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ADENYLYL CYCLASE IN SH-SY5Y HUMAN NEUROBLASTOMA CELLS IS REGULATED BY INTRA- AND EXTRACELLULAR CALCIUM

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Abstract—Adenylyl cyclase exists as a family of closely related subtypes which differ in their tissue distribution and regulatory properties. Submicromolar rises in $[Ca^{2+}]_i$ produced via activation of phospholipase C (PLC) or Ca^{2+} channel opening, provide a mechanism by which Ca^{2+}/calmodulin (CaM) or protein kinase C (PKC)-sensitive isoforms of adenylyl cyclase can be regulated. In this study we have examined, in detail, the muscarinic (M_3) regulation of adenylyl cyclase in SH-SY5Y cells and report a role for both $[Ca^{2+}]_e$ and $[Ca^{2+}]_i$. Carbachol (1 mM) and potassium (100 mM) caused a time ($T_{1/2}$ = 3 and 4 min, respectively) and dose (EC_{50} = 6.95 μM and 34.7 mM respectively) related increase in cAMP formation. This amounted to an approximate two-fold increase over basal levels. Carbachol and potassium also caused a biphasic increase in $[Ca^{2+}]_i$ with basal, peak and plateau values of 118.4 nM, 697.6 nM, 253.0 nM and 104.0 nM, 351.6 nM, 181.5 nM, respectively. Calcium channel blockade with nickel (2.5 mM) abolished potassium-stimulated cAMP formation and rises in $[Ca^{2+}]_i$. However, carbachol-stimulated cAMP formation was significantly decreased only at the later time points, where rises in $[Ca^{2+}]_i$ were also essentially abolished. Further evidence for a role for $[Ca^{2+}]_e$ and $[Ca^{2+}]_i$ is provided by the stimulation of cAMP formation by carbachol in the absence of added Ca^{2+}, followed by a further increase on its re-addition. Carbachol- and potassium-stimulated cAMP formation were inhibited by the CaM antagonist trifluoperazine (100 μM). The μ-opiate agonists, morphine and fentanyl also inhibited carbachol-stimulated cAMP formation. In addition, cAMP formation in SH-SY5Y cell membranes was significantly increased in the presence of Ca^{2+} (1.46 μM), CaM (200 nM) and forskolin (1 μM). PKC inhibition with Ro 31 8220 did not affect carbachol-stimulated cAMP formation. Taken collectively, these data suggest that SH-SY5Y cells express type 1, and possibly type 8 isoforms of adenylyl cyclase, which can be regulated by intra- and extracellular Ca^{2+}.

Key words: calcium; cAMP; adenylyl cyclase; SH-SY5Y human neuroblastoma cells

cAMP† serves as an important second messenger, transmitting extracellular signals to modulate a diverse array of cellular responses [1]. We have previously reported that SH-SY5Y human neuroblastoma cells express a homogeneous population of M_3 muscarinic receptors [2], which preferentially couple to PLC [3] probably via Gq [4], and cause a biphasic increase in intracellular calcium concentration ($[Ca^{2+}]_i$) [3]. This well-characterized rise in $[Ca^{2+}]_i$ is due to rapid IP_3-induced calcium release from an internal store and simultaneous ROCC opening [5]. SH-SY5Y cells also possess L- and N-type VSCC which can be opened under K^+ depolarizing conditions to increase $[Ca^{2+}]_i$ [6].

Current molecular and pharmacological classification of the muscarinic receptor family indicates that $M_{1/3/5}$ subtypes couple to PLC and increase $[Ca^{2+}]_i$, and $M_{2/4}$ couple to adenylyl cyclase to decrease cAMP [7]. However, the reported $M_{1/3/5}$ stimulated increase in $[Ca^{2+}]_i$ represents a mechanism by which Ca^{2+}-sensitive isoforms of adenylyl cyclase can be indirectly regulated, allowing cross-talk between phosphatidyl inositol and adenylyl cyclase coupled receptors.

To date, eight different isoforms of mammalian adenylyl cyclase have been cloned [8]. Types 1–8 are all activated by G_s-α and forskolin but vary in their sensitivity to different regulatory molecules, these include G_i-α, G-$\beta\gamma$, Ca^{2+}/CaM, PKC and PKA [8]. The predominant adenylyl cyclase isoform to be expressed in neuronal tissue is type 1 [9] which is inhibited by G-$\beta\gamma$ and activated by Ca^{2+}/CaM [8]. Moreover, Choi *et al.* demonstrated muscarinic stimulation of transfected type 1 adenylyl cyclase in HEK 293 [10], but there is still some debate surrounding its regulation by PKC [8, 11]. Recent characterization of type 8 adenylyl cyclase has shown that it is also neuronal in origin and can be regulated by Ca^{2+}/CaM [12].

