Inhibitory effects of caffeine on secretagogue-induced catecholamine secretion from adrenal chromaffin cells of the guinea-pig

¹Yoshikazu Nakazato, Yuichiro Tani, *Hiroki Teraoka, Takeshi Sugawara, Tadashi Asano, Toshio Ohta & Shigeo Ito

Department of Pharmacology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, and *Department of Veterinary Pharmacology, Faculty of Dairy Science, Rakuno Gakuen University, Ebetsu 069, Japan

- 1 The inhibitory action of caffeine on catecholamine secretion induced by secretagogues was investigated in perfused adrenal glands and dispersed chromaffin cells of the guinea-pig.
- 2 Caffeine (10 mM) caused a reversible inhibition of catecholamine secretion evoked by acetylcholine (ACh, 50 μ M), KCl (56 mM, high K^+) and veratridine (100 μ M) and that induced by muscarinic receptor activation in the absence of extracellular Ca^{2+} in perfused adrenal glands.
- 3 In dispersed chromaffin cells, caffeine caused a dose-dependent inhibition of the secretory responses to 100 μ M ACh and veratridine. Forskolin (30 μ M), dibutyryl cyclic AMP (1 mM) and 8-bromo cyclic AMP (1 mM) did not mimic the action of caffeine.
- 4 In the voltage-clamp, whole-cell recording mode (at a holding potential of -60 mV or -70 mV), ACh (100 μ M) evoked an inward current, and depolarizing pulses elicited inward Na^+ , Ca^{2+} and outward K^+ currents. All these responses were partially inhibited by caffeine (20 mM).
- 5 ACh rapidly increased the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in fura-2-loaded cells in either the presence or the absence of external Ca^{2+} , though its magnitude was decreased by about 50% in Ca^{2+} -free conditions. Caffeine (20 mM) inhibited these ACh-induced increases in $[Ca^{2+}]_i$.
- 6 In permeabilized chromaffin cells, caffeine (20 mM) caused an inhibition of catecholamine secretion evoked by Ca^{2+} (10 μ M).
- 7 These results suggest that caffeine inhibits evoked catecholamine secretion through mechanisms such as the blockade of voltage-dependent Na^+ and Ca^{2+} currents and ACh receptor current, and reduction of the release of intracellularly stored Ca^{2+} and/or Ca^{2+} -sensitivity of the secretory apparatus.

Keywords: Acetylcholine currents; caffeine; Ca^{2+} transient; intracellular concentration of Ca^{2+} ; whole cell patch clamp

Introduction

Caffeine is known to be a potentiator of Ca^{2+} -induced Ca^{2+} release (CICR) in muscle cells (Endo, 1977). It has been demonstrated that caffeine causes a much larger increase in catecholamine secretion in the absence of extracellular Ca^{2+} and/or Na^+ than in their presence in perfused adrenal glands of the cat and guinea-pig (Yamada *et al.*, 1988; 1989; Teraoka *et al.*, 1990; Nakazato *et al.*, 1992), and in cultured bovine adrenal chromaffin cells (Teraoka *et al.*, 1991). Caffeine was also found to increase the cytosolic free Ca^{2+} level ($[Ca^{2+}]_i$) and catecholamine secretion, both of which were abolished after combined treatment with caffeine and ryanodine (Teraoka *et al.*, 1991; Nakazato *et al.*, 1992). These results suggest that caffeine stimulates Ca^{2+} mobilization from ryanodine-sensitive intracellular Ca^{2+} stores through the CICR mechanism, resulting in an increase in catecholamine secretion from adrenal chromaffin cells. Apart from these stimulant actions, we also found that caffeine decreased catecholamine secretion evoked by Ca^{2+} in digitonin-permeabilized bovine adrenal chromaffin cells, suggesting that caffeine reduces the susceptibility of the secretory apparatus to Ca^{2+} (Teraoka *et al.*, 1991). However, there has been no further information about the mode of inhibitory action of caffeine on adrenal catecholamine secretion.

