Muscarinic (M_1) receptor-mediated inhibition of K^+ -evoked [3H]-noradrenaline release from human neuroblastoma (SH-SY5Y) cells via inhibition of L- and N-type Ca^{2+} channels

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1 Human neuroblastoma (SH-SY5Y) cells were preincubated with [3H]-noradrenaline ([3H]-NA) in the presence of 0.2 mM pargyline to examine the modulation of K^+ -evoked [3H]-NA release by muscarinic agonists.

2 Release of [3H]-NA evoked by 4 min exposure to 100 mM K^+ could be partially inhibited by 5 μM nifedipine and partially inhibited by 100 nM ω -conotoxin GVIA (ω -CgTx). When nifedipine and ω -CgTx were added together, evoked release was inhibited by approximately 93%.

3 K^+ -evoked [3H]-NA release was inhibited by >90% by pretreatment of cells for 2 min with muscarine, carbachol or oxotremorine methiodide (each at 300 μM). For muscarine, inhibition of evoked release was both time- and concentration-dependent and was reversible. Muscarine also inhibited [3H]-NA release evoked by veratridine (28 μM) and replacement of extracellular Ca^{2+} with Ba^{2+} , but not that evoked by the Ca^{2+} ionophore, A23187 (19 μM).

4 Residual K^+ -evoked [3H]-NA release measured in the presence of either nifedipine (5 μM) or ω -CgTx (100 nM) was inhibited by muscarine with a similar potency as release evoked in the absence of either Ca^{2+} channel blocker. Pretreatment of cells for 16–24 h with pertussis toxin (200 ng ml⁻¹) did not affect K^+ -evoked release *per se* or the ability of muscarine to inhibit such release.

5 Muscarinic inhibition of K^+ -evoked [3H]-NA release was potently antagonized by pirenzepine (pA₂ 8.14) and by hexahydrosiladiphenidol (pA₂ 9.03), suggesting the involvement of an M_1 receptor.

6 Our results demonstrate that 100 mM K^+ -evoked release of [3H]-NA from the human neuroblastoma is mediated by activation of both L- and N-type Ca^{2+} channels. Activation of muscarinic M_1 receptors can inhibit release via a pertussis toxin-insensitive mechanism which involves non-selective inhibition of L- and N-type Ca^{2+} channels.

Keywords: SH-SY5Y; calcium channels; noradrenaline release; muscarinic receptor; nifedipine; ω -conotoxin GVIA; pirenzepine; hexahydrosiladiphenidol

Introduction

Many transmitters and hormones regulate neuronal functioning by modifying the activity of voltage-gated Ca^{2+} channels (Tsien *et al.*, 1988; Anwyl, 1991; Swandulla *et al.*, 1991). These channels exert important influences on neuronal excitability and provide the pathway for Ca^{2+} influx which is required to stimulate neurotransmitter release. Numerous studies have used patch clamp techniques to demonstrate both inhibitory and stimulatory actions of neurotransmitters on neuronal Ca^{2+} channels (e.g. Anwyl, 1991; Caulfield *et al.*, 1992; Cox & Dunlap, 1992; Yang & Tsien, 1993), but for reasons of experimental ease or necessity, Ca^{2+} channels are often recorded from the cell soma rather than at neurotransmitter release sites (see for example Lipscombe *et al.*, 1989). Consequently, less direct information is available concerning the influence of Ca^{2+} channel regulation by transmitters and hormones on neurosecretion. It is apparent that in different neurones, different sub-types of Ca^{2+} channels mediate Ca^{2+} influx required to trigger neurosecretion: for example, in dorsal root ganglion cells, L-type (dihydropyridine-sensitive) Ca^{2+} channels mediate release of substance P (Perney *et al.*, 1986), and in neurohypophysial cells, both L-type and N-type Ca^{2+} channels can mediate neurosecretion (N-type being defined as ω -conotoxin GVIA (ω -CgTx)-sensitive; Nowicky, 1991). In sympathetic neurones, N-type is the dominant Ca^{2+} channel (Hirning *et al.*, 1988), but dihydropyridine agonists can enhance noradrenaline release (Perney *et al.*, 1986). In chromaffin cells, three types of Ca^{2+} channel (L-, N-, and ω -agatoxin-IVA-sensitive P-type) have been shown to influ-

ence catecholamine release to varying degrees, depending on the electrical parameters used to stimulate cells (Artalejo *et al.*, 1994).