Recent conflicting reports can be found in the literature showing that carbachol can stimulate cAMP formation in SK-N-SH/SH-SY5Y cells by both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms [13–15]. In this study we have examined

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† Abbreviations: cAMP, 3′,5′-cyclic adenosine monophosphate; PLC, phospholipase C; IP_3, inositol-1,4,5-triphosphate; ROCC, receptor operated calcium channel; VSCC, voltage-sensitive calcium channel; CaM, calmodulin; PKC, protein kinase C; PKA, protein kinase A; HEK, human embryonic kidney cells; [^{3}H]NMS, 1-[N-methyl-^{3}H]scopolamine methyl chloride; IBMX, 4-isobutyl-1-methylxanthine; TFP, trifluoperazine; Fura-2AM, Fura-2-acetoxymethylester; BAPTA, 1,2-bis(*O*-aminophenoxy)ethane-*N*′*N*′*N*′*N*-tetracetic acid; PMA, 4-β-phorbol-12-myrisate-13-acetate.

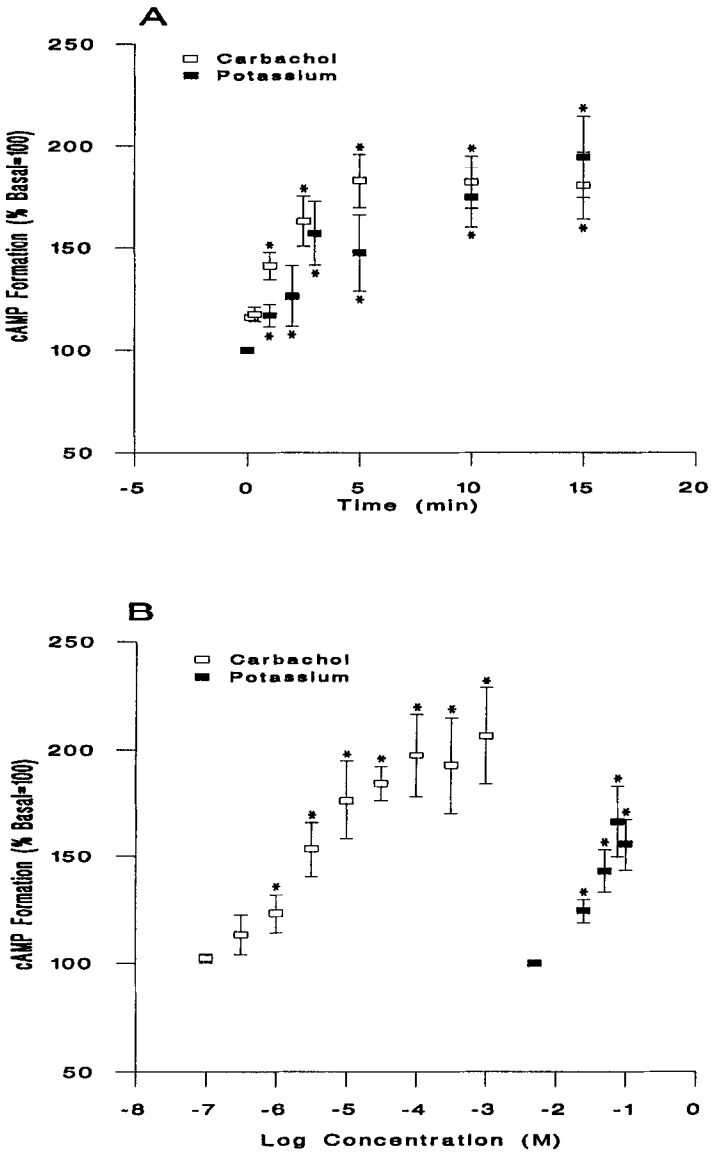

Fig. 1. (□) Carbachol and (■) potassium caused (A) a time, and (B) a dose-related stimulation of cAMP formation in SH-SY5Y cells. Data are mean ± SEM (N = 6–10). Dose response curves and time courses were significant ($P < 0.05$) by ANOVA. *Significantly ($P < 0.05$) increased compared with basal levels.

in detail the regulation of adenylyl cyclase in SH-SY5Y cells and report a role for both $[Ca^{2+}]_e$ and $[Ca^{2+}]_i$.