The primary object of this study was to determine whether caffeine caused inhibition of catecholamine secretion evoked by various secretagogues in perfused adrenal glands and dispersed chromaffin cells of the guinea-pig. The results showed that caffeine decreased the evoked catecholamine secretion irrespective of the kind of secretagogue used.

Therefore, possible mechanisms underlying this inhibitory action were examined by studying the effects of caffeine on membrane currents and $[Ca^{2+}]_i$ using the whole-cell mode of the patch clamp technique and monitoring $[Ca^{2+}]_i$ by fura-2, respectively.

Methods

Experiments with perfused adrenal glands

Guinea-pigs of either sex, weighing 350–450 g, were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}) intraperitoneally. Both adrenal glands were perfused and isolated following the general procedure described previously (Ito *et al.*, 1979). The glands were perfused at a flow rate of 0.5 to 0.6 ml min^{-1} and maintained at room temperature (approximately 25°C). The adrenal effluent was collected continuously in 5 min aliquots into glass tubes kept on ice.

The standard perfusion medium was modified Locke solution of the following composition (mM): NaCl 154, KCl 5.6, $CaCl_2$ 2.2, $MgCl_2$ 1.2, Na_2HPO_4 - NaH_2PO_4 buffer 3 (pH 7.0). When ACh was used as a secretagogue, perfusion solutions contained 0.2 μ M physostigmine. All solutions used were bubbled with 100% O_2 throughout the experiments.

Experiments were started about 40 min after isolation of adrenal glands. Secretagogues were administered for 1 min, beginning 1 min before the following 5 min collection periods because of the 1 min dead time of the arterial cannula. Samples collected were acidified with 8 M perchloric acid to a final concentration of 0.4 M and stored on ice until assayed.

¹ Author for correspondence.

Experiments with dispersed chromaffin cells

Guinea-pigs of either sex weighing 250–400 g were stunned and bled to death. Adrenal glands were isolated and freed from surrounding tissues in Ca^{2+} -free Krebs solution and then sliced with a razor blade. Sliced adrenal glands were incubated with Ca^{2+} -free Krebs solution containing collagenase ($1.5\text{--}2\text{ mg ml}^{-1}$) according to the methods of Hochman & Perlman (1976). The isolated chromaffin cells were suspended in modified Krebs solution (1 ml) containing bovine serum albumin (5 mg ml^{-1}) and incubated in a shaking bath ($150\text{ oscillations min}^{-1}$) with drugs at 37°C for 20 min under 100% O_2 . Cells were then rapidly chilled on ice and removed by centrifugation, after which the catecholamine content in the supernatant and pellet was measured. Catecholamine release from the isolated cells was expressed as a percentage of catecholamine content in the cells. The modified Krebs solution contained (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, MOPS 25 (pH 7.2 adjusted with NaOH), CaCl_2 2.5, MgSO_4 1.2 and glucose 10.

Experiments with permeabilized chromaffin cells

Permeabilized adrenal chromaffin cells of guinea-pigs were prepared by the method of Dunn & Holz (1983). Cells were preincubated with a solution of the following composition (mM): potassium glutamate 139, PIPES 20 (pH 6.8, adjusted with KOH), MgSO_4 5, ATP- Na_2 5, EGTA 5, glucose 5, (K^+ glutamate solution) at 37°C for 30 min. Then the cells were washed twice with K^+ glutamate solution and incubated with this solution containing β -escin ($20\text{ }\mu\text{M}$) and caffeine (20 mM) with or without Ca^{2+} ($10\text{ }\mu\text{M}$) at room temperature for 20 min.

Assay of catecholamine

Adrenaline and noradrenaline were separated by high performance liquid chromatography (h.p.l.c., Jasco, Japan) and detected by an electrochemical detector (840-EC, Jasco). The detection limit was 10 nM (0.01 nmol ml^{-1}) for either adrenaline or noradrenaline. The treatment of samples for h.p.l.c. was carried out according to the method of Salzman & Sellers (1982).