In this study, we have used the clonal cell line SH-SY5Y to investigate the modulation of K^+ -evoked [3H]-noradrenaline ([3H]-NA) release caused by activation of muscarinic receptors. SH-SY5Y cells are derived from human sympathetic tissue (Pahlman *et al.*, 1990) and retain the ability to release noradrenaline in response to a variety of stimuli (Murphy *et al.*, 1991a,b; Vaughan *et al.*, 1993a,b; McDonald *et al.*, 1994). Our results indicate that both L- and N-type Ca^{2+} channels mediate release of [3H]-NA in response to raised $[K^+]_o$ and that activation of muscarinic M_1 receptors reduces evoked release by inhibition of both types of Ca^{2+} channel.

Methods

Culture of SH-SY5Y cells

The human neuroblastoma SH-SY5Y (kindly provided by Dr J. Beidler, Sloan-Kettering Institute for Cancer Research, Rye, New Jersey, U.S.A.) was cultured in a 1:1 mixture of Ham's F-12 and Eagle's minimal essential media containing nonessential amino acids supplemented with 10% foetal calf serum in a 98% air, 2% CO₂ humidified incubator at 37°C. When confluent, cell layers were harvested by incubation at 37°C for 10 min in Ca^{2+} and Mg^{2+} -free phosphate-buffered saline (PBS) in the absence of trypsin, to select against possible contamination of epitheloid SH-EP cells (Ciccarone *et al.*, 1989; Ogino & Costa, 1992). The cell suspension was

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trituted by 3 passes through a 0.8 mm guage needle, and cell number was estimated with a Coulter Counter. Cells were then subcultured in 24-well plates at a seeding density of 10^5 cells ml^{-1} , 0.4 ml being added to each plate.

[^3H]-noradrenaline release assays

Release of [^3H]-NA was measured after 4–5 days in subculture using previously described methodology (Murphy *et al.*, 1991b; Vaughan *et al.*, 1993a). In brief, culture medium was removed from the 24-well plates and cell monolayers were washed twice with PBS. To each well was then added 0.4 ml of HEPES-buffered saline (HBS) of composition (in mM): NaCl 135, KCl 5, MgCl₂ 0.6, CaCl₂ 2.5, HEPES 10, glucose 6, pargyline 0.2, ascorbic acid 0.2 (pH 7.4) containing 40 nM [^3H]-NA. After incubation for 1 h at 37°C, unaccumulated [^3H]-NA was removed by 4 consecutive washes with 0.4 ml aliquots of HBS, each applied to cells for 10 min. Cell layers were preincubated with 0.4 ml HBS alone or containing muscarine, carbachol or other drugs for the times indicated in the relevant figures. These solutions were then replaced with 100 mM K⁺-containing solutions to evoke [^3H]-NA release (which contained the same concentration of drugs used during the preincubation period) and incubations continued for 4 min. In solutions of high K⁺ concentration (100 mM) the Na⁺ concentration was reduced correspondingly to maintain isoosmolarity. Unreleased [^3H]-NA was extracted with 0.4 M perchloric acid and the amount of [^3H]-NA released during a 4 min incubation in 100 mM K⁺-containing solutions in a given experiment was expressed as a percentage of the total amount of radioactivity in the cell layer at the beginning of the incubation period.

For some experiments, cells were pretreated for 16–24 h with pertussis toxin (PTX). PTX was added to culture medium from a 50% glycerol stock solution of 50 $\mu\text{g ml}^{-1}$ to give a final concentration of 200 ng ml^{-1} . In these studies, control cells were treated with the same volume of an identical solution, but lacking the PTX.