METHODS

Sources of reagents

Tissue culture media, supplements, trypsin/EDTA, sera and plasticware (Nunc) were from Life Technologies (Uxbridge, U.K.). [^{3}H]NMS (84 Ci/mmol) and [2,8-^{3}H]cAMP (28.4 Ci/mmol) were from Amersham (U.K.) and NEN DuPont (Boston, MA, U.S.A.), respectively. cAMP, IBMX, atropine, fentanyl, morphine, carbachol, muscarine, TFP, GTP, ATP, phosphocreatine, creatine kinase, Fura-2AM, fura-2 free acid and calmodulin were from Sigma Chemical Co. (Poole, U.K.). Ro 31 8220 was a kind gift from Roche Pharmaceuticals (Welwyn Garden City, U.K.). Any other reagents used were of the highest purity available. Radioactivity was measured by liquid scintillation spectroscopy using either Optiphase Safe or Optiphase Hi-safe III (Wallac, U.K.) as the scintillant.

Cell culture and miscellaneous methods

SH-SY5Y cells (a gift from Dr J. Biedler, Sloane-Kettering Institute, NY, U.S.A.), passages 68–98, were cultured in minimum essential medium

supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone and 10% foetal calf serum at 37° in 5% CO_2/humidified air. The cells were passaged weekly (1:7) and fed twice weekly. When confluency was reached (6–7 days), the cells were harvested by brief exposure to HEPES (10 mM) buffered (pH 7.4) saline (0.9%) containing EDTA (0.5 mM).

Whole cells were washed twice (15 mL/flask/wash) with Krebs/HEPES buffer pH 7.4 at 37° with the following composition (mM): Na^+ (143.3), K^+ (4.7), Ca^{2+} (2.5), Mg^{2+} (1.2), Cl^- (125.6), $H_2PO_4^{2-}$ (1.2), SO_4^{2-} (1.2), glucose (11.7) and HEPES (10). Na^+ concentrations were adjusted to maintain tonicity in K^+ depolarization experiments.

[^{3}H]*NMS binding studies*

The binding of [^{3}H]NMS to whole cell suspensions (approx. 200 μg) was performed at 37° in 1 mL for 60 min in Krebs/HEPES buffer at pH 7.4. Calcium was omitted from the buffer for incubations with nickel, and non-specific binding was assessed in the presence of atropine (1 μM). In saturation experiments a range of concentrations was used (0.1–3 nM). For displacement studies a fixed concentration of [^{3}H]NMS (0.2 nM) was used [2]. Bound and free [^{3}H]NMS were separated by rapid vacuum filtration using a Brandel cell harvester onto Whatman GF/B filters and washed with 2 × 5 mL of ice-cold buffer. Filters were placed in scintillation vial inserts and covered with 4 mL of Optiphase Safe. Radioactivity was extracted overnight.

Measurement of cAMP

Whole cells. Whole cell suspensions (approx. 200 μg) were incubated in 300 μL volumes of Krebs/HEPES buffer containing 1 mM IBMX, and combinations of other drugs for varying times at 37°. Reactions were terminated by the addition of 20 μL of HCl (10 M). The pH was equilibrated by the addition of 20 μL of NaOH (10 M) and 180 μL of Tris-HCl buffer (1 M, pH 7.4). Following centrifugation (13,000 rpm for 2 min in a Sarstedt microfuge), cAMP was measured in the supernatant by a radioreceptor assay [16] using a binding protein from bovine adrenal cortex.

Membranes. SH-SY5Y cell membranes were prepared freshly for each experiment as follows; complete homogenization of whole cell suspensions (3 mL) was achieved using a tissue tearor (setting 5, for 5 × 10 sec). The membranes were sedimented at 15,000 rpm for 15 min (Sarstedt microfuge), and then resuspended in 3 ml of nominally Ca^{2+}-free ATP regenerating buffer pH 7.4 at 37° with the following composition (mM): ATP (0.5), phosphocreatine (10), GTP (0.1), HEPES (10), Na^+ (140), Cl^- (143), Mg^{2+} (3), IBMX (0.5) and creatine kinase (30 EU/mL) [17]; this constituted the first wash. Membranes were washed four times before use. Reactions were terminated and cAMP was measured as described above.

Measurement of Ca^{2+}

Confluent (6–7 days old) cells were harvested and washed in Krebs/HEPES buffer (10 mL). Cell suspensions were loaded with 5 μM Fura-2AM (3 mL) for 30 min at 37°. The cells were then sedimented and resuspended in Krebs/HEPES buffer and incubated for 15 min at room temperature to allow for de-esterification of the Fura-2AM. $[Ca^{2+}]_i$ was measured at 37°, using a Perkin-Elmer LS50B fluorimeter [18, 19]. R_{min} (EGTA, 3 mM), R_{max} (Triton-X100, 0.1%) and Sfb were 0.61 ± 0.01, 5.10 ± 0.1 and 3.24 ± 0.07 (N = 24), respectively. Membrane buffer $[Ca^{2+}]$ was determined fluorometrically using 1 μM Fura-2 free acid; R_{max} and R_{min} were established by the addition of Ca^{2+} (1 mM) and EGTA (3 mM), respectively.

