Inhibitory effects of capsaicin on acetylcholine-evoked responses in rat phaeochromocytoma cells

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1 The effects of capsaicin on cellular responses evoked by acetylcholine (ACh) and those by adenosine 5'-triphosphate (ATP) were investigated in rat phaeochromocytoma PC12 cells.

2 Capsaicin (1 to 30 μM) suppressed dopamine release and the intracellular Ca^{2+} increase evoked by 100 μM ACh. The concentration-dependence of the ACh-evoked release of dopamine was not shifted but the maximal response was reduced by capsaicin. Dopamine release evoked by 80 mM KCl was also suppressed by capsaicin (3 and 30 μM), but the extent of suppression was smaller than that of the ACh-evoked release.

3 Under whole-cell voltage-clamp, capsaicin (1 to 30 μM) reversibly inhibited the inward current activated by ACh (30 to 300 μM). The inhibition exhibited dependence on the concentration of ACh, and the current activated by a higher concentration of ACh was less inhibited. Voltage-dependence of block by capsaicin was not observed when it was tested either by applying a ramp pulse during the current activation by ACh or by eliciting the current in cells held at various potentials.

4 High concentrations of capsaicin (30 to 100 μM) enhanced the inward current as well as dopamine release evoked by 30 μM ATP.

5 The results suggest that low concentrations of capsaicin selectively antagonize responses mediated by nicotinic receptor-channels without affecting those mediated by purinoceptor-coupled channels. As the antagonism by capsaicin of the ACh-evoked responses was observed at concentrations as low as 1 μM , the influence on nicotinic receptors should be taken into account when this compound is used as a pharmacological tool to deplete neuropeptides.

Keywords: Capsaicin; acetylcholine; nicotinic receptor-channels; extracellular ATP; rat phaeochromocytoma cells; dopamine release; intracellular Ca^{2+} concentration; voltage clamp

Introduction

Capsaicin has been shown to excite a subpopulation of primary afferent sensory neurones (Bevan & Szolcsanyi, 1990). As this neurone population has characteristics in common with neurones that mediate inflammatory responses, the excitation is believed to be responsible for the chemical irritation observed with this compound (Bevan & Szolcsanyi, 1990). Capsaicin has also been shown to block neurotransmission not mediated by adrenoceptors or acetylcholine receptors in various tissues. This block of 'non-adrenergic non-cholinergic' transmission is considered to be due to neuronal excitation and subsequent depletion of neuropeptides such as substance P (Szolcsanyi & Bartho, 1978; Ellis & Burnstock, 1989) or calcitonin gene-related peptide (CGRP; Franco-Cereceda & Lundberg, 1985; Saito *et al.*, 1987), which exist, presumably, in sensory neurones.

Effects of capsaicin on ion channels have been reported. Capsaicin evoked an inward current through non-selective cation channels in rat vagal (Marsh *et al.*, 1987) or dorsal root ganglion neurones (Docherty *et al.*, 1991), monkey trigeminal ganglion neurones (Ingram *et al.*, 1993), and a rat dorsal ganglion cell line, F-11 cells (Kusano & Gainer, 1993). In contrast, no such inward current was evoked by capsaicin in guinea-pig or chick dorsal root ganglion neurones (Petersen *et al.*, 1987). Capsaicin also affects voltage-gated channels. For Ca^{2+} channels, inhibition was observed in rat dorsal root ganglion neurones (Docherty *et al.*, 1991) whereas both inhibition and potentiation were observed in guinea-pig dorsal root ganglia (Petersen *et al.*, 1989) and F-11 cells (Kusano & Gainer, 1993). Inhibition of Na^{+} and K^{+} channels were also reported in guinea-pig dorsal root ganglion cells (Petersen *et al.*, 1987). In contrast to the results observed on voltage-gated channels, effects of capsaicin on ligand-gated channels have not been reported.

The present study was aimed at examining the effects of

capsaicin on responses mediated through two types of ligand-gated channels: nicotinic receptor channels and ATP-activated channels in PC12 cells. The PC12 cell line was derived from a rat phaeochromocytoma (Greene & Tischler, 1976) and has been widely used to investigate mechanisms underlying catecholamine release and properties of ion channels. We found that capsaicin inhibited ACh-evoked responses at concentrations as low as 1 μM and that it facilitated ATP-evoked responses at higher concentration.