Membrane current recording

Whole-cell membrane currents were measured with standard patch clamp techniques (Hamill *et al.*, 1981) using a patch clamp amplifier (CEZ-2300, Nihon-Koden, Japan) at room temperature. The patch pipette resistance ranged from 3 to $5\text{ M}\Omega$. Data recording and illustration were carried out as described previously (Ohta *et al.*, 1992). The ionic composition of the external solution used for this experiment was as follows (mM): NaCl 126, KCl 6, MgCl_2 1.2, CaCl_2 1.7, HEPES 10, glucose 14. The K^+ -containing pipette solution contained (mM): KCl 132, MgCl_2 1.2, HEPES 10 (pH 7.2, with KOH), EGTA 10, ATP- Na_2 2. The K^+ -free, Cs^+ -containing pipette solution was prepared by substitution of equimolar CsCl for KCl.

Flourescence measurements

The intracellular concentration of Ca^{2+} , $[\text{Ca}^{2+}]_i$, was estimated from fura-2 fluorescence by the ratio method using dual-wavelength excitation and single emission (Grynkiewicz *et al.*, 1985). Dispersed chromaffin cells attached to a cover glass were loaded with fura-2 by incubating with the external solution described above, containing fura-2AM ($5\text{ }\mu\text{M}$) and bovine serum albumin (0.05%) at room temperature for 30 min. Then the cells and the cover glass were transferred to a small chamber (volume 0.6 ml) placed on the stage of a fluorescence microscope (TMD, Nikon, Japan) attached to a fluorimeter (CAM-200, Jasco). The cells were superfused with

the external solution at a flow rate of $1.5\text{--}2\text{ ml min}^{-1}$ and waste solution was continuously drained. The fluorescence signal was detected with a Nikon CF u.v. (Fluor, $\times 40$ oil-immersion objective) lens and the emission light, which was passed through a pinhole diaphragm slightly larger than a cell, was collected by a photomultiplier through a 500 nm filter. The ratio of the fluorescence due to excitation at 340 nm to that at 380 nm (F340/F380) was calculated from successive illumination periods and stored on videotape through a PCM data recording system (RP-880, NF Electronic Instruments, Japan).

Chemicals

The following chemicals were used: acetylcholine chloride (Ovisort, Daiichi, Tokyo), adenosine-5'-triphosphate (ATP, Boehringer-Manheim-Yamanouchi, Tokyo), β -escin, bovine serum albumin, forskolin, N6,2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate (dibutyryl cyclic AMP), 8-bromo-adenosine 3',5'-cyclic monophosphate (8-bromo cyclic AMP), veratridine (Sigma, St. Louis, MO), ethylenediaminetetraacetic acid (EDTA), glycoethyldiamine- N,N,N',N' -tetraacetic acid (EGTA), N-2-hydroxyethyl-piperazine- N' -2-ethanesulphonic acid (HEPES), 3-(N-morpholino) propane-sulphonic acid (MOPS), fura-2AM (Dojindo Laboratories, Kumamoto), caffeine monohydrate, collagenase, theophylline (Wako Pure Chem, Tokyo), sodium 1 octanesulphonate (Nacalai Tesque, Kyoto) and tetrodotoxin (Mitsui, Tokyo).

Statistics

The data are presented as arithmetic mean $\pm$ s.e.mean. Significance was assessed with Student's *t* test.

Results

Effects of caffeine on catecholamine secretion induced by secretagogues in perfused adrenal glands

The effect of caffeine on catecholamine secretion evoked by various secretagogues was investigated with perfused adrenal glands. Acetylcholine (ACh, $50\text{ }\mu\text{M}$), KCl (56 mM , high K^+) and veratridine ($100\text{ }\mu\text{M}$) were sequentially applied to the adrenal gland for 1 min at 20 min intervals before, during and after infusion of caffeine (10 mM). Caffeine showed almost no consistent effect on the resting catecholamine secretion during perfusion with standard Locke solution as reported previously (Teraoka *et al.*, 1990; Nakazato *et al.*, 1992). However, the secretory responses to ACh, high K^+ and veratridine all were partially inhibited in the presence of caffeine (Figure 1).

