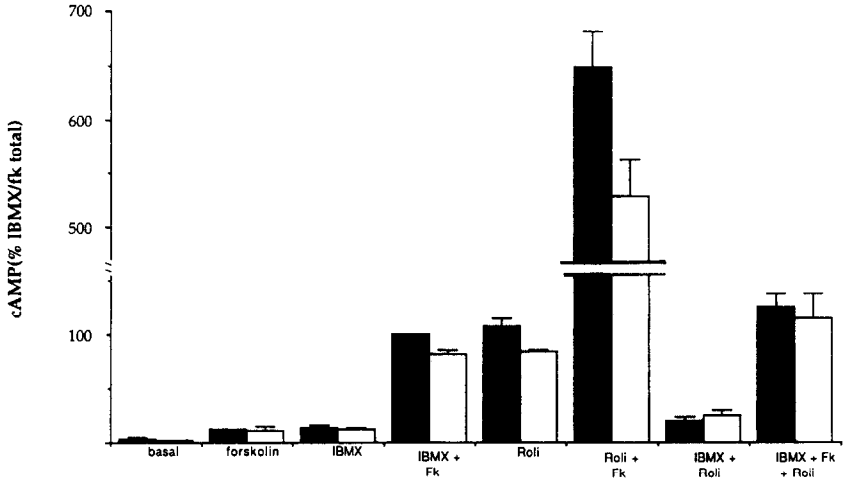

Fig. 3. Assessment of localization of cAMP after PDE inhibition. Experiments were performed in microfuge tubes, otherwise the protocol essentially followed that for earlier experiments, i.e. preincubation for 20 min with DMSO (1.6% v/v) or inhibitor (rolipram = 100 μM; IBMX = 1 mM). Forskolin (Fk) (1 μM) was added for 2 min where indicated. For intracellular cAMP determination (open columns), samples were centrifuged for 15 sec at 11,000 g, the supernatant was aspirated and acid was added to the cell pellet. For "total" samples (intra- plus extracellular cAMP; filled columns), tubes were centrifuged as above, but the supernatant was not removed when acid was applied. Basal cAMP: total = 4.48 ± 1.63, intra = 3.65 ± 1.77; IBMX: total = 13.46 ± 5.26, intra. = 11.99 ± 3.83 pmol/mg protein. Data are means ± SEM of four experiments performed in duplicate or triplicate.

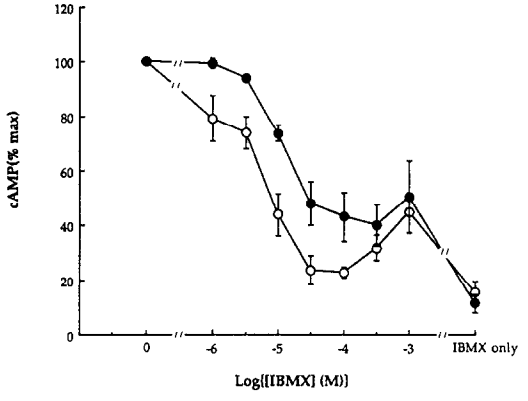

Fig. 4. Effect of IBMX on rolipram-elicited increases in cAMP. Cells were incubated for 20 min with 100 μM rolipram plus various concentrations of IBMX (○). Forskolin (1 μM) was then added for another 10 min where indicated (●). For "IBMX alone", a 1 mM concentration was used. Rolipram alone = 77.3 ± 41.95, rolipram + forskolin = 1057 ± 653 pmol cAMP/mg protein. Results are expressed as means ± SEM of three to four experiments performed in triplicate.

pre-elevation was examined. Clearly, IBMX potently inhibited the accumulation elicited by the other compounds (IC_{50} 4–10 μM). In these experiments, the curve was biphasic when forskolin was absent, lower IBMX concentrations (≤100 μM) inhibiting the response to that with IBMX alone, and higher concentrations of IBMX once again increasing cAMP levels above IBMX alone ($P < 0.01$), probably a result of its PDE-inhibiting properties starting to dominate.