Table 1. Muscarinic regulation of SH-SY5Y adenylyl cyclase was reversed by atropine and TFP (mean ± SEM, N = 3–6)

Treatment	[cAMP] (% basal = 100)
Muscarine (1 mM)	178.9 ± 10.2*
+Atropine (1 μM)	79.8 ± 4.6
Carbachol (1 mM)	245.7 ± 18.6*
+Atropine (1 μM)	79.8 ± 5.5
+TFP (100 μM)	96.3 ± 11.0
Forskolin (10 μM)	3302.8 ± 138.2*
+Carbachol (1 mM)	5691.4 ± 113.7*†
+Atropine (1 μM)	3658.1 ± 202.3*
Carbachol (30 mM)	221.3 ± 14.2*
+Atropine (1 μM)	138.4 ± 14.8
+TFP (100 μM)	108.2 ± 11.8

* Significantly ($P < 0.05$) increased compared with basal.
† Significantly ($P < 0.05$) increased compared with forskolin.

Data analysis

Data are expressed as mean ± SEM of at least three determinations. EC_{50}, IC_{50}, K_i, and slope values were all obtained by computer-assisted curve fitting using GRAPHPAD. Where appropriate statistical comparisons were made using Student's paired or unpaired *t*-test (following ANOVA where appropriate) and considered significant when $P < 0.05$.

RESULTS

Carbachol and potassium caused a time (approx. $T_{1/2}$ = 3 and 4 min, respectively) and dose (EC_{50} = 6.95 ± 1.97 μM, N = 6 and 34.75 ± 4.09 mM, N = 8, respectively) related increase in cAMP formation (Fig. 1(A) and 1(B)), amounting to an approximate two-fold increase in basal cAMP formation. There was considerable variation in absolute values of cAMP formation, for example, mean basal and 1 mM carbachol-stimulated cAMP formation at 15 min were 9.6 ± 1.3, range 1.3–23.3 and 20.9 ± 2.7, range 6.6–57.8 pmols/mg protein (N = 21) respectively. Unless stated otherwise all subsequent values are expressed as % basal.

Carbachol (1 mM) and muscarine (1 mM) stimulated cAMP formation was reversed by atropine (1 μM) (Table 1). Carbachol (1 mM) stimulated cAMP formation was also inhibited by TFP (100 μM). Direct activation of adenylyl cyclase with forskolin