Drugs used were muscarine chloride, carbachol chloride, oxotremorine methiodide, atropine sulphate, pirenzepine dihydrochloride, hexahydrostiladiphenidol hydrochloride (HHSiD), nifedipine, veratridine, A23187, yohimbine hydrochloride (from Sigma Chemical Co., Poole, Dorset) and omega-conotoxin GVIA (ω -CgTx; from Calbiochem, Nottingham). To examine the effects of these drugs on [^3H]-NA release from SH-SY5Y cells evoked by solutions of raised [K⁺]_o, drugs were added to the cells at varying periods (30 s to 8 min) before exposure to high [K⁺] solutions, and were also usually present in the solution of high [K⁺]. Cells were always exposed to high [K⁺] solutions for 4 min. Since application times varied according to each experiment, further details are given in the Results section and figure legends. All results are expressed as means $\pm$ standard error of the mean (s.e.mean), taken from the number of experiments indicated. One experiment constitutes the mean of results obtained from two culture dishes i.e. experiments were always performed in duplicate. Statistical comparisons were made as indicated in the Results using the unpaired Student's *t* test.

Results

K⁺-evoked [^3H]-NA release is coupled to L- and N-type Ca²⁺ channels

Figure 1 shows that raising extracellular [K⁺] from 5 mM to 100 mM evoked [^3H]-NA release from SH-SY5Y cells, as previously described (Murphy *et al.*, 1991a,b). In the presence of the L-type Ca²⁺ channel blocker, nifedipine (which was applied 8 min before the 100 mM K⁺ solution), at a supramaximal but selective concentration (Reeve *et al.*, 1994) of 5 μM , K⁺-evoked release was reduced by approximately 76%. The N-type Ca²⁺ channel blocker ω -CgTx

Figure 1 Release of [^3H]-noradrenaline ([^3H]-NA) from SH-SY5Y cells, expressed as mean ($\pm$ s.e.mean, taken from 4 experiments in each case) percentage of total radioactivity taken up by cell monolayers (see Methods). Release determined in the presence of 5 mM K⁺ represents basal release, and in all subsequent figures basal release has been subtracted. Nifedipine was added at a concentration of 5 μM , for 8 min prior to exposure to 100 mM K⁺-containing solutions. ω -Conotoxin GVIA (ω -CgTx) was applied at 100 nM for 20 min prior to exposure to 100 mM K⁺-containing solutions. Both blockers were also present during the 4 min exposure to 100 mM K⁺-containing solutions.

(applied 20 min prior to the 100 mM K⁺ solution) also inhibited evoked [^3H]-NA release, by approximately 47%. In the presence of both blockers, K⁺-evoked [^3H]-NA release was reduced by over 90% (Figure 1). These data indicate that K⁺-evoked release involves Ca²⁺ influx through both dihydropyridine-sensitive L-type and ω -CgTx-sensitive N-type Ca²⁺ channels in SH-SY5Y cells. It is conceivable that removal of Na⁺ (which is required to maintain isoosmolarity in solutions of high K⁺ concentration) might in itself evoke release, for example by reversal of Na-Ca exchange. However, such a possibility is not likely to cause a large effect on release since release was almost fully inhibited by blockers of voltage-gated Ca²⁺ channels. The inhibitory actions of the Ca²⁺ channel blockers, applied alone or together, were highly statistically significant ($P < 0.0001$).