Methods

Cell culture

PC12 (passage 55 to 65) were cultured as previously described (Inoue & Kenimer, 1988; Inoue *et al.*, 1989). Cells cultured in 35 mm-polystyrene dishes for 1 to 3 days were used for experiments.

Dopamine release

Dopamine release was measured by a procedure described previously by Ohara-Imaizumi *et al.* (1991). In brief, the dishes were preincubated with 1 ml of balanced salt solution (BSS) containing (in mM): NaCl 150, KCl 5, CaCl_2 1.8, MgCl_2 1.2, NaH_2PO_4 1.2, D-glucose 10, ethylenediaminetetraacetic acid (EDTA) 0.1, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 25 (pH was adjusted to 7.4 with NaOH) for 1 h. The preincubation solution was removed and 1 ml BSS was added. After 1 min, the BSS was aspirated and the cells were stimulated for 1 min by ACh or ATP at indicated concentrations in BSS or by 80 mM KCl with or without capsaicin. The solution containing 80 mM KCl was prepared by replacing equimolar NaCl in the BSS. When the effect of capsaicin was examined, an additional

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1 min-treatment with the BSS and stimulation were carried out in the presence of this compound. At the end of the incubation, the incubation solution was transferred to test tubes containing 0.25 ml of 1 M HClO₄. The dopamine remaining in the cells was extracted by sonication with 0.2 M HClO₄. After centrifugation (at 5°C for 2 min, 1000 g), supernatants of both the incubation solution and the sonicated cellular solution were collected for measurement of dopamine content. Dopamine content was determined with a high-performance liquid chromatography-electrochemical detector (h.p.l.c.-e.c.d.) system (Bioanalytical Systems). Percentage of release was calculated using the values obtained for the dopamine content in the incubation solution and the content remaining in the cells. All procedures except centrifugation were performed at room temperature (about 25°C).

Intracellular Ca²⁺ concentration

Intracellular Ca²⁺ levels were measured from individual cells using fura-2 (Grynkiewicz *et al.*, 1985) under the conditions described by Ohara-Imaizumi *et al.* (1991) and Nakazawa & Inoue (1992). Cells loaded with fura-2/AM were placed on an inverted microscope (Olympus IMT-2) equipped with a xenon lamp and band pass filters. Fluorescent intensities were recorded by a silicon intensifier target camera (C-2741-08, Hamamatsu Photonics). The intracellular Ca²⁺ concentration was calculated from the ratio of fluorescence intensity measured at wave lengths of 340 and 360 nm (F340 and F360). Drugs were applied by superfusion.

Membrane current measurements

Current recordings were obtained by conventional whole-cell voltage-clamp method (Hamill *et al.*, 1981) under the conditions described elsewhere (Nakazawa *et al.*, 1990). The cells were bathed in an extracellular solution containing (in mM): NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1.0, HEPES 10, D-glucose 11.1 (pH was adjusted to 7.4 with NaOH). Tip resistances of fire-polished patch pipettes ranged between 3 to 5 MΩ when the pipettes were filled with an intracellular solution containing (in mM): CsCl 150, HEPES 10, ethyleneglycos-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 5, (pH 7.3 with CsOH). Application of ACh or ATP was made from an emitting tube (2 mm in inner diameter). Cells located near the mouth of the emitting tube (about 1 mm in distance) were selected for recordings, and the ACh- or ATP-containing extracellular solution was applied rapidly (ca. 0.4 ml s⁻¹). To avoid significant desensitization of the nicotinic receptor-channels, the period of each ACh-application was brief (4 to 5 s), and each application was separated by 1 min. Before examining the effects of capsaicin, ACh was applied two or three times and the stability of the response to ACh was confirmed in individual cells. Experiments were performed at room temperature (about 25°C). Data were filtered at 1 kHz and stored on magnetic tape for later analysis.

Statistical analysis

Statistical analysis was made by Scheffe's test for multiple comparison. In the case of the comparison of the current responses obtained from the same cells, the data were analyzed by the paired *t* test. Significant differences are indicated with asterisks (**P* < 0.05, ***P* < 0.01; two-tailed test) in figures.