Extending these studies, concentration–response curves for rolipram were constructed in the presence and absence of 10 μM IBMX, a concentration giving ~50% inhibition; note the different Y axis scales for either control or IBMX-treated cells (Fig. 5A and B). No apparent change in the potency of rolipram was observed when methylxanthine was present, irrespective of forskolin stimulation. Furthermore, the effect of IBMX was apparently rapid: the elevation in cAMP induced by pretreatment with 100 μM rolipram was inhibited by 10 μM IBMX with a $T_{1/2}$ = 5.5 min ($N \geq 3$; not shown).

The potency of IBMX for inhibiting the rolipram response was reminiscent of its affinity for adenosine receptors (K_i at A_2 receptors = 5 μM) [7–9]. Should endogenous adenosine be produced by the cells and be acting in an autocrine fashion at receptors coupled positively to adenylyl cyclase, then receptor antagonism by IBMX would block cyclase stimulation and cAMP accumulation would be attenuated. With the non-adenosine receptor PDE inhibitors (rolipram and Ro 20 1724), this receptor-mediated enhancement would actually be potentiated. In an attempt to eliminate endogenous adenosine effects, the receptor antagonist theophylline (a weak PDE inhibitor [8] and see Fig. 2) was applied to naive and forskolin-stimulated cells (Fig. 6). A dramatic lowering of cAMP levels was observed, significant only in forskolin-treated cells, with a concentration dependency (IC_{50} 17 μM) not dissimilar to its affinity for adenosine receptors ($K_i \sim 5$ μM at either A_1 or A_2 receptors) [7–9]. It has been noted previously

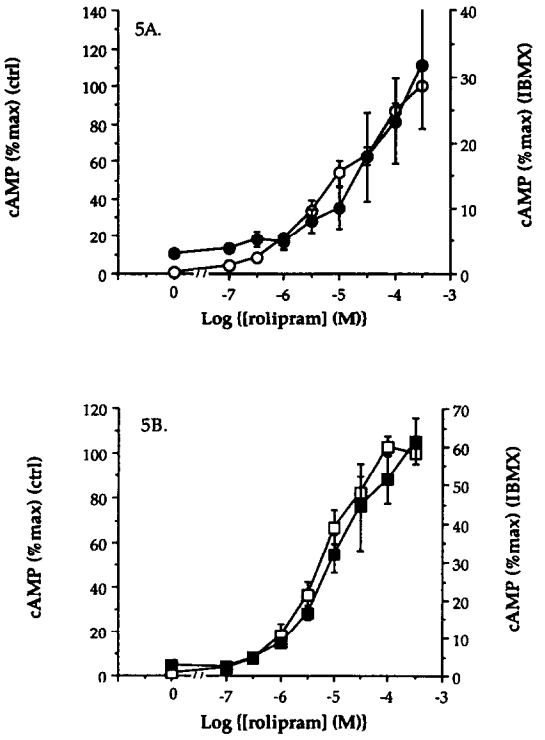

Fig. 5. Effect of IBMX on rolipram concentration–response relationship. Cells were incubated with varying inhibitor concentrations for 20 min. IBMX (10 μM, filled symbols) or DMSO (open symbols) was co-incubated with varying rolipram concentrations (A) and stimulated cells were exposed to 1 μM forskolin for 10 min (B). Data in each panel are expressed as means ± SEM of three to four experiments performed in triplicate.