Muscarine inhibits K⁺-evoked [^3H]-NA release

Exposure of SH-SY5Y cells to muscarine inhibited 100 mM K⁺-evoked [^3H]-NA release from SH-SY5Y cells in a time- and concentration-dependent manner (Figure 2). When muscarine was applied to cells simultaneously with the high [K⁺]-containing solutions, inhibition of evoked release was only seen at concentrations $> 30 \mu\text{M}$, and appeared to saturate at approximately 25% inhibition with 300 μM muscarine ($\square$, Figure 2a). By contrast, if cells were exposed to muscarine for 2 min prior to application of 100 mM K⁺ (and muscarine was also present in the 100 mM K⁺-containing solution), release could be inhibited by up to 90–95%, with an IC₅₀ of 3 μM ($\bullet$, Figure 2a) as determined by eye from the data shown. This inhibition-response curve was shifted further to the left (i.e. to lower muscarine concentrations) when cells were pretreated for 4 min prior to application of 100 mM K⁺ (IC₅₀ 0.5 μM , $\circ$, Figure 2a). The ($\blacksquare$) symbols of Figure 2a show that exposure of cells to 100 nM atropine 8 min prior to muscarine exposure completely abolished the inhibition of K⁺-evoked release caused by 2 min pretreatment with muscarine. We also observed inhibition of K⁺-evoked [^3H]-NA release with carbachol (300 μM causing $88.5 \pm 0.8\%$ inhibition, $n = 4$ experiments) and oxotremorine methiodide (300 μM causing $92.4 \pm 0.3\%$ inhibition, $n = 4$). Again, these agonists were present 2 min prior to, and during the 4 min exposure to 100 mM K⁺. Muscarinic agonists can themselves evoke [^3H]-NA release from SH-SY5Y cells via

activation of M₃ receptors (Murphy *et al.*, 1991b), and so it was conceivable that the small release of [³H]-NA evoked by pretreatment of cells with muscarine ($0.30 \pm 0.15\%$ above basal release in the present studies, $n = 7$) could act to inhibit K⁺-evoked release by stimulating α_2 receptors and hence inhibiting Ca²⁺ channels coupled to noradrenaline release. However, the ability of muscarine to inhibit K⁺-evoked release was not significantly affected by the α_2 antagonist, yohimbine: in a series of 4 experiments conducted in parallel, muscarine (applied 4 min prior to, and during, 100 mM K⁺ application) inhibited K⁺-evoked release by $38.9 \pm 0.6\%$ and $89.3 \pm 0.5\%$ at concentrations of 0.3 μ M and 30 μ M respectively. In the presence of 1 μ M yohimbine (added 8 min prior to, and during, 100 mM K⁺ application) release was inhibited by $36.6 \pm 0.2\%$ and $90.5 \pm 0.5\%$ by these concentrations of

muscarine. Yohimbine alone did not affect release during the 8 min exposure in the presence of 5 mM K⁺.

The time-dependence of muscarine inhibition of K⁺-evoked release was studied in greater detail using two muscarine concentrations of 0.3 and 30 μ M (Figure 2b). At the higher concentration, muscarine rapidly inhibited K⁺-evoked release of [³H]-NA, and reached a plateau level of 90–95% within 2–4 min. At the lower concentration, muscarine inhibition developed more slowly and appeared to approach a lower plateau level of approximately 50%. To investigate the reversibility of muscarine's action, we examined the release of [³H]-NA evoked by 100 mM K⁺ in cells which were exposed to muscarine for 2 min and then repeatedly washed in muscarine-free solution for 4 min prior to application of 100 mM K⁺ solutions. In cells treated this way, K⁺ evoked release of $12.5 \pm 0.3\%$ of the total cellular ³H content ($n = 3$). This value represents $87.4 \pm 0.4\%$ of release seen in cells which were never exposed to muscarine but were simply repeatedly washed before exposure to 100 mM K⁺, which released $14.3 \pm 0.7\%$ of total content ($n = 3$). This contrasts with the marked suppression of release evoked in the continued presence of muscarine (Figure 2) and indicates that the actions of muscarine were largely reversible.

Muscarinic inhibition of [³H]-NA release occurs via pertussis toxin-insensitive block of L- and N-type Ca²⁺ channels

Muscarinic agonists are known to inhibit neuronal Ca²⁺ channels (see Introduction), and given that K⁺-evoked [³H]-NA release in SH-SY5Y cells is mediated via L- and N-type Ca²⁺ channels (Figure 1) it seemed likely that muscarine acted to inhibit evoked release via inhibition of Ca²⁺ channels. To examine this possibility, we investigated the ability of muscarine to inhibit [³H]-NA release evoked by other stimuli. We have previously shown that Ba²⁺ evokes release in these cells via depolarization and Ca²⁺ influx through voltage-gated Ca²⁺ channels (Vaughan *et al.*, 1993b). Figure 3 shows that release evoked by substitution of extracellular Ca²⁺ with Ba²⁺ (2.5 mM) for 4 min, and also by 4 min exposure to 28 μ M veratridine (another commonly used depolarizing secretagogue) can be inhibited to similar degrees as K⁺-evoked release with 300 μ M muscarine. By contrast, release evoked by 4 min exposure to the Ca²⁺ ionophore, A23187 (19 μ M), which still involves Ca²⁺ influx but not via