Drugs

Drugs used were capsaicin, (Wako Pure Chemistry, Osaka, Japan), acetylcholine chloride (ACh; Wako), adenosine 5'-triphosphate disodium salt (ATP; Sigma St. Louis, MO, U.S.A.), resiniferatoxin (Wako), guanosine 5'-O(2-thiotriphosphate) trilithium salt (GDPβS; Sigma) and K-252a (8R*,

9S*, 11S*)-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H, 11H-2,7b,11a-triazadibenzo (a,g)cycloocta (cde)trinden-1-one; Kyowa Hakko, Tokyo, Japan). Fura-2/AM was from Dojin (Kumamoto, Japan). Capsaicin was dissolved in ethanol and diluted in the extracellular media so that final concentration of ethanol was <2%. The dopamine release or the ACh-activated current in the present study was not affected by 2% ethanol. K-252a or resiniferatoxin was dissolved in dimethylsulphoxide (DMSO) and added to the intracellular or extracellular solution, respectively (the final concentration of DMSO was ≤ 0.1%).

Results

Effects of capsaicin on dopamine release and increase in intracellular Ca²⁺ concentration

ACh (10 to 300 μM) evoked dopamine release from PC12 cells. The ACh-evoked release was regarded as a nicotinic response previously characterized in these cells (e.g., Greene & Rein, 1977) because our preliminary experiments showed that the release was inhibited by 70% by 100 μM hexamethonium and was almost abolished by 300 μM CdCl₂, which is a characteristic of nicotinic responses in these cells (Inoue & Kenimer, 1988; see also below). Capsaicin itself (1 to 30 μM) did not affect basal dopamine release, but inhibited the ACh-evoked dopamine release in a concentration-dependent manner (Figure 1a). The concentration-response curve for the ACh-evoked dopamine release was not shifted toward a higher concentration-range, but the maximal response was suppressed (Figure 1b), suggesting that the antagonism by capsaicin is non-competitive.

The influence of capsaicin on the ACh-induced increase in intracellular Ca²⁺ concentration was also tested. When repeated applications of 100 μM ACh were made to control cells, the response to the second application was not significantly different from that to the first response (paired *t* test, *P* > 0.05; Figure 2a). In the presence of 3 or 30 μM capsaicin (Figure 2b), however, the response to the second application was less than the response to the first application, and the ratio of the second response to the first response (S₁/S₂) was significantly smaller than that under control conditions (Figure 2c). Thus, the inhibition by capsaicin of the dopamine release by ACh may have resulted from the attenuation of increase in intracellular Ca²⁺ concentration.

Voltage-gated Ca²⁺ channels secondarily activated by depolarization contribute to nicotinic responses in PC12 cells (Ritchie, 1979; Inoue & Kenimer, 1988). As for ACh-stimulated catecholamine release, Ca²⁺ channels play an obligatory role because the release was abolished by Ca²⁺ channel antagonists (Inoue & Kenimer, 1988). Thus, the inhibition by capsaicin of the ACh-evoked release may be due to its reported blockade of Ca²⁺ channels (Petersen *et al.*, 1989; Kusano & Gainer, 1993). We tested whether mechanisms mediated through Ca²⁺ channels are involved in the inhibition of the ACh-evoked responses by stimulating the cells with 80 mM KCl, which evokes dopamine release sensitive to Ca²⁺ channel antagonists (Ritchie, 1979; Nakazawa & Inoue, 1992). Although capsaicin (3 and 30 μM) suppressed the dopamine release evoked by 80 mM KCl (Figure 1c), the magnitude of inhibition was smaller than that of the ACh-evoked release (Figure 1a). For example, the ACh-evoked dopamine release was roughly halved by 3 μM capsaicin (Figure 1a) whereas the KCl-evoked dopamine release was reduced by only 17% by 3 μM capsaicin (Figure 1c). These results suggest that mechanisms involving voltage-gated Ca²⁺ channels do not play a major role in the inhibition of the ACh-evoked release.

Inhibition by capsaicin of ACh-activated current

ACh (30 to 300 μM) elicited a transient inward current in PC12 cells as was previously described (Ifune & Steinbach,

1990). Capsaicin (1 and 3 μM) suppressed this ACh-elicited current (Figure 3). The suppression was reversible, and the ACh-activated current almost fully recovered after a 1 min