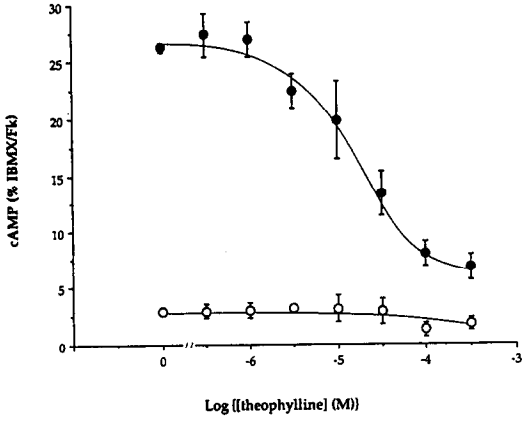

Fig. 6. Concentration–response relationship between theophylline and cAMP accumulation. Preincubation for 20 min with theophylline/DMSO (○) or IBMX reference (1 mM). Forskolin (Fk) (1 μM) was added thereafter for 10 min (●). Basal cAMP = 3.34 ± 0.45, 300 μM; theophylline = 1.96 ± 0.38; Fk = 30.89 ± 4.59, Fk/300 μM theophylline = 9.40 ± 2.21 pmol/mg protein. Data represent means ±SEM of three experiments performed in triplicate.

that agonists (or G proteins) synergize with the diterpene in activating adenylyl cyclase [32].

Adenosine involvement can be further inferred from the experiments performed with adenosine deaminase (ADA). Figure 7 shows that preincubation of cells with the adenosine-metabolizing enzyme significantly blunted the cAMP responses to rolipram and forskolin ($P < 0.05$), but not the cAMP levels in basal or IBMX-treated cells. Moreover, the ADA effect appeared to be maximal at 1 U ADA/mL, although most notably, it did not reduce the rolipram response to that with IBMX alone ($P < 0.01$).

Further experiments (Fig. 8) gave support for adenosine receptors on SH-SY5Y neuroblastoma cells that are coupled positively to cAMP accumulation. Upon application of the receptor agonist, 2-chloroadenosine (which only marginally discriminates between receptor subtypes [33]), cAMP levels increased in cells pretreated with ADA, irrespective of PDE inhibition by rolipram. With a more marked increase in cAMP elicited in the presence of the PDE inhibitor, this suggests that receptors are indeed present which couple to adenylyl cyclase serving to enhance cAMP synthesis.

DISCUSSION

In SH-SY5Y neuroblastoma cells it has been indicated that addition of PDE inhibitors can elevate cAMP, even in the absence of exogenous agonist, suggesting that apparent "basal" cAMP flux is significant in these cells. However, what was not expected was that the xanthine-derived inhibitor IBMX was a far less effective agent than the non-xanthine, chemically disparate inhibitors papaverine, Ro 20 1724 and rolipram. Assuming that IBMX would inhibit *all* isoforms of PDE whilst Ro 20 1724 and rolipram would selectively reduce cAMP hydrolysis effected by the low K_m cAMP-specific (type IV) PDE [1–3], the only difference that could have been predicted was a *greater* increase evoked by a maximally effective concentration of the non-selective inhibitor; however, the opposite was observed.

Cyclic AMP hydrolytic activity in cell homogenates displayed its normal pattern of susceptibility to the PDE inhibitors, refuting the notion that the PDEs in these cells were somewhat less sensitive to IBMX inhibition when compared to other inhibitors. Moreover, the maximal reduction of cAMP hydrolysis by rolipram and Ro 20 1724 appears to suggest that at more physiological cAMP concentrations ~70% of cAMP hydrolysis is carried out by type IV PDE, although a still significant 30% capacity is effected by an undefined PDE population. Although it is highly unlikely to be the type III (cGMP-inhibitable) PDE [1], given the lack of effect of milrinone and the lack of evidence for cGMP-inhibitable PDE in nervous tissue [1–3], the experimental protocol was not optimized to demonstrate its presence. The existence of a type III PDE cannot, therefore, be completely ruled out.