Figure 2 Inhibition of K⁺-evoked [³H]-noradrenaline ([³H]-NA) release by muscarine: (a) percentage inhibition of 100 mM K⁺-evoked release by muscarine added simultaneously with 100 mM K⁺ (□) or 2 min (●) or 4 min (○) prior to application of 100 mM K⁺. Muscarine was also always present during the 4 min exposure to 100 mM K⁺. Atropine (100 nM), added for 8 min prior to (and during) 100 mM K⁺ exposure, abolished the inhibition of evoked release caused by 2 min pretreatment with muscarine (■). (b) Time course of inhibition of K⁺-evoked release by muscarine at 0.3 μ M (○) and 30 μ M (●), applied for varying periods before application of 100 mM K⁺ as indicated (muscarine was also present during the 4 min exposure to 100 mM K⁺-containing solutions). Points plotted in (a) and (b) are means with s.e.mean taken from 4 to 14 experiments in each case.

Figure 3 Release of [³H]-noradrenaline ([³H]-NA) (expressed as percentage of total cellular ³H content; mean values $\pm$ s.e.mean, taken from 3 to 5 experiments in each case, basal release subtracted) from SH-SY5Y cells evoked by 4 min exposures to 100 mM K⁺, 28 μ M veratridine, replacement of extracellular Ca²⁺ with Ba²⁺ (2.5 mM) and 19 μ M A23187 as indicated, in the absence or presence (+ Musc) of 300 μ M muscarine (applied 2 min prior to stimulus and present during the 4 min period of exposure to the stimulus), as indicated. Note lack of effect of muscarine on A23187-evoked release.

voltage-gated Ca^{2+} channels, was unaffected by pretreatment of cells with muscarine (Figure 3). For all studies shown in Figure 3, 300 μM muscarine was added to cells 2 min prior to each stimulus, and was also present during the 2 min exposure to the stimulus. These data indicate that muscarine inhibits depolarization-mediated release via inhibition of Ca^{2+} channels.

Figure 4 indicates that muscarinic inhibition of K^{+} -evoked $[\text{H}]\text{-NA}$ release involves non-selective inhibition of both L-type and N-type Ca^{2+} channels. In the presence of nifedipine (5 μM , applied for 8 min prior to and also during exposure to 100 mM K^{+} solutions), residual, $\omega\text{-CgTx}$ sensitive release can be inhibited by 300 μM muscarine and, conversely, residual, nifedipine-sensitive release evoked in the presence of 100 nM $\omega\text{-CgTx}$ (applied for 8 min prior to and also during exposure to 100 mM K^{+} solutions) was also inhibited (Figure 4a).

Figure 4 (a) Release of $[\text{H}]\text{-noradrenaline}$ ($[\text{H}]\text{-NA}$) (means $\pm$ s.e.mean, taken from 3 experiments in each case) caused by a 4 min exposure to 100 mM K^{+} in the presence of 5 μM nifedipine (Nif) or 100 nM $\omega\text{-conotoxin GVIA}$ ($\omega\text{-CgTx}$) from untreated cells (hatched columns) or cells pretreated for 4 min with 300 μM muscarine prior to and during application of 100 mM K^{+} (solid columns). Nifedipine and $\omega\text{-CgTx}$ were applied for 8 min prior to (and during) exposure to 100 mM K^{+} -containing solutions. Release is expressed as percentage of release measured in the absence of muscarine, nifedipine or $\omega\text{-CgTx}$, taken as 100% (open column). Actual release was $16.6 \pm 0.7\%$ of total cellular ^3H content in the absence of muscarine or Ca^{2+} channel blockers. (b) Percentage inhibition of K^{+} -evoked $[\text{H}]\text{-NA}$ release caused by muscarine. Release was measured in the presence of 5 μM nifedipine (○), 100 nM $\omega\text{-CgTx}$ (■) or in the absence of Ca^{2+} channel blockers (●). Nifedipine, $\omega\text{-CgTx}$ and muscarine were applied for the same time periods as in (a). In each of these 3 cases, release was normalized to 100% in the absence of muscarine, and each point plotted is a mean (with s.e.mean) taken from 3 to 5 experiments. No significant differences were found between the 3 conditions at any muscarine concentration studied.