Figure 1 Inhibition by capsaicin of dopamine release evoked by acetylcholine (ACh) (a,b) or 80 mM KCl (c) from PC12 cells. The cells were pretreated with capsaicin 1 min before secretagogue applications. Each symbol or column is the mean with s.e. from three dishes in the same batch. The data are representative of two to three experiments. Asterisks indicate significant difference from the dopamine release without capsaicin (Scheffe's test; * $P < 0.05$, ** $P < 0.01$). (a) Concentration-dependence of inhibition by capsaicin of dopamine release evoked by 100 μM ACh. A smooth curve was fitted to the data with a EC_{50} value of 3.7 μM and a Hill coefficient of 1. (b) Comparison of concentration-response relationship for the ACh-evoked dopamine release in the absence (○) and presence of 3 μM capsaicin (●). Curves were fitted to the data assuming that the EC_{50} value (40 μM) and Hill coefficient (3.0) are not changed, but only the maximal response is reduced to 60% of control in the presence of capsaicin. (c) Inhibition by 3 and 30 μM capsaicin of the dopamine release evoked by 80 mM KCl.

wash with drug-free solution (Figure 3a). The magnitude of inhibition of the ACh-evoked current (Figure 3b) was roughly the same as that of the inhibition of dopamine release (Figure 1a). A higher concentration (30 μM) of capsaicin abolished the current activated by 100 μM ACh ($n = 2$). Resiniferatoxin (10 nM to 1 μM), a plant product that has structural similarity to capsaicin and is about 10,000 times more potent in activation of the capsaicin-sensitive non-selective cation channels (Bevan & Szolcsanyi, 1990), did not suppress the ACh-activated current in five cells tested: for example, the current activated by 100 μM ACh in the

Figure 2 Attenuation by capsaicin of the acetylcholine (ACh)-evoked increase in intracellular Ca^{2+} concentration in PC12 cells. (a,b) Intracellular Ca^{2+} response to repeated applications of 100 μM ACh to the same cells in the absence (a) or presence of 3 μM capsaicin (b). Time course of continuous recordings are illustrated. Each symbol represents the mean with s.e. from 11 (control) or 12 (3 μM capsaicin) cells tested. (c) Comparison of the increase in intracellular Ca^{2+} concentration upon the second application of 100 μM ACh. The Ca^{2+} increase upon the second application (S_2) and that upon the first application (S_1) were measured as shown in (a) and (b), and the ratio of S_2/S_1 was determined in individual cells. Each column represents mean with s.e. The data without capsaicin and those with 3 μM capsaicin were obtained from the cells shown in (a) and (b), and those with 30 μM capsaicin were from 14 cells tested. Asterisks indicate significant difference from the Ca^{2+} response without capsaicin (Scheffe's test; * $P < 0.05$, ** $P < 0.01$).

presence of $1\text{ }\mu\text{M}$ resiniferatoxin was $99.6 \pm 8.6\%$ of the current in the absence of this compound (mean $\pm$ s.e., $n = 3$).

The magnitude of the current inhibition by capsaicin was increased when the current was activated by a lower concentration of ACh (Figure 4). In the case shown in Figure 4a, the current elicited by $30\text{ }\mu\text{M}$ ACh was decreased to 62% whereas the current elicited by $300\text{ }\mu\text{M}$ ACh was decreased only to 86% of control in the presence of $1\text{ }\mu\text{M}$ capsaicin. The dependence on the concentration of ACh was also obvious with the current inhibition by $3\text{ }\mu\text{M}$ capsaicin (Figure 4c). The current evoked by 30 or $100\text{ }\mu\text{M}$ ACh was more significantly suppressed by $3\text{ }\mu\text{M}$ capsaicin than the current evoked by $300\text{ }\mu\text{M}$ ACh, and the inhibition by $1\text{ }\mu\text{M}$ capsaicin was also significantly larger with the current evoked by $30\text{ }\mu\text{M}$ ACh than with the current evoked by $300\text{ }\mu\text{M}$ ACh (Scheffe's test, $P < 0.01$).

