Effects of Caffeine on Phosphatidylinositol Turnover and Calcium Mobilization in Human Neuroblastoma SK-N-SH Cells

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Abstract

The effects of caffeine on receptor-controlled Ca²⁺ mobilization and turnover of inositol phosphates in human neuroblastoma SK-N-SH cells were studied. Caffeine inhibited both the rise in cytosolic Ca²⁺ concentration ([Ca²⁺]ᵢ) evoked by muscarinic receptor agonists and the total production of inositol phosphates in a dose-dependent manner, but to different extents. At 10 mM, caffeine inhibited agonist-evoked generation of inositol phosphates almost completely, whereas the agonist-evoked [Ca²⁺]ᵢ rise remained observable after caffeine treatment, in the absence or presence of extracellular Ca²⁺. Raising the cytosolic cAMP concentration increased the carbachol-induced [Ca²⁺]ᵢ rise, and this effect was abolished in the presence of caffeine. Our data suggested that caffeine may exert two effects on receptor-controlled Ca²⁺ mobilization: 1) inhibition of inositol phosphate production, 2) augmentation of the size of the releasable Ca²⁺ pool by elevating cytosolic cAMP concentration.

Key Words: SK-N-SH cells, caffeine, cytosolic Ca²⁺ concentration

Introduction

Caffeine, a methyl xanthine alkaloid, is known to be able to activate the Ca²⁺-induced Ca²⁺ release (CICR) pathway via the ryanodine receptor (20, 31, 33) in many cell systems. Therefore, caffeine has frequently been used as a tool to study the physiological role of CICR. However, caffeine also exerts effects other than inducing Ca²⁺ release from intracellular stores. For example, it inhibits phosphodiesterase (2), and is an antagonist of the adenosine receptor (13). It has been reported that caffeine inhibits the sustained Ca²⁺ signals evoked by acetylcholine (25) or cholecystokinin (35) in mouse pancreatic acinar cells, by vasopressin in smooth muscle cells (26), and by direct application of inositol 1,4,5-trisphosphate (IP₃) in both pancreatic cells and *Xenopus* oocytes (27). It has been proposed that caffeine exerts its inhibitory effect directly on the IP₃ receptor (27).

However, caffeine does not inhibit IP₃-evoked Ca²⁺ release from pancreatic endoplasmic reticulum vesicles (7). In addition, caffeine blocks IP₃ production in mouse pancreatic acinar cells and then inhibits the subsequent agonist-evoked Ca²⁺ signal (34). Therefore, the effects of caffeine on cells are very complicated.

In this study, we used human neuroblastoma SK-N-SH cells as a model system to study the effects of caffeine on IP₃-induced Ca²⁺ mobilization and the possible mechanisms involved. Human neuroblastoma SK-N-SH cells possess a high density of M3 muscarinic receptors (11) which are effectively coupled to phosphoinositide metabolism and Ca²⁺ homeostasis (9, 10). We showed that caffeine inhibited both the Ca²⁺ mobilization and inositol phosphate (IP) generation stimulated by muscarinic receptor agonists. However, the extents of the inhibition by caffeine on these two related events were different. We suggested that in addition to inhibiting IP generation, caffeine
increased agonist-induced Ca\(^{2+}\) signals by its elevation of cytosolic cAMP concentration.

**Materials and Methods**

**Materials**

SK-N-SH cells were obtained from ATCC. Myo-[\(^2\)H\] Inositol (15 Ci/mmol) was obtained from DuPont-New England Nuclear Co. Forskolin, 3-isobutyl-1-methylxanthine (IBMX), dibutyryl cyclic AMP (dBcAMP), carbachol, methacholine, muscarine, caffeine and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fura-2 acetoxymethyl ester was obtained from Molecular Probes, Inc. (Eugene, OR, USA). Other chemicals were obtained from Merck.

**Cell Culture**

Human SK-N-SH neuroblastoma cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in an atmosphere of 90% air/10% CO\(_2\) under conditions described by Fisher and Snider (9).