Although $\omega\text{-CgTx}$ was applied for less time than in the experiments shown in Figure 1, the degree of inhibition of release it caused was not significantly affected. If release in the presence of either Ca^{2+} channel blocker is normalized to 100% in the absence of muscarine (Figure 4b), it can be seen that muscarine inhibits release mediated by L-type and N-type Ca^{2+} channels with similar potency, and release in the presence of either Ca^{2+} channel blocker is similarly sensitive to muscarine as in the absence of either. Thus muscarine is non-selective in its ability to inhibit 100 mM K^{+} -evoked release via Ca^{2+} influx through L-type or N-type Ca^{2+} channels.

To investigate whether muscarine inhibited Ca^{2+} channels via a pertussis toxin- (PTX-) sensitive G-protein, we investigated the effects of muscarine in inhibiting K^{+} -evoked $[\text{H}]\text{-NA}$ release in cells pretreated for 16–24 h with 200 ng ml^{-1} PTX. Such pretreatment did not affect the inhibitory action of muscarine: for example, in 4 experiments conducted in parallel, 30 μM muscarine inhibited K^{+} -evoked release by $89.4 \pm 2.0\%$ in untreated cells, and in PTX-treated cells the same concentration inhibited release by $90.8 \pm 2.1\%$. These findings suggest that the actions of muscarine are not mediated by PTX-sensitive G-proteins.

Muscarine acts at M_1 receptors to inhibit K^{+} -evoked $[\text{H}]\text{-NA}$ release

SH-SY5Y cells are known to express muscarinic M_1 , M_2 and M_3 receptors. To examine whether the inhibition of K^{+} -evoked release by muscarine is coupled to a specific muscarinic sub-type, we tested the effects of two muscarinic receptor antagonists, pirenzepine and HHSiD. Figure 5a plots the inhibitory effects of muscarine on K^{+} -evoked $[\text{H}]\text{-NA}$ release in the absence and presence of three concentrations of pirenzepine. Pirenzepine was added 4 min prior to muscarine, then both drugs were incubated with the cells for 4 min prior to addition of 100 mM K^{+} . Both drugs were also present during the 4 min exposure to 100 mM K^{+} . From these data, a Schild plot was obtained (Figure 5b) which yielded a pA_2 value for pirenzepine of 8.14. Thus pirenzepine acted as a potent antagonist at the receptors mediating the effects of muscarine on K^{+} -evoked $[\text{H}]\text{-NA}$ release, suggesting that the receptors involved were of the M_1 or M_4 sub-type (see Discussion). Figure 5c plots the effects of three concentrations of HHSiD (applied as described above for pirenzepine) on the actions of muscarine to reduce K^{+} -evoked release, and from these results a Schild plot was obtained (Figure 5d) which yielded a pA_2 of 9.03 for HHSiD. Since HHSiD has been shown to be a potent antagonist at M_1 and M_3 receptors (Lambrecht *et al.*, 1988; Richards, 1991), and pirenzepine was also found to be potent in the current studies, the results presented in Figure 5 suggest that muscarinic inhibition of K^{+} -evoked $[\text{H}]\text{-NA}$ release is mediated by M_1 receptors. Neither pirenzepine nor HHSiD altered basal $[\text{H}]\text{-NA}$ release in the presence of 5 mM K^{+} .