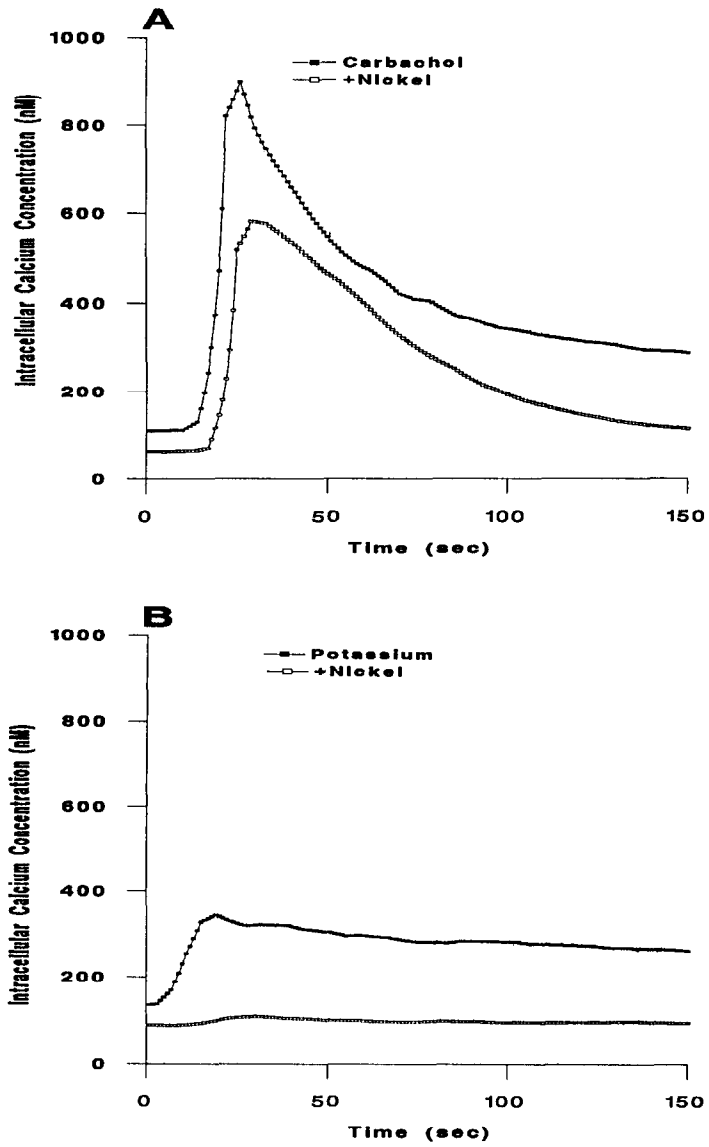

Fig. 2. (A, ■) 1 mM carbachol, and (B, ■) 100 mM potassium caused a biphasic rise in $[Ca^{2+}]_i$. (□) In the presence of 2.5 mM nickel the rise in $[Ca^{2+}]_i$ was reduced for both stimuli. Data are for a single experiment from six others.

(10 μM) caused a 33-fold stimulation of cAMP formation. This was increased to 56.9-fold in the presence of carbachol (1 mM) with the increase being reversed by atropine (1 μM) (Table 1). At supramaximal concentrations of carbachol (30 mM), cAMP was also increased in an atropine (1 μM) and TFP (100 μM) reversible fashion (Table 1).

Carbachol and potassium caused a biphasic increase in $[Ca^{2+}]_i$ (Fig. 2). The potassium peak (351.6 ± 40.2 nM) and plateau (measured at 2 min, 181.5 ± 19.6 nM) were lower ($P < 0.05$) than that produced by carbachol (peak = 697.6 ± 64.0 nM and plateau = 253.0 ± 12.7 nM) (Table 2). In the presence of nickel, carbachol evoked increases in $[Ca^{2+}]_i$ were significantly ($P < 0.05$) reduced at basal and peak phases, with the plateau being essentially abolished. However, potassium increases in $[Ca^{2+}]_i$ were abolished in the presence of nickel (2.5 mM) (Table 2 and Fig. 2).

Preincubation with nickel (2.5 mM) did not affect the initial (at 1 min) carbachol mediated rise in cAMP but significantly ($P < 0.05$) reduced the later (at 5 and 10 min) cAMP levels (Fig. 3). Nickel (2.5 mM) and TFP (100 μM) also inhibited potassium (100 mM, 15 min) stimulated cAMP formation by 175 ± 23.2 and 120 ± 25.9%, respectively.

The binding of [^{3}H]NMS was dose-dependent and saturable with K_D and B_{max} values of 0.3 ± 0.01 nM and 74.7 ±5.2 fmol/mg protein, respectively (N = 6). Atropine, and surprisingly nickel and TFP caused

Table 2. Carbachol and potassium caused a biphasic increase in $[Ca^{2+}]_i$ (mean ± SEM, N = 6 or 7)

Treatment	Intracellular Ca^{2+} concentration (nM)		
	Basal	Peak	Plateau
Carbachol (1 mM)	118.4 ± 9.2	697.6 ± 64.0*	253.0 ± 12.7*
+Nickel (2.5 mM)	84.8 ± 12.2†	498.0 ± 35.2*†	131.2 ± 12.5*†
Potassium (100 mM)	104.0 ± 10.6	351.6 ± 40.2*	181.5 ± 19.6*
+Nickel (2.5 mM)	77.6 ± 7.6‡	99.0 ± 8.1‡	88.7 ± 9.3‡

* Significantly ($P < 0.05$) increased compared with basal.
† Significantly ($P < 0.05$) decreased compared with carbachol.
‡ Significantly reduced compared with potassium.

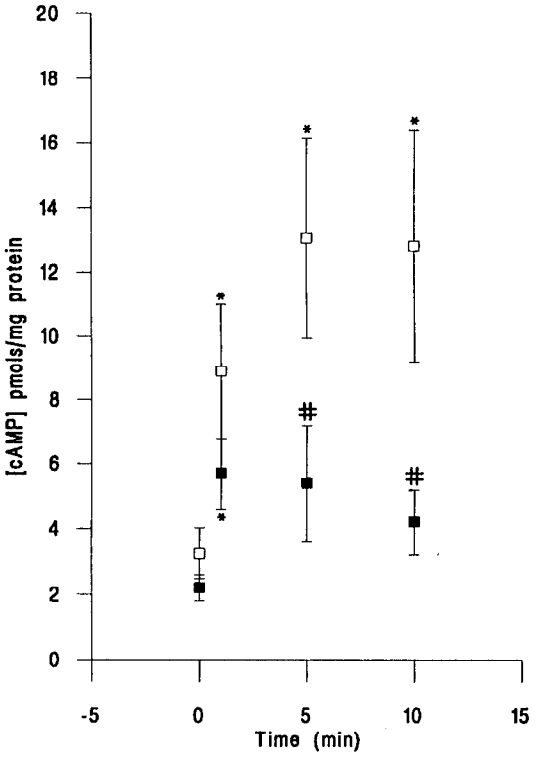

Fig. 3. Time course for 1 mM carbachol stimulated cAMP formation in (■) the presence, or (□) absence of 2.5 mM nickel. Data are mean ± SEM (N = 6). *Significantly ($P < 0.05$, paired *t*-test) increased compared with basal. # Significantly ($P < 0.05$, paired *t*-test) decreased compared with carbachol.