**\([\text{Ca}^{2+}]\), Measurements**

Changes in \([\text{Ca}^{2+}]\) were measured by fura-2. SK-N-SH cells were loaded with fura-2 by incubating the cells (5\(\times\)10\(^6\) cells/ml) at 37°C for 30 min with 7 \(\mu\)M fura-2 acetoxymethyl ester in a loading buffer containing 150 mM NaCl, 5 mM KCl, 2.2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM glucose and 10 mM HEPES, pH 7.4. After loading, cells were washed twice with loading buffer. All \([\text{Ca}^{2+}]\) measurements were carried out in this loading buffer unless otherwise stated. The solution containing caffeine was made isotonic (300 ±5 mOsm) by decreasing the concentration of NaCl to avoid any nonspecific osmotic effect on the cells. Fluorescence at 340 nm and 380 nm as excitation and 505 nm as emission of the cell suspensions were measured using a dual-excitation fluorometer (SPEX, CM system). \([\text{Ca}^{2+}]\) was calculated using the ratio of the fluorescence at 340 nm to that at 380 nm, as previously described (20). \(R_{\text{max}}\) was obtained by adding 0.01 % digitonin to the cuvette at the end of experiments and excessive EGTA was subsequently added to obtain \(R_{\text{min}}\). A \(K_d\) value of 224 nM for fura-2/\(\text{Ca}^{2+}\) complex was used (15). All experiments were carried out at least three times using different batches of cells and were performed in duplicate each time.

**Determination of Inositol Phosphate Generation**

SK-N-SH cells were cultured in 6-well plates till confluent and prelabelled with [\(^3\)H]inositol (1 \(\mu\)Ci/ml) for 18-24 hours at 37°C in Dulbecco’s modified Eagle’s medium/10% fetal calf serum in an atmosphere of 90% air/10% CO\(_2\). After loading, cells were washed with loading buffer containing 0.5% bovine serum albumin and 20 mM LiCl three times and for 15 min each time. After washing, the cells were treated with stimulants for 30 min, after which stimulation was terminated by adding methanol to each well. Chloroform (4 ml) and loading buffer (1 ml) were added to the methanol-extracted cells, and the total water-soluble IP fraction was separated and quantitated by anion-exchange chromatography (3). The radioactivity of fractions from chromatography was measured by \(\beta\)-counter (Beckman). Data on IP generation included levels of inositol phosphate, inositol bisphosphate, and inositol trisphosphate. Control experiments were carried out in the loading buffer without stimulants. The maximum response was defined as the response in the cells stimulated with agonist at optimal dose.

**Determination of cAMP Generation**

The cells were cultured in 25 cm\(^2\) culture flask and washed with loading buffer three times. Then, the cells were treated with or without carbachol in the presence of various concentrations of caffeine for 10 min. After the treatment, the cells were lysed in HCl 0.1 N and heated in a water bath at 37°C for 2 hr, and then neutralized. Cyclic AMP was determined by enzymeimmunoassay (EIA) using a cyclic AMP EIA kit (Amersham).

**Results**

Carbachol, methacholine or muscarine were used to stimulate the muscarinic receptor in human neuroblastoma SK-N-SH cells at the concentration to trigger a maximum response as 0.3 mM, 0.3 mM and 10 \(\mu\)M, respectively. We had previously determined that these concentrations of agonists gave maximum changes in \([\text{Ca}^{2+}]\). The peak level of the \([\text{Ca}^{2+}]\), transient induced by methacholine (392±17 nM, n=15; the basal was included) was similar to that induced by carbachol (398±34 nM, n=30). However, the \([\text{Ca}^{2+}]\), rise in response to 10 M muscarine was slightly smaller, about 82±10 % (n=25) of that induced by 0.3 mM carbachol.

Caffeine at 40 mM inhibited the \([\text{Ca}^{2+}]\), rise induced by stimulation of muscarinic receptors either in the presence or absence of extracellular \(\text{Ca}^{2+}\) (Fig. 1 and 2). However, the extents of inhibition by caffeine on the \([\text{Ca}^{2+}]\), transients induced by the three agonists differed: 40 mM caffeine almost completely inhibited the muscarine-induced \([\text{Ca}^{2+}]\), transient,
whereas it inhibited only about half of those induced by carbachol or methacholine. The basal \([\text{Ca}^{2+}]_i\) were unchanged when cells were added to a solution in the presence or absence of 40 mM caffeine (Fig. 1). Fig. 3 shows that the inhibition by caffeine on carbachol- or muscarine-induced \([\text{Ca}^{2+}]_i\) rise was concentration-dependent. The potency of caffeine on methacholine-induced \([\text{Ca}^{2+}]_i\) rise was similar to the carbachol-induced one. In the presence of extracellular \(\text{Ca}^{2+}\), 40 mM caffeine reduced the carbachol- and muscarine-induced \([\text{Ca}^{2+}]_i\), rises to 50±8% and 8±5%, respectively, of the responses obtained in the absence of caffeine. In the absence of extracellular \(\text{Ca}^{2+}\), 40 mM caffeine reduced the carbachol- and muscarine-induced \([\text{Ca}^{2+}]_i\), rises to 64±14% and 22±2%, respectively, of the control responses obtained in the absence of caffeine.