Discussion

Raised extracellular K^{+} concentrations can stimulate release of $[\text{H}]\text{-NA}$ from SH-SY5Y cells via depolarization and the consequent activation of voltage-gated Ca^{2+} channels. This is in agreement with previous reports (Murphy *et al.*, 1991a,b) and the present study indicates that both L-type and N-type Ca^{2+} channels are involved in this release mechanism, since 100 mM K^{+} -evoked release was sensitive to both nifedipine and $\omega\text{-CgTx}$ (Figure 1). These channels account for the whole-cell Ca^{2+} channel current seen in electrophysiological studies (Kennedy & Henderson, 1992; Reuveny & Narahashi, 1993; Reeve *et al.*, 1994). Muscarinic agonists inhibited 100 mM K^{+} -evoked release in a time- and concentration-dependent manner (Figure 2) that can be attributed to a mechanism involving inhibition of both L- and N-type Ca^{2+}

Figure 5 (a) Percentage inhibition of K⁺-evoked [³H]-noradrenaline ([³H]-NA) release measured in cells pretreated with muscarine for 4 min in the absence of pirenzepine (●) and in the presence of 30 nM (○), 300 nM (□) and 1 μM (■) pirenzepine (pirenzepine was added for 4 min prior to application of muscarine). Cells were then incubated in the presence of muscarine and pirenzepine for 4 min prior to exposure to 100 mM K⁺, and both drugs were also present during 4 min exposure to 100 mM K⁺-containing solutions. (b) Schild plot obtained from data in (a), taking dose-ratios (r) at 50% inhibition. Values were determined by eye from the data shown in (a). The line was fitted by linear regression ($r^2 = 0.97$) and has a slope of 0.89. Intercept gives a pA₂ value of 8.14. (c) Percentage inhibition of K⁺-evoked [³H]-NA release measured in cells pretreated with muscarine for 4 min in the absence of hexahydrosiladiphenidol (HHSiD; ●) and in the presence of 10 nM (○), 100 nM (□) and 1 μM (■) HHSiD (in each case added for 4 min prior to application of muscarine; protocols were exactly as for pirenzepine, in a). (d) Schild plot obtained from data in (c), taking dose-ratios (r) at 50% inhibition. The line fitting the data by linear regression ($r^2 = 0.99$) has a slope of 0.80. Intercept gives a pA₂ value of 9.03. Each plotted point in (a) and (c) is the mean ($\pm$ s.e.mean) taken from 3 to 5 experiments in each case.

channels: release evoked by Ba²⁺ which, like K⁺ and veratridine, stimulates release via depolarization and consequent Ca²⁺ entry (Vaughan *et al.*, 1993b) was also strongly inhibited by muscarine, whereas release evoked by the Ca²⁺ ionophore, A23187 (which effectively bypasses Ca²⁺ channels to allow Ca²⁺ entry) was unaffected by muscarine (Figure 3).

Exposure of cells to either nifedipine or ω -CgTx caused partial inhibition of K⁺-evoked [³H]-NA release (Figure 1) which was always less than the maximal inhibition of release caused by muscarine. Residual release of [³H]-NA measured in the presence of either nifedipine or ω -CgTx could also be inhibited by muscarine (Figure 4), indicating that the inhibition of Ca²⁺ channels by muscarine was non-selective i.e. muscarine could inhibit Ca²⁺ influx through both L- and N-type Ca²⁺ channels. Furthermore, the sensitivity to inhibition by muscarine was similar for the two channel types (Figure 4b).

The lack of effect of PTX on muscarinic inhibition of K⁺-evoked release is unlikely to be due to the fact that muscarine acts via a pathway that does not involve G proteins, since all known muscarinic receptors are believed to be G protein-coupled. Instead, muscarine could be acting via a PTX-insensitive G protein, and in this respect it is noteworthy that the G protein subunit G₁₂ has recently been identified in SH-SY5Y cells (Ammer & Schulz, 1994). This subunit is classified as belonging to the G_i family of G proteins but is known to be insensitive to PTX (Hepler & Gilman, 1992). We can discount the possibility that PTX was for some reason ineffective on SH-SY5Y cells since in other parallel studies conducted in our laboratory, SH-SY5Y cells were treated with the same batch of PTX in an identical manner, and such pretreatment inhibited the suppressive actions of neuropeptide Y (NPY) on Ca²⁺ channel currents recorded with the patch-clamp technique (see Reeve *et al.*,

1994, for recording details): 100 nM NPY caused $36.5 \pm 5.1\%$ inhibition of peak inward Ca^{2+} channel currents in 8 cells which were not exposed to PTX, but this inhibitory action of 100 nM NPY was significantly reduced ($P < 0.02$) to $16.8 \pm 5.6\%$ in 7 cells pretreated with 200 ng ml^{-1} PTX for 16–24 h.