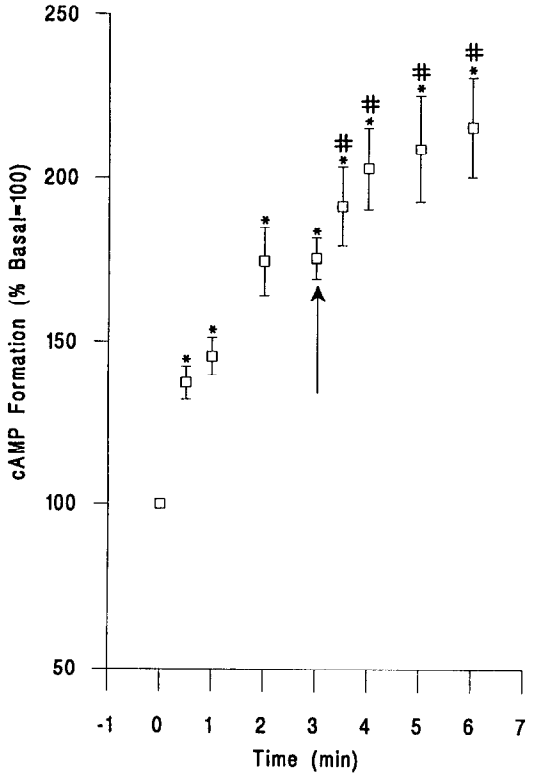

Fig. 4. 1 mM carbachol stimulated cAMP formation in the absence then presence (arrow) of 2.5 mM Ca^{2+}. * Significantly ($P < 0.05$, paired *t*-test) increased compared with basal. # Significantly ($P < 0.05$, paired *t*-test) increased compared with 3 min time point. Data are mean ± SEM (N = 4).

displacement of 0.2 nM [^{3}H]NMS with K_i values of 0.78 nM, 0.69 mM and 1.02 μM, respectively (Table 3). Using the Cheng–Prussoff equation [20] the concentration of nickel and TFP required to inhibit 1 mM carbachol stimulated cAMP formation by 50% was 182 mM and 269 μM, respectively.

Carbachol (1 mM) challenge, under Ca^{2+}-free conditions, stimulated cAMP formation with a significant ($P < 0.05$) increase occurring from 1 min. This amounted to an approximate 75% enhancement at 3 min. Subsequent Ca^{2+} addition at 3 min was followed by a significant elevation in cAMP formation at 3.5 min ($P < 0.05$), such that by 6 min the increase in cAMP formation was approximately two-fold basal (Fig. 4.).

In SH-SY5Y membranes, Ca^{2+} (1.46 μM), CaM (200 nM) and forskolin (1 μM) caused a significant ($P < 0.05$) stimulation of cAMP formation when

Table 3. Affinity of atropine, nickel and TFP for the muscarinic receptor on SH-SY5Y cells (mean ± SEM, N = 3–6)

	IC_{50}	K_i	Slope
Atropine	2.12 ± 0.08 nM	0.78 ± 0.03 nM	0.94 ± 0.03
Nickel	1.67 ± 0.12 mM	0.69 ± 0.06 mM	1.06 ± 0.08
TFP	2.34 ± 0.07 μM	1.02 ± 0.10 μM	1.37 ± 0.23

Table 4. Effects of EGTA, Calmodulin (CaM) and forskolin on adenylyl cyclase activity in SH-SY5Y membranes (mean ± SEM, N = 10 or 11)

Treatment	cAMP (pmol/mg protein)	Stimulation (% basal = 100)
EGTA (100 μM)	10.4 ± 0.7	100.0
Ca^{2+} (1.4 μM)	12.7 ± 0.7*	122.1
Ca^{2+} + CaM (200 nM)	14.2 ± 0.7*	136.5
Forskolin (1 μM)	45.1 ± 4.3*	437.7

* Significantly ($P < 0.05$) increased compared with EGTA.

compared with EGTA (0.1 mM) (Table 4). Nominally Ca^{2+}-free ATP regenerating buffer contained 1.46 ± 0.02 μM (N = 4) Ca^{2+}.

Carbachol stimulated cAMP formation was inhibited by the opiate agonists Fentanyl and morphine with IC_{50} values of 8.5 nM and 69.2 nM, respectively (Fig. 5).