Previously we demonstrated that both nicotinic and muscarinic receptors almost equally contribute to catecholamine secretion evoked by ACh with concentrations of $50\text{ }\mu\text{M}$ and $100\text{ }\mu\text{M}$ (Nakazato *et al.*, 1988). Furthermore, unlike nicotinic receptor activation, muscarinic activation mobilizes intracellular stored Ca^{2+} to increase catecholamine secretion (Nakazato *et al.*, 1984; 1988; Yamada *et al.*, 1988; Teraoka *et al.*, 1990). Thus, the effect of caffeine on ACh-evoked catecholamine secretion through muscarinic receptor activation was studied in the adrenal glands perfused with Ca^{2+} -free Locke solution containing hexamethonium (0.5 mM). ACh ($100\text{ }\mu\text{M}$) applied for 1 min caused increases in catecholamine secretion repeatedly during perfusion with Ca^{2+} -free solution, as long as the adrenal glands were loaded with 2.2 mM Ca^{2+} for 20 to 25 min between stimuli. It was also noted that the Ca^{2+} loading produced a tonic increase in catecholamine secretion which declined on repetition (Figure 2a). In Figure 2b, ACh ($100\text{ }\mu\text{M}$) was first applied during perfusion with the standard Locke solution, and then the same protocol of experiment as shown in Figure 2a was carried out in the presence of caffeine (10 mM) during the

Figure 1 Inhibitory effect of caffeine on secretagogue-induced catecholamine secretion. Acetylcholine (ACh, 50 μ M) (a), KCl (K, 56 mM) (b) and veratridine (Vera, 100 μ M) (c) were sequentially applied for 1 min before, during and after infusion of caffeine (10 mM) ($\downarrow$ — $\downarrow$) in perfused adrenal glands of the guinea-pig. The standard Locke solution was used for perfusion. Representative data are shown. Columns show the amount of adrenaline (open) and noradrenaline (hatched) released in nmol 5 min⁻¹.

second and third exposure to Ca^{2+} -free solution. The magnitude of ACh-induced catecholamine secretion in the absence of extracellular Ca^{2+} was about 25% of that in the presence of Ca^{2+} . Caffeine caused a significant inhibition of ACh-induced catecholamine secretion which completely recovered after its removal. In addition, there was almost no increase in catecholamine secretion in response to the Ca^{2+} loading.

Effects of caffeine on catecholamine secretion induced by ACh and veratridine in dispersed chromaffin cells

By use of dispersed chromaffin cells, the potency of caffeine to inhibit the agonist-induced catecholamine secretion was estimated. The dispersed chromaffin cells were incubated for 20 min at 37°C with ACh (100 μ M) or veratridine (100 μ M) in combination with various concentrations of caffeine (1–40 mM). Caffeine caused a dose-dependent inhibition of both secretory responses (Figure 3). The rate of inhibition was about 10% at 1 mM caffeine and increased as its concentration increased, attaining a maximum at 20 mM for both the responses to ACh and those to veratridine. The IC_{50} values for caffeine were about 4.4 mM in ACh- and 6.0 mM in veratridine-induced catecholamine secretions.

Figure 2 Inhibitory effect of caffeine on catecholamine secretion induced by acetylcholine in the absence of extracellular Ca^{2+} . The adrenal glands were perfused with the standard Locke and Ca^{2+} -free Locke solutions alternately as indicated below the records. Acetylcholine (ACh, 100 μ M) was applied for 1 min as indicated by vertical heavy bars. (a) Shows control experiment and in (b) caffeine (10 mM) was present for the periods indicated ($\downarrow$ — $\downarrow$). Representative data are illustrated. Columns show the amount of adrenaline (open) and noradrenaline (hatched) released in nmol 5 min⁻¹.

Figure 3 Dose-dependent inhibition of secretagogue-induced catecholamine secretion by caffeine. Dispersed chromaffin cells were incubated with acetylcholine (ACh, 100 μ M) (a) and veratridine (100 μ M) (b) for 20 min at 37°C in the absence and presence of various concentrations of caffeine (1–40 mM). Modified Krebs solution was used for incubation. Symbols indicate the mean $\pm$ s.e. mean (if vertical lines are present) of adrenaline released as a percentage of total cell content in the presence of the secretagogues obtained from 8 experiments.