SH-SY5Y cells have been shown to express M_1 , M_2 and M_3 receptors (Adem *et al.*, 1987; Serra *et al.*, 1988; Lambert *et al.*, 1989; Mei *et al.*, 1989; Cioffi & Fisher, 1990; Murphy *et al.*, 1991b). Our observation that pirenzepine potently antagonized the actions of muscarine suggested that muscarine acted via a M_1 or M_4 receptor (e.g. Richards, 1991). We were able to discriminate between these two receptor types by finding that HHSiD was also a potent muscarinic antagonist in our cells: HHSiD has previously been shown to act potently at M_1 and M_3 receptors (Lambrecht *et al.*, 1988), but is a poor antagonist at M_4 receptors. Thus the muscarinic receptor mediating inhibition of K^+ -evoked $[\text{H}^3]\text{-NA}$ release from SH-SY5Y cells appeared to be the M_1 type.

Different presynaptic muscarinic receptor subtypes have been shown to be coupled to modulation of noradrenaline release from sympathetic nerves. For example, Costa & Majewski (1991) demonstrated inhibitory M_2 and facilitatory M_1 receptors in atrial sympathetic nerves. Facilitatory M_1 receptors have also been reported in sympathetic nerves innervating blood vessels (Casado *et al.*, 1992). By contrast, in a series of studies, Hille and colleagues described two distinct pathways by which a muscarinic agonist inhibits Ca^{2+} currents in rat sympathetic neurones (Beech *et al.*, 1991; Bernheim *et al.*, 1991; Mathie *et al.*, 1992). One pathway was rapid, mediated by M_4 receptors, blocked by PTX treatment of cells and caused selective inhibition of N-type Ca^{2+} channels. The second pathway, mediated by M_1 receptors, was slower, insensitive to PTX treatment, involved an unidentified diffusible second messenger and caused non-selective

inhibition of both L- and N-type channels. Features of this second pathway (namely M_1 receptor-mediation, PTX-insensitivity, non-selective inhibition of L- and N-type Ca^{2+} channels) are remarkably similar to the data presented here, and we would suggest that the results described by those workers can account for the effects of muscarine on K^+ -evoked $[\text{H}^3]\text{-NA}$ release in SH-SY5Y cells. Unfortunately, it is at present impossible to compare these findings further: the second messenger involved in M_1 receptor-mediated non-selective inhibition of L- and N-type channels was not identified despite detailed investigations (Bernheim *et al.*, 1991). Ca^{2+} channel inhibition was reduced by intracellular Ca^{2+} buffers (Beech *et al.*, 1991), but we are unable to test whether Ca^{2+} buffering alters muscarinic inhibition of noradrenaline release since exposure of SH-SY5Y cells to membrane permeable Ca^{2+} buffers is likely in itself to inhibit K^+ -evoked release. Nevertheless, as far as we can determine, our results are likely to occur as a consequence of Ca^{2+} channel inhibition as described earlier (Beech *et al.*, 1991; Bernheim *et al.*, 1991). Our results do not provide evidence that a fast, PTX-sensitive pathway for muscarinic receptor inhibition is involved in the suppression of K^+ -evoked neurosecretion. However, we cannot at present discount such a possible mechanism in our cells. Furthermore, it must be remembered that the contribution of Ca^{2+} channel sub-types to the regulation of neurotransmitter release and therefore the importance of selective regulation of Ca^{2+} channel sub-types, depends critically on the nature and intensity of the stimulus (Artalejo *et al.*, 1994).

The support of The British Heart Foundation is gratefully acknowledged.

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(Received May 26, 1994

Revised June 7, 1994

Accepted June 9, 1994)