Unlike type IV inhibitors, IBMX can also inhibit cGMP PDEs [1], and whilst it was possible that potentially antagonistic increases in cGMP could be responsible for a lowering of the cAMP response

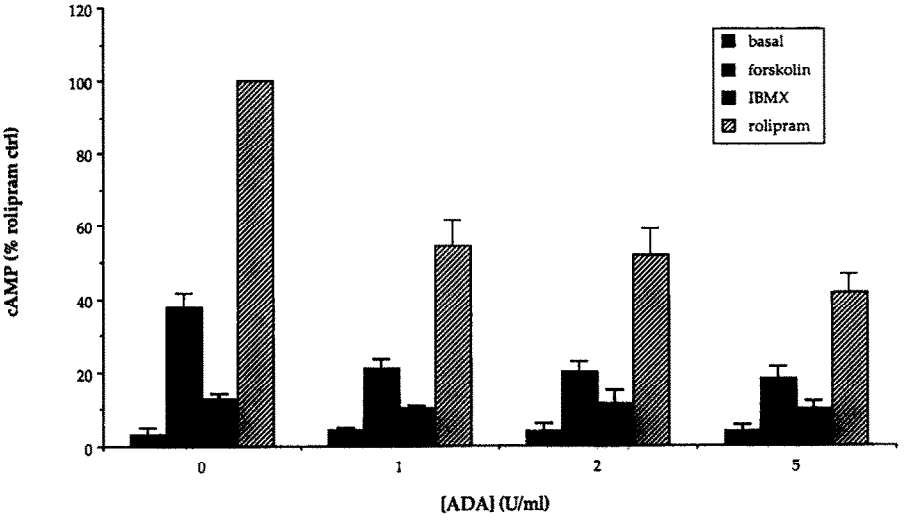

Fig. 7. Effect of ADA upon cAMP levels following stimulation or PDE inhibition. Cells were preincubated for 20 min in the presence of varying ADA concentrations or its vehicle, glycerol (1.6%, v/v); PDE inhibitors or their DMSO vehicle were likewise included (rolipram = 100 μM, IBMX = 1 mM). Forskolin (1 μM) was added for 10 min. Rolipram = 79.56 ± 36.84, rolipram/ADA = 31.37 ± 11.88 pmol cAMP/mg protein. Data represent means ± SEM of three to four experiments performed in triplicate.

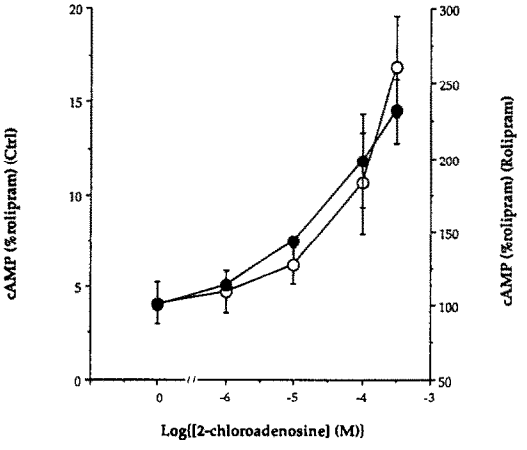

Fig. 8. Effect of 2-chloroadenosine upon basal and rolipram-elevated cAMP levels in the presence of ADA. After a 20 min preincubation with (●) or without (○) 100 μM rolipram in the presence of 2 U/mL ADA, cells were stimulated for 10 min with a range of 2-chloroadenosine concentrations. 2-Chloroadenosine was dissolved in 50% DMSO (1.6% v/v final). Basal = 6.79 ± 0.02; rolipram = 104.85 ± 4.57 pmol cAMP/mg protein (mean ± range). Data represent means ± range of two experiments performed in triplicate.

with the non-selective agent, it was shown in Table 1 that increases in cGMP were elicited with IBMX. This implies a lower basal turnover of this second messenger when compared with cAMP in these cells. Additional experiments confirmed that cAMP was not extruded from these cells, such that the majority of the cyclic nucleotide routinely measured was found in the intracellular compartment, and that the IBMX effect therefore could not be due to interference with the trans-plasmalemmal distribution of cAMP.

When cAMP levels were increased by prior application of rolipram (or rolipram plus forskolin), IBMX could potently reduce this elevation, essentially to that observed with 1 mM IBMX alone, and this was not a consequence of altered cAMP egress. Since the potency of rolipram for raising cAMP was relatively unchanged in the presence of IBMX, alteration of the accessibility of rolipram to its binding site(s) did not seem to be affected by the non-selective PDE inhibitor, and was supportive of IBMX acting at a distinct site.