Because in SK-N-SH cells stimulation of muscarinic receptors induce a \([\text{Ca}^{2+}]_i\) rise mainly via IP3, the effect of caffeine on IP generation was then examined. Caffeine inhibited the IP production induced by muscarinic receptor agonists (Table 1). Caffeine at 40 mM by itself unaffected or only affected little basal IP generation (to 78±17% of the control). Optimum concentrations of carbachol, methacholine, and muscarine required for inducing IP generation were similar to that required for \([\text{Ca}^{2+}]_i\), mobilization (data not shown). IP generation induced by muscarine was about half of which induced by carbachol and methacholine, which may explain why a lower \([\text{Ca}^{2+}]_i\) rise was induced by muscarine than that induced by carbachol (Fig. 1). In the presence of 40 mM caffeine, IP production induced by the three agonists was almost completely inhibited, and IP production evoked by muscarine was reduced to a level lower than the basal IP production (Table 1). Fig. 4 shows the dose-
dependent curve for the inhibitory effect of caffeine on carbachol-induced IP generation, with an ID50 around 4 mM. It is interesting to find that caffeine inhibited agonist-induced IP generation and $[Ca^{2+}]_i$ rise to different extents; 10 mM caffeine greatly reduced carbachol-induced IP generation (12 ± 2.6% (n=12) of the control) but the carbachol induced $[Ca^{2+}]_i$ rise almost remained the same (95 ± 1% (n=18) as the controls which were the responses obtained in the absence of caffeine).

Table 1. Effect of caffeine on inositol phosphate generation upon stimulation of muscarinic receptors in human neuroblastoma SK-N-SH cells. Cultured SK-N-SH cells were stimulated by 0.3 mM carbachol, 0.3 mM methacholine, or 10 µM muscarine in the absence (control) or presence of 40 mM caffeine. Data represent fold increases over the basal inositol phosphate generation in the absence of agonist. $^3$H-radioactivity (included IP$_1$, IP$_2$, IP$_3$) of unstimulated SK-N-SH cells (10$^6$ cells/well) was 186±14 (n=25) cpm/well.

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<tr>
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<th>Carbachol</th>
<th>Methacholine</th>
<th>Muscarine</th>
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<tr>
<td>Control</td>
<td>27.8±3.3</td>
<td>25.3±1.5</td>
<td>12.4±1.4</td>
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<tr>
<td>40 mM Caffeine</td>
<td>2.1±0.7</td>
<td>1.9±0.4</td>
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Fig. 3. Dose-dependent effect of caffeine on agonist-evoked $[Ca^{2+}]_i$ rise in SK-N-SH cells. Fura-2-loaded SK-N-SH cells were stimulated by 0.3 mM carbachol (circles) or 10 M muscarine (triangles) in $Ca^{2+}$-free (open symbols) or $Ca^{2+}$-containing (filled symbols) loading buffer containing various concentrations of caffeine as indicated. The $Ca^{2+}$-free loading buffer had no added $Ca^2+$ and contained 0.5 mM EGTA. Maximal $Ca^{2+}$ response was the $[Ca^{2+}]_i$ change induced by the agonist in the absence of caffeine. Data are presented as mean ± SD (n=12-24) from different batches of cells.

Fig. 4. Dose-dependent effects of caffeine on carbachol-evoked inositol phosphate generation. Human SK-N-SH cells which had been preloaded with $^3$H-inositol were stimulated with 0.3 mM carbachol in the presence of various concentrations of caffeine as indicated. Total inositol phosphate production was measured as described in Materials and Methods. Data represent the percentage of the maximal response that was induced by 0.3 mM carbachol in the absence of caffeine. Data shown are mean±SD (n=9-18) from different batches of cells.