Kusano & Gainer (1993) reported that the inhibition by capsaicin of Ca^{2+} channels in F-11 cells is mediated through guanosine 5'-triphosphate (GTP)-binding proteins. A test was made by including 1 mM GDP β S, a non-hydrolysable analogue of GTP, in the intracellular solution to clarify whether GTP-binding proteins are involved in the block by capsaicin of the ACh-activated current. The current activated by $100\text{ }\mu\text{M}$ ACh in the presence of GDP β S was also inhibited by $3\text{ }\mu\text{M}$ capsaicin in four out of four cells tested. We next tested the contribution of protein kinases by involving $1\text{ }\mu\text{M}$ K-

Figure 3 Inhibition by capsaicin of the current evoked by $100\text{ }\mu\text{M}$ acetylcholine (ACh). The cells were held at -60 mV . (a) Reversible inhibition by $3\text{ }\mu\text{M}$ capsaicin of the ACh-activated current. Capsaicin was continuously present 30 s before and during the ACh-application (middle panel). (b) Concentration-dependence of the current inhibition by capsaicin. Current responses were obtained as in (a), and the peak amplitude of the current during the application of capsaicin was normalized to that before the application. Each column represents mean with s.e. from 6 ($1\text{ }\mu\text{M}$ capsaicin) or eight cells ($3\text{ }\mu\text{M}$ capsaicin) tested. Asterisks indicate significant difference from the current before the application of capsaicin when the values before normalization were compared in individual cells (paired t test; $*P < 0.05$).

252a, a non-selective protein kinase inhibitor (Kase *et al.*, 1987), in the intracellular solution. The inhibition by $30\text{ }\mu\text{M}$ capsaicin of the ACh ($100\text{ }\mu\text{M}$)-activated current was not abolished by K-252a. The current remaining in the presence of $3\text{ }\mu\text{M}$ capsaicin with intracellular GDP β S ($48.1 \pm 6.6\%$ of control; mean $\pm$ s.e., $n = 4$) or K-252a ($53.9 \pm 5.6\%$ of control, $n = 5$) was not significantly different from that obtained without these compounds ($38.7 \pm 1.8\%$ of control, $n = 8$) when compared using Scheffe's test ($P > 0.05$). The inability of GDP β S or K-252a to affect the inhibition by capsaicin may not be due to poor accessibility of these compounds to target intracellular molecules because the enhancement of the ATP-activated current by adenosine was blocked by intracellular GDP β S (Inoue *et al.*, 1994), and that by dopamine was blocked by K-252a (Nakazawa *et al.*, 1993) under similar conditions. The results suggest that neither GTP-binding proteins nor protein kinases are involved in the block by capsaicin of the ACh-activated current.

Figure 4 Dependence of the capsaicin-induced inhibition on acetylcholine (ACh) concentration. The cells were held at -60 mV . The cells were pretreated with capsaicin 30 s before the ACh-application. (a,b) Inhibition by $1\text{ }\mu\text{M}$ capsaicin of the current activated by $30\text{ }\mu\text{M}$ (a) or $300\text{ }\mu\text{M}$ ACh (b). The extent of inhibition was larger with the current activated by $30\text{ }\mu\text{M}$ ACh than that by $300\text{ }\mu\text{M}$ ACh. (c) Summarized data for the current inhibition by 1 or $3\text{ }\mu\text{M}$ capsaicin. The ACh-evoked current was measured as in (a) and (b), and the peak current amplitude with capsaicin was normalized to that without capsaicin before the application. Each column represents the mean with s.e. from five to seven cells tested.

We examined whether the inhibition of the ACh-evoked current by capsaicin exhibits voltage-dependence. Figure 5a compares current-voltage relationships for ACh ($100\ \mu\text{M}$)-activated currents in the absence and presence of $3\ \mu\text{M}$ capsaicin, which was determined using a ramp pulse. The current trace before ACh-application was almost linear between -70

Figure 5 Lack of voltage-dependence of the current inhibition by capsaicin. The cells were pretreated with capsaicin 30 s before the acetylcholine (ACh)-application. (a) Change in instantaneous current-voltage relation by $100\ \mu\text{M}$ ACh in the absence (ACh alone) or presence of $3\ \mu\text{M}$ capsaicin (ACh + capsaicin). A ramp pulse of $+30$ to -70 mV ($-100\ \text{mV}\ 200\ \text{ms}^{-1}$) was applied to a cell every 300 ms and the current traces near the peak current were compared with those in the absence of ACh. (b,c) The current inhibition by $1\ \mu\text{M}$ capsaicin of the ACh-activated current at different holding potentials. The cells were continuously held at a potential and exposed to $100\ \mu\text{M}$ ACh. The current responses in a cell held at -40 and -80 mV (b) and summarized data at -40 , -60 and -80 mV (c). Each column in (c) is the mean with s.e. from 5 cells tested.