Preincubation (5 min) with the PKC inhibitor, Ro 31 8220 [21] (10^{-5}–10^{-10} M) failed to significantly inhibit carbachol-stimulated cAMP formation (data not shown).

DISCUSSION

In SH-SY5Y cells, cAMP formation was increased by potassium depolarization and by M_3 muscarinic receptor stimulation, in a time and dose dependent manner. cAMP was increased approximately two-fold over basal for both stimuli, with carbachol stimulated levels being consistent with two reports [13, 14]. Carbachol stimulated cAMP formation was inhibited by TFP, G_i linked opioid receptors and appeared to be driven by both intra- and extracellular Ca^{2+}. Potassium-stimulated cAMP formation was also reversed by TFP, and was dependent on extracellular Ca^{2+} alone.

Taken collectively, these data indicate the presence of a Ca^{2+}/CaM-sensitive isoform of adenylyl cyclase in SH-SY5Y cells, in that both agents (carbachol and potassium) also increased $[Ca^{2+}]_i$. Analysis of dose–response curves for carbachol stimulated rises in peak phase IP_3 (EC_{50}, 9.4 μM) [22] and $[Ca^{2+}]_i$ (EC_{50} 7.6 μM) [23] show close correlation with the EC_{50} for carbachol stimulated cAMP formation (6.95 μM), suggesting a tight coupling of these events. A similar correlation for potassium stimulated cAMP formation (EC_{50} 34.8 mM) and potassium stimulated increases in $[Ca^{2+}]_i$ (EC_{50} 34.0 mM) [6] was also observed. There are conflicting reports on which pools of Ca^{2+} are required for stimulation of the Ca^{2+}-sensitive isoforms of adenylyl cyclase [10, 14, 24, 25]. In SH-SY5Y cells, Jansson and co-workers showed that preincubation with the $[Ca^{2+}]_i$ chelator BAPTA, caused inhibition of carbachol stimulated cAMP formation by approximately 50% [15]. Yet, Baumgold and colleagues reported that carbachol stimulated cAMP formation in the parent SK-N-SH cell line was not affected by BAPTA pretreatment [24]. In addition, when adenylyl cyclase type 1 isoform was transfected into HEK cells, capacitative Ca^{2+} entry following thapsigagin pretreatment was sufficient to stimulate cAMP formation, indicating a role for $[Ca^{2+}]_e$ [25]. Yet, Choi *et al.* reported that BAPTA pretreatment abolished carbachol stimulated cAMP formation in HEK 293 cells transfected with type 1 adenylyl cyclase [10]. These latter data imply that intracellular Ca^{2+} release is capable of activating type 1 adenylyl cyclase.

Molecular studies have revealed eight different isoforms of adenylyl cyclase which exist in a variety of tissues [8]. The predominant isoform expressed in neuronal tissue is type 1; type 8 is also neuronal and both can be stimulated by Ca^{2+}/CaM [9, 12]. The purification of adenylyl cyclase from bovine brain and the identification of a phosphorylation site on the catalytic subunit has revealed a discrepancy in its regulation by PKC [26]. PKC activation with phorbol esters stimulates cAMP formation in HEK 293 cells transfected with type 1 isoform of adenylyl cyclase [11]. However, in cultured SH-SY5Y cells, Nakagawa-Yagi and colleagues have shown that incubation with the PKC inhibitor Calphostin C had no effect on forskolin stimulated cAMP formation, and that in the same study PKC activation with PMA-enhanced forskolin-stimulated cAMP formation [13]. However, in another study also using SH-SY5Y cells, PKC inhibition with Staurosporine

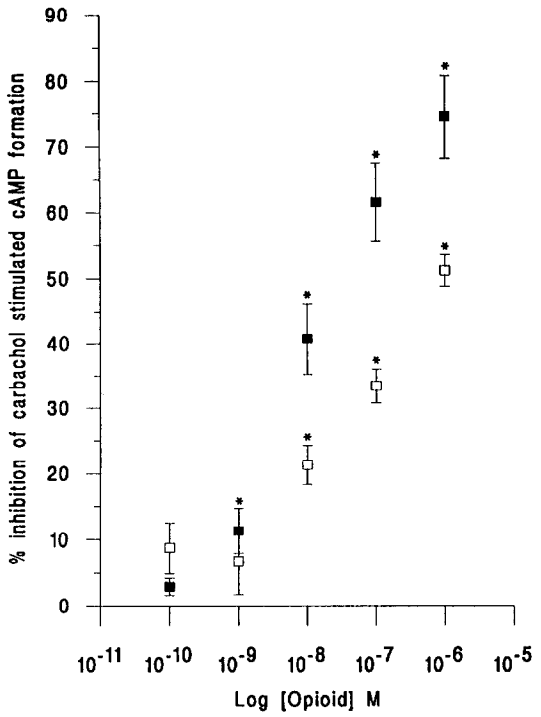

Fig. 5. Dose-related inhibition of 1 mM carbachol stimulated cAMP formation by (□) morphine, and (■) Fentanyl. Curves were significant ($P < 0.05$) by ANOVA. * Significantly ($P < 0.05$, paired t-test) inhibited compared with carbachol alone. Data are mean ± SEM ($N = 10$).