Table 1 Catecholamine secretion evoked by acetylcholine and veratridine in the presence or absence of dibutyryl cyclic AMP, 8-bromo cyclic AMP and forskolin

	Catecholamine secretion (%)		
	Resting	Acetylcholine	Veratridine
Control	0.75 ± 0.46 (4)	9.11 ± 0.86 (5)	8.66 ± 0.67 (5)
Dibutyryl cyclic AMP	1.88 ± 0.70 (5)	14.07 ± 1.24 (5)	9.10 ± 1.64 (4)
Control	0.61 ± 0.36 (5)	12.91 ± 0.72 (6)	14.88 ± 1.44 (4)
8-Bromo cyclic AMP	1.23 ± 0.49 (5)	13.35 ± 0.80 (4)	11.76 ± 1.67 (4)
Control	2.00 ± 0.49 (5)	15.10 ± 0.74 (5)	18.20 ± 0.43 (5)
Forskolin	2.70 ± 0.29 (5)	19.59 ± 0.76 (5)	16.59 ± 0.79 (5)

Dispersed chromaffin cells were incubated for 20 min at 37°C with modified Krebs solutions containing 100 µM acetylcholine or veratridine in the absence (Control) or presence of 1 mM dibutyryl cyclic AMP, 8-bromo cyclic AMP and 30 µM forskolin. Numbers indicate the mean ± s.e.mean of adrenaline released as a percentage of total cell contents (no. of experiments in parentheses).

Figure 4 Dose-dependent inhibition of acetylcholine (ACh)-induced inward current in single chromaffin cells. Cells were dialyzed with K⁺-rich (KCl, 132 mM) pipette solution and superfused with the normal external solution at a holding potential of -60 mV. ACh (100 µM) was sequentially applied for 10 s (horizontal bars) through a pipette placed close to the cell in the presence and absence of caffeine, which was added to the superfused solution in the concentrations indicated above the current responses. Representative records are shown in (a) and the data obtained from different experiments are summarized in (b). The symbols indicate the mean ± s.e.mean of peak amplitude, if vertical lines are present, as a percentage of control in the presence (●) and absence (○) of caffeine.

Like caffeine, theophylline (5–10 mM) also caused a dose-dependent inhibition of ACh-evoked catecholamine secretion (data not shown).

Effects of membrane-permeant analogues of cyclic AMP and forskolin

To determine whether caffeine or theophylline caused inhibition of secretagogue-induced catecholamine secretion by increasing the amount of cyclic AMP, chromaffin cells were stimulated with ACh (100 µM) and veratridine (100 µM) in the presence or absence of dibutyryl cyclic AMP (1 mM), 8-bromo cyclic AMP (1 mM) or forskolin (30 µM). The results are summarized in Table 1. Both membrane-permeant cyclic AMP analogues and forskolin caused a small increase in the resting catecholamine secretion, but no consistent effect on ACh- and veratridine-evoked catecholamine secretions. Forskolin (30 µM) also failed to inhibit sequentially evoked

Figure 5 Inhibitory effect of caffeine on depolarization-induced membrane currents in single chromaffin cells. Traces from top to bottom: inward Na⁺ (I_{Na^+}), Ca²⁺ ($I_{Ca^{2+}}$), outward K⁺ currents (I_{K^+}) and depolarization pulse. Depolarizing pulses from holding potential, -70 mV to 0 mV were applied using a patch pipette which contained CsCl (132 mM) instead of KCl for Na⁺ and Ca²⁺ currents and KCl (132 mM) for K⁺ current before (control), during and after (after control) the addition of caffeine (20 mM). The duration of the pulse was 30 ms for Na⁺ and K⁺ currents and 100 ms for Ca²⁺ current. When K⁺ and Ca²⁺ currents were recorded, tetrodotoxin (0.5 µM) was added to the bathing solution to prevent the initiation of Na⁺ current.

catecholamine secretion by ACh (100 µM) or high K⁺ in perfused adrenal glands (data not shown).