Fig. 5 Elevation of cAMP induced by caffeine. SK-N-SH cells were stimulated with (solid bars) or without (open bars) 0.3 mM carbachol for 5 min at room temperature in the presence or absence of caffeine at 40 mM.
EFFECTS OF CAFFEINE ON SK-N-SH CELLS

A fold increase in the cellular cAMP level (Fig. 5). Carbachol also induced a small increase in the cAMP level, possibly via muscarinic receptor, which was not affected by caffeine. To determine whether caffeine exerted its effect through its inhibition of phosphodiesterase and the subsequent increase in cellular level of cAMP, we examined the effect of increasing cytosolic cAMP on carbachol-induced [Ca$^{2+}$]$_i$ rise. Several agents which are known to elevate intracellular cAMP, including forskolin (an adenylate cyclase activator), IBMX (an inhibitor of phosphodiesterase), and dBcAMP, were used to increase the cytosolic cAMP. In contrast to the inhibitory effect of caffeine, forskolin, IBMX, and dBcAMP enhanced carbachol-induced [Ca$^{2+}$]$_i$ rise (Fig. 6). Similar enhancement was observed in the absence of extracellular Ca$^{2+}$ (Fig. 6B).

We then further examined the synergistic effects of cAMP and caffeine on carbachol-induced [Ca$^{2+}$]$_i$ rise. When SK-N-SH cells were preincubated with dBcAMP for 10 min, an increase in carbachol-induced [Ca$^{2+}$]$_i$ rise at all concentrations of carbachol used (Fig. 7) was found. This data reinforced our suggestion that the elevation of intracellular cAMP level can raise the calcium signal coupled with muscarinic receptor stimulated by carbachol. It also showed that the enhancing effects of dBcAMP exited at all concentrations of carbachol used. In the presence of 40 mM caffeine, the enhancement by dBcAMP was abolished (Fig. 7). The decrease of carbachol-induced calcium signal in the presence of caffeine can be accounted by the inhibitory effects of caffeine on IP production, while there is no synergistic effects of dBcAMP and caffeine on carbachol-induced [Ca$^{2+}$]$_i$ rise. Similar results can be observed when we used methacholine to stimulate the cells. The abolishment of the enhancing effects of dBcAMP in the presence of caffeine might be due to caffeine itself which can elevate cAMP level, thus the effects of dBcAMP and caffeine can not be additive. This data illustrated that the effects of cAMP on carbachol-induced [Ca$^{2+}$]$_i$ rise was included into the multiple effects of caffeine so the enhancement by dBcAMP was abolished in the presence of caffeine.

Discussion

In this paper we reported that caffeine inhibited
both IP generation and [Ca$$^{2+}$$], elevation induced by stimulation of muscarinic receptors. Inhibition in both cases was dose-dependent, but IP generation was inhibited to a greater extent than [Ca$$^{2+}$$], rise. Our data suggested that caffeine exerts two effects on receptor-activated Ca$$^{2+}$$ mobilization: first, caffeine inhibited the receptor-triggered [Ca$$^{2+}$$], transients by inhibiting IP production. Second, caffeine increased the size of releasable Ca$$^{2+}$$ pools by elevating cytosolic cAMP concentration. Therefore, when caffeine was present at a concentration that suppresses almost all IP generation, the [Ca$$^{2+}$$], transient induced by stimulation of muscarinic receptor was only partly suppressed.

The Ca$$^{2+}$$-releasing function of caffeine has been demonstrated in a wide variety of cell systems including both electrically excitable, e.g. neurons (14, 19, 21), chromaffin cells (1, 16, 20), and non-excitable cells (12, 18, 23). In human neuroblastoma SK-N-SH cells, our result that caffeine alone did not affect [Ca$$^{2+}$$], suggested that caffeine-sensitive intracellular Ca$$^{2+}$$ pools were absent in these cells. Caffeine has been known for inactivating ryanodine receptor-mediated Ca$$^{2+}$$ release (30). Thus, the inhibitory effects of caffeine on agonist-induced [Ca$$^{2+}$$], rise are due to effects other than depletion of the intracellular Ca$$^{2+}$$ stores.