reduced carbachol-stimulated cAMP formation by approx. 50% [14]. Yet complete downregulation of PKC expression or PKC activation with PMA, had no overall effect on carbachol stimulated cAMP formation in parent SK-N-SH cells [15]. The reason for these differences are unclear but the finding that PKC inhibition in this study using Ro 31 8220 had no effect on carbachol stimulated cAMP formation supports the exclusion of PKC.

Carbachol-stimulated cAMP formation in SH-SY5Y cells appeared to be due to Ca^{2+} release from intracellular stores and Ca^{2+} entry across the plasma membrane. In the presence of the calcium channel antagonist Ni^{2+}, the time course for carbachol stimulated cAMP formation was altered such that the later 5 and 10 min time points were significantly decreased. The 1 min time point was not changed. Use of Ni^{2+} clearly indicates a role for $[Ca^{2+}]_e$ in adenylyl cyclase activation in these cells. Moreover, Ni^{2+} decreases carbachol stimulated Ca^{2+} entry, without affecting IP_3 induced Ca^{2+} release [6]. Our data indicate a role for both $[Ca^{2+}]_i$ and $[Ca^{2+}]_e$ in carbachol-stimulated cAMP formation. Further evidence for this is that in the absence of extracellular Ca^{2+}, carbachol was still able to stimulate cAMP formation, and when Ca^{2+} was replaced there was a further significant rise. In the absence of extracellular calcium, carbachol stimulated cAMP formation reached a plateau at between 2 and 3 min; this was probably caused by a lack of cAMP degradation resulting from phosphodiesterase inhibition with IBMX. Nevertheless, there remains a discrepancy in the Ca^{2+} and cAMP time courses to carbachol in that $[Ca^{2+}]_i$ is increased more rapidly than cAMP, but this may reflect a lag required for Ca^{2+} and CaM complexes to form, prior to binding and activation of adenylyl cyclase.

When TFP and nickel are used alongside muscarinic receptor driven processes there are problems with data interpretation due to their muscarinic antagonistic effects. Application of the Cheng-Prusoff equation [20] to our data indicates that in order to inhibit 1 mM carbachol stimulated cAMP formation by 50%, nickel and TFP concentrations of 182.2 mM and 269.4 μM would be required. However, by using supramaximal concentrations of carbachol (30 mM) the problems associated with significant muscarinic receptor displacement by TFP were overcome such that 30 mM carbachol stimulated cAMP formation was still abolished by 100 μM TFP. In addition, potassium stimulated (receptor independent) cAMP formation was also reversed by 100 μM TFP, giving strong evidence of a role for CaM. However, non-specific actions of TFP should also be considered, for example with PKC. Yet our data does not support a role for PKC in carbachol stimulated cAMP formation. In the presence of the G_i linked μ-opiate agonists fentanyl and morphine [27], carbachol-stimulated cAMP formation was inhibited, in a dose-dependent manner, indicating that whatever the mechanism of adenylyl cyclase activation, G_i linked receptors are still functionally coupled.

Our data strongly suggest that a Ca^{2+}/CaM-sensitive isoform of adenylyl cyclase is expressed in SH-SY5Y cells. Indeed, use of well washed SH-SY5Y membranes revealed that basal turnover of cAMP was Ca^{2+} dependent, in that addition of 100 μM EGTA (to reduce basal Ca^{2+} from 1.4 μM) inhibited cAMP formation. In the presence of 200 nM CaM, cAMP formation was increased; this observation again suggests the presence of a Ca^{2+}/CaM-sensitive isoform of adenylyl cyclase. Using Western blotting techniques, Gilman and colleagues have shown that SH-SY5Y cells express detectable levels of Type 1 adenylyl cyclase only (Tang WJ and Gilman AG, 1994, pers. comm.) in keeping with the neuronal origin of SH-SY5Y cells. However, co-expression of type 8 isoform of adenylyl cyclase cannot be excluded. In summary, our data show that in SH-SY5Y cells extracellular Ca^{2+} entry and intracellular Ca^{2+} release are both able to regulate cAMP formation, and indicate the presence of type 1 and possibly type 8 adenylyl cyclase in these cells.

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