Effects of caffeine on membrane currents

The effects of caffeine on membrane current were observed using single chromaffin cells under voltage clamp conditions. After achieving the whole-cell configuration by rupture of the membrane patch, ACh (100 µM) was added to the external solution for 10 s every 3 or 4 min. At a holding potential of -60 mV, ACh repeatedly evoked a transient inward current together with an increase in the background noise. This transient current was abolished by hexamethonium, indicating that the current was mediated by the activation of nicotinic receptors. When caffeine was added to the external solution, ACh-evoked inward current was inhibited depending on the concentration of caffeine (1–20 mM). The inhibitory action of caffeine started to occur at 1 mM and progressively increased with the concentration of caffeine, attaining a maximum, a little less than 50% of the control, at 20 mM and recovered after its removal. A representative

Table 2 Effects of caffeine on Ca^{2+} transient and membrane current induced by acetylcholine (ACh) and on depolarization-evoked membrane currents

	n	Control	Caffeine	After control
ACh-induced				
Inward current	8	100	37.3 ± 2.3	87.9 ± 3.7
Ca^{2+} transient	9	100	44.8 ± 6.7	86.6 ± 2.7
Voltage-dependent				
Na^+ current	7	100	62.8 ± 4.2	101.7 ± 9.1
Ca^{2+} current	6	100	44.5 ± 6.3	77.4 ± 6.5
K^+ current	5	100	70.9 ± 2.6	100.6 ± 3.1

Numbers indicate the mean $\pm$ s.e. mean of peak amplitude of Ca^{2+} transient and current responses, as a percentage of each control, obtained before (Control), during (Caffeine) and after (After control), application of caffeine (20 mM). Numbers of experiments (*n*) are shown.

result and the summarized data from different experiments are shown in Figure 4a and b, respectively.

To study the effect of caffeine on voltage-dependent membrane currents, depolarization pulses of 30 ms duration to evoke inward Na^+ and outward K^+ currents, and of 100 ms duration to elicit inward Ca^{2+} current from a holding potential of -70 mV to test potential of 0 mV were applied at 20 s intervals before, during and after addition of caffeine (20 mM). Figure 5 shows that all current responses were partially, but reversibly inhibited by caffeine. The effects of different preparations on the inhibitory action of caffeine are summarized as percentages of control values in Table 2. The data show that caffeine is more effective in decreasing Ca^{2+} current than Na^+ and K^+ currents.

Effects of caffeine on the intracellular concentration of Ca^{2+}

The effect of caffeine on ACh-induced changes in $[\text{Ca}^{2+}]_i$ was observed on single chromaffin cells loaded with fura-2. ACh ($100 \mu\text{M}$) added to the external solution for 10 s rapidly increased $[\text{Ca}^{2+}]_i$. In about half of the cells tested, the addition of caffeine (20 mM) to the external solution also caused an increase in $[\text{Ca}^{2+}]_i$ which progressively declined toward the resting level even if caffeine was still infused. When the caffeine-induced increase in $[\text{Ca}^{2+}]_i$ attained the resting level, ACh ($100 \mu\text{M}$) was applied. Regardless of whether or not caffeine increased $[\text{Ca}^{2+}]_i$, subsequent ACh-induced Ca^{2+} transient was reversibly inhibited by caffeine to about 45% of the control (Figure 6a, b and Table 2). In agreement with previous results (Teraoka *et al.*, 1991), caffeine-induced increase in $[\text{Ca}^{2+}]_i$ was blocked by pretreatment with caffeine plus ryanodine (data not shown).

Next, it was determined whether ACh was effective in increasing $[\text{Ca}^{2+}]_i$ in the absence of external Ca^{2+} and if so, whether it was affected by caffeine. ACh ($100 \mu\text{M}$) was applied for 10 s at 3 min intervals before and during exposure to Ca^{2+} -free solution containing EGTA (0.5 mM). As shown in Figure 6c and d, ACh still increased $[\text{Ca}^{2+}]_i$ in the absence of external Ca^{2+} , though the magnitude was decreased by about 50% of the control value. After readmission of Ca^{2+} , the ACh-induced Ca^{2+} transient recovered completely and then caffeine (20 mM) was added to the external medium. Two minutes after the start of exposure to caffeine, external Ca^{2+} was replaced by EGTA (0.5 mM) and ACh was applied.