There have also been reports of IBMX and other xanthines actually *stimulating* PDE activity (by activation of the type II, cGMP-stimulated PDE [34, 35]) which could conceivably explain the inhibition of rolipram-stimulated cAMP levels. However, it is unlikely to pertain in this instance for several reasons: first, at higher IBMX levels (>100 μM), the expected inhibitory actions of the xanthine predominate [34, 35], and even at 1 mM IBMX, its effect on accumulation is poor in these cells. Second, this activation by xanthines is only observed with low substrate concentrations (~1 μM) [34, 35], whilst in SH-SY5Y cells, IBMX dramatically inhibits the elevation of cAMP evoked by 100 μM rolipram (which raises cAMP about 50-fold).

Adenosine released by cells has previously been established to have an auto- or paracrine effect upon cAMP levels in a variety of systems [7, 8]. An attractive explanation of the data was that the effects

of endogenous adenosine were potentiated by rolipram and Ro 20 1724, whilst IBMX, an adenosine receptor antagonist, was blocking the effect of the nucleoside; hence the disparity in effectiveness. The similarity of the K_i of IBMX at A_2 receptors (~5 μM) [8] with its potency for inhibiting rolipram responses (IC_{50} 4–10 μM) is consistent with this notion. Moreover, addition of the adenosine receptor antagonist, theophylline, did ablate cAMP accumulation in cells at concentrations incapable of inhibiting cAMP PDEs (Fig. 2) or affecting cellular cGMP levels (Table 1).

More importantly, conversion of cell-derived adenosine to inosine by ADA treatment significantly reduced rolipram and forskolin stimulatory effects, whilst IBMX-elevated cAMP levels were unchanged. To strengthen further the argument for the involvement of adenosine, the metabolically resistant agonist 2-chloroadenosine was employed to elicit cAMP responses in SH-SY5Y cells. Its success suggested that these cells do express purinoceptors functionally coupled to increases in cAMP synthesis as has been suggested previously [22], thus providing a means by which the released adenosine can act on these cells. It must be noted, however, that the potency of 2-chloroadenosine appears to be quite low in this regard (cf. K_d 1 μM [8]). Whether this reflects expression of both A_1 and A_2 receptors on SH-SY5Y cells coupled antagonistically to adenylyl cyclase is unclear at present.

What was not expected was the fact that ADA, at an apparently maximal activity, was unable to completely reduce the rolipram response to that of IBMX alone. It may be that this ADA-insensitive component reflects adenosine-*independent* effects of IBMX [4–6]. Indeed, in further experiments, the size of the ADA-sensitive component appeared to depend on whether or not adenylyl cyclase was stimulated: ADA inhibited the maximal rolipram elevation of cAMP 70 ± 7%, but only 34 ± 7% when 1 μM forskolin was also present (mean ± SEM, $N \geq 3$). Furthermore, the ADA-insensitive component was still inhibited by IBMX. That the adenosine potentiation of the rolipram response is fully responsible for the difference between IBMX and the type IV inhibitor is not easily reconciled with such a small effect of ADA under conditions of forskolin stimulation. Given recent reports that methylxanthines and similar compounds can modulate adenylyl cyclase activity indirectly via G proteins [6, 36], it may be that IBMX also impairs cAMP synthesis at a post-receptor level (though probably not the P-site of adenylyl cyclase itself [7]). However, the ability of ADA to remove adenosine completely has recently been questioned [37], so further experiments will be required to strengthen such a hypothesis.

In conclusion, we believe that the data demonstrate that IBMX is a less effective agent for elevating cAMP in these neuroblastoma cells than other non-xanthine PDE inhibitors, a phenomenon which is in part accounted for by its antagonism of endogenously released adenosine that acts to stimulate adenylyl cyclase. However, our data do not exclude the possibility that a lesser appreciated action of IBMX may affect cAMP metabolism in addition to the effect upon adenosine receptors.

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