Carbachol, methacholine and muscarine showed different potencies in inducing [Ca$$^{2+}$$], rise and IP generation. Although M3 is the dominant subtype of muscarinic receptor, M1 and M2 have been shown to be present in SK-N-SH cells (9). Possibly different agonists may act on different subtypes of receptor with different potencies. The possibility that nicotinic receptors may involve in inducing the [Ca$$^{2+}$$], rise was excluded by the absence of effect when SK-N-SH cells were stimulated by the nicotinic receptor agonist DMPP (data not shown).

IP$$\_3$$, which is generated upon receptor stimulation, is known to release Ca$$^{2+}$$ from intracellular stores. Our results show a correlation between the level of IP generated and [Ca$$^{2+}$$], rise. Among the three agonists used, muscarine generated the least IP (Table 1) and the subsequent [Ca$$^{2+}$$], rise was also the smallest. In addition, muscarine-induced IP generation was inhibited to below the basal level by 40 mM caffeine, and the [Ca$$^{2+}$$], rise was also completely inhibited. In contrast, carbachol-induced IP generation in the presence of caffeine remained about double the basal level and the [Ca$$^{2+}$$], rise was halved (Table 1, Fig. 3). Therefore, the inhibition of the muscarinic agonist-induced [Ca$$^{2+}$$], rise by caffeine was caused at least in part by caffeine inhibiting receptor-stimulated IP generation. Similar results have been found in mouse pancreatic acinar cells (28, 34). Under our experimental conditions, it could not be the main target through that caffeine exerts its inhibitory effects by inhibiting IP$$\_3$$ binding to its receptor or by inhibiting the operation of the IP$$\_3$$-sensitive Ca$$^{2+}$$ channel, as suggested by others (4, 27).

The extent of inhibition by caffeine was greater on carbachol-induced IP generation than on [Ca$$^{2+}$$], rise. Caffeine is known to increase the cytosolic cAMP concentration by inhibiting PDE (Fig 5). Raising the cytosolic cAMP concentration did increase the carbachol-induced [Ca$$^{2+}$$], rise, an effect abolished in the presence of caffeine (Fig. 7), probably due to caffeine’s acting via the same pathway as cAMP. The enhancement of [Ca$$^{2+}$$], transients by cAMP elevating agents has been suggested to be caused by cAMP increasing the size of the releasable Ca$$^{2+}$$ pools (5).

In muscle cells, increasing cAMP concentration would activate a cAMP-dependent protein kinase, which is proposed to phosphorylate phospholamban and then stimulate Ca$$^{2+}$$ pump activity (6, 17, 29, 32). The increase in Ca$$^{2+}$$ pump activity causes an increase in total Ca$$^{2+}$$ content in the sarcoplasmic reticulum. Similar results have been shown in non-muscle cells (8). Forskolin is reported to increase the caraccol induced calcium responses in CHO cells expressing the M3 subtype of the muscarinic acetylcholine receptors (22). Moreover, cAMP-dependent phosphorylation has been proven to increase Ca$$^{2+}$$ flux through IP$$\_3$$ receptors (24). Our data that cAMP increased the size of the releasable Ca$$^{2+}$$ pools sensitive to IP$$\_3$$ signal were correlated with the above studies. In SK-N-SH cells, it is conceivable that caffeine simultaneously inhibits receptor-triggered [Ca$$^{2+}$$], transients by inhibiting IP$$\_3$$ production while increasing the size of the Ca$$^{2+}$$ pools through its ability to raise cAMP concentration. Therefore, at concentrations (e.g. 10 mM) at which IP generation was almost completely abolished by caffeine, the carbachol-evoked [Ca$$^{2+}$$], transient caused by the residual IP$$\_3$$ remained largely unaffected. However, there was still a possibility that the potency of caffeine on abolishing IP$$\_3$$ generation might not be similar to IP generation; hence, further investigation is needed.

In summary, caffeine inhibited muscarinic agonist-induced [Ca$$^{2+}$$], transients in SK-N-SH cells predominantly by its inhibitory effects on IP generation. Caffeine also appeared to exert its effect by elevating and thus increase the size of cytosolic Ca$$^{2+}$$ pools. These effects of caffeine on SK-N-SH cells were not related to its effects on release of Ca$$^{2+}$$ from intracellular stores.

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References