Under these conditions, ACh completely failed to evoke Ca^{2+} transient regardless of whether or not caffeine itself caused increase in $[\text{Ca}^{2+}]_i$.

Effect of caffeine on Ca^{2+} -sensitivity of the secretory apparatus

In cultured bovine adrenal chromaffin cells permeabilized by treatment with digitonin, caffeine decreased catecholamine

Figure 6 Inhibitory effect of caffeine on acetylcholine (ACh)-induced Ca^{2+} transients in a single chromaffin cell. Traces show intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). ACh ($100 \mu\text{M}$) was sequentially applied for 10 s (■) in the presence (a, b) and absence (c, d) of external Ca^{2+} through a pipette placed close to the cell before, during and after the addition of caffeine (20 mM) (↓). Caffeine itself caused a Ca^{2+} transient in (a) and (c), but not in (b) and (d). In Ca^{2+} -free solution, Ca^{2+} was omitted and 0.5 mM EGTA was added (0Ca, 0.5G; □).

Figure 7 Inhibitory effect of caffeine on catecholamine secretion evoked by Ca^{2+} in permeabilized chromaffin cells. Dispersed chromaffin cells were incubated in K^+ glutamate solution containing β -escin ($20 \mu\text{M}$) with (+) or without (–) Ca^{2+} ($10 \mu\text{M}$) for 20 min at room temperature (approximately 25°C) in the absence (a) and presence (b) of caffeine (20 mM). Columns indicate the mean $\pm$ s.e. mean of adrenaline released as a percentage of total cell contents obtained from 19 experiments. * $P < 0.01$, when compared with the response to Ca^{2+} in control (a).

secretion directly evoked by Ca^{2+} , suggesting a reduction of the Ca^{2+} -sensitivity of the secretory apparatus in the presence of caffeine (Teraoka *et al.*, 1991). We therefore examined whether the same was true in the case of the guinea-pig. Dispersed chromaffin cells were incubated with K^+ glutamate solution containing β -escin ($20 \mu\text{M}$) for 20 min with or without Ca^{2+} ($10 \mu\text{M}$) in the presence and absence of caffeine (20 mM). Figure 7 shows that Ca^{2+} caused an increase in catecholamine secretion, which was significantly inhibited by caffeine.

Discussion

The present study showed that caffeine causes a marked inhibition of catecholamine secretion evoked by ACh, veratridine and high K^+ in perfused adrenal glands and isolated chromaffin cells of the guinea-pig. The mechanism of action inducing catecholamine secretion is different among the secretagogues. ACh, veratridine and high K^+ have been considered to depolarize the chromaffin cell and to increase Ca^{2+} influx through voltage-dependent Ca^{2+} channels, resulting in an increase in catecholamine secretion (Douglas & Rubin, 1961; Ito *et al.*, 1978; 1979; Kilpatrick *et al.*, 1981; Knight & Baker, 1983; Knight & Kesteven, 1983; Wakade & Wakade, 1983). However, the mechanism underlying the depolarization is different between stimulants, ACh and veratridine cause depolarization resulting mainly from the activation of nicotinic receptor channels and voltage-dependent, tetrodotoxin-sensitive Na^+ channels, respectively. High K^+ depolarizes the membrane directly by changing the equilibrium potential for K^+ . On the other hand, muscarinic receptor activation is assumed to mobilize intracellularly stored Ca^{2+} responsible for catecholamine secretion (Nakazato *et al.*, 1984; 1988; Yamada *et al.*, 1988; Teraoka *et al.*, 1990).

In the present experiments, caffeine was found to decrease Na^+ , Ca^{2+} and K^+ currents evoked by depolarizing voltage steps. The inhibition of the Ca^{2+} current was much greater than that of Na^+ and K^+ currents. Caffeine is also reported to block voltage-operated Ca^{2+} channels in smooth muscle

cells (Hughes *et al.*, 1990; Watanabe *et al.*, 1992). Therefore, the inhibitory effect of caffeine on the secretory responses to veratridine and high K^+ may be due partly to the action on voltage-dependent Na^+ and/or Ca^{2+} channels. Caffeine also inhibited both ACh-induced inward currents through nicotinic receptor channels and Ca^{2+} transients, indicating that the inhibitory action of caffeine on catecholamine secretion induced by ACh can be attributed mainly to the inhibition of increase in $[\text{Ca}^{2+}]_i$ through nicotinic receptor channels and voltage-dependent Ca^{2+} channels. However, it was noted that the rate of inhibition by caffeine was much larger in catecholamine secretion than in either inward currents or Ca^{2+} transients, suggesting the involvement of some additional mechanisms in the inhibition of the evoked catecholamine secretion.

Caffeine was found to block catecholamine secretion induced by muscarinic receptor activation in adrenal glands perfused with Ca^{2+} -free Locke solution in the present experiments. In addition, caffeine abolished the ACh-induced increase in $[\text{Ca}^{2+}]_i$ in the absence of external Ca^{2+} regardless of whether or not caffeine itself evoked Ca^{2+} transient. It therefore seems unlikely that caffeine inhibits ACh-induced Ca^{2+} transient by depleting intracellular Ca^{2+} stores. It has been reported that caffeine causes non-specific inhibition of inositol 1,4,5-trisphosphate (InsP_3)-induced Ca^{2+} release from rat cerebellar microsomes (Brown *et al.*, 1992) and mouse pancreatic acinar cells (Toescu *et al.*, 1992). One of the possible mechanisms involved in the inhibitory effect of caffeine on secretagogue-induced catecholamine secretions is the inhibition of Ca^{2+} release from intracellular Ca^{2+} stores. Another possibility is that caffeine may decrease the Ca^{2+} -sensitivity of the secretory apparatus, because caffeine reduced catecholamine secretion induced by Ca^{2+} in permeabilized chromaffin cells of the guinea-pig. Our results are in accord with reports that caffeine decreases the Ca^{2+} -sensitivity of secretory apparatus in bovine adrenal chromaffin cells (Teraoka *et al.*, 1991) and of contractile proteins in smooth muscle cells (Sato *et al.*, 1988; Ahn *et al.*, 1988; Savineau & Mironneau, 1990). The inhibitory effect of caffeine on the Ca^{2+} -sensitivity of the secretory apparatus may account for the failure of a continuous infusion of caffeine to increase catecholamine secretion, even if $[\text{Ca}^{2+}]_i$ rose in response to the caffeine infusion.

Xanthine derivatives are well known to increase the cyclic AMP concentration as a result of inhibition of cyclic AMP phosphodiesterase activity (Butcher & Sutherland, 1962). In the present experiments theophylline was also shown to cause inhibition of ACh-evoked catecholamine secretion. However, neither membrane-permeant analogues of cyclic AMP, such as dibutyryl cyclic AMP and 8-bromo cyclic AMP nor forskolin showed consistent effects on evoked catecholamine secretion from both perfused adrenal glands and isolated chromaffin cells, indicating that cyclic AMP does not play a role in the inhibitory action of caffeine.

Furthermore, the inhibitory effect of caffeine on various ionic channels should not be due to a secondary action of increased cytosolic free Ca^{2+} level, because the inhibitory effects still occurred long after the end of the Ca^{2+} transient evoked by caffeine. The inhibitory action of caffeine observed in this study was observed to recover quickly after washout of the drug. Such rapid recovery seems to provide an important clue for determining the mechanism of inhibitory action of xanthine derivatives on ionic channels. Further studies are certainly required to solve this problem.

In conclusion, caffeine causes inhibition of secretagogue-induced catecholamine secretion by inhibiting nicotinic receptor channel currents, voltage-dependent Ca^{2+} and Na^+ currents and by decreasing the mobilization of intracellularly stored Ca^{2+} and/or the sensitivity of the secretory apparatus to Ca^{2+} .

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