and $+30$ mV (Figure 5a). ACh added an inward current component at potentials more negative than 0 mV, but did not add an outward component at potentials more positive than 0 mV (Figure 5a), suggesting that the ACh-activated current exhibits strong inward rectification. This voltage-dependence was in accord with that previously obtained by

Figure 6 Effects of capsaicin on ATP ($30\ \mu\text{M}$)-evoked inward current (a,b,c) and dopamine (DA) release (c). (a) The enhancement of the ATP-activated current. The current was recorded from a cell held at -60 mV in the absence (left) and presence of $100\ \mu\text{M}$ capsaicin (right). Capsaicin was present 30 s before and during the ATP-application. (b) Summarized data of enhancement by capsaicin of peak amplitude of the ATP-activated current. The current was recorded as in (a) and normalized to control current. Data were shown as mean $\pm$ s.e. of five cells tested with each concentration of capsaicin. An asterisk indicates significant difference from ATP alone by paired *t* test ($*P < 0.05$). (c) Effect of capsaicin on the ATP-evoked dopamine release. The data were obtained and indicated as in Figure 1. Each column represents mean and s.e. from three dishes in the same batch. Data are representative of two experiments. Asterisks indicate significant difference from ATP alone by Scheffe's test for multiple comparison ($**P < 0.01$).

Ifune & Steinbach (1990) in PC12 cells. Capsaicin did not affect the voltage-dependence of the ACh-activated current in four out of four cells tested: the current activated in the presence of capsaicin also exhibited inward rectification and the inward component in the negative potential range was uniformly suppressed (Figure 5a).

The voltage-dependence of current inhibition was also tested by applying ACh (100 μ M) to cells held at different membrane potentials (Figure 5b,c). The magnitude of the current inhibition by 1 μ M capsaicin was not different among the three potentials (–40, –60 and –80 mV; Figure 5c).

Effects of capsaicin on ATP-evoked responses

Extracellular ATP activates non-selective cation channels in PC12 cells (Nakazawa *et al.*, 1990). We investigated the influence of capsaicin on inward currents activated by 30 μ M ATP. Capsaicin (1 to 10 μ M) did not affect the ATP-activated currents (not shown). A higher concentration of capsaicin (30 and 100 μ M), however, enhanced the ATP-activated current (Figure 6a,b). The peak amplitude of the ATP-activated current was increased by about 30% in the presence of 30 μ M capsaicin (Figure 6b), and by 60% in the presence of 100 μ M capsaicin.

Dopamine release evoked by 30 μ M ATP was also affected by capsaicin. ATP (30 μ M) evoked a dopamine release of $3.00 \pm 0.67\%$ of the total content ($n = 9$). Capsaicin (100 μ M) significantly potentiated the ATP-evoked dopamine release by about 130% (Figure 6c; Scheffé's test, $P < 0.01$). The potentiation was not observed with 30 μ M capsaicin (Figure 6c).

Discussion

We have investigated the effects of capsaicin on responses mediated by nicotinic receptor-channels and on those mediated by ATP-activated channels in PC12 cells. Capsaicin inhibited dopamine release, the increase in intracellular Ca^{2+} concentration and inward current evoked by ACh at concentrations of 1 to 3 μ M, and enhanced dopamine release and inward current evoked by ATP at higher concentrations (30 to 100 μ M). These results are novel findings that indicate ligand-gated channels are targets of capsaicin as well as voltage-gated channels (Petersen *et al.*, 1987; 1989; Kusano & Gainer, 1993). Reduction by capsaicin of ACh-evoked accumulation of Ca^{2+} and Na^+ , which was recently reported in bovine adrenal medullary cells (Toyohira *et al.*, 1992), may also support our findings although the ACh-evoked ion accumulation may have been the result of ion permeation through voltage-gated channels, secondarily activated by depolarization.

The ACh-activated current was inhibited by capsaicin at concentrations as low as 1 μ M (Figure 3). Although this potency is weaker than that reported to open the non-selective cation channels in rat sensory neurones (EC_{50} was about 0.3 μ M; Bevan & Szolcsanyi, 1990), it was considerably stronger than that found to inhibit Na^+ , K^+ , or Ca^{2+} currents in guinea-pig sensory neurones (Petersen *et al.*, 1987; 1989), where capsaicin blocked only a smaller part of the currents at 50 μ M. The inhibition of the ACh-activated current was insensitive to K-252a, suggesting that protein kinases are not involved in the channel inhibition. Direct interaction of GTP-binding proteins with the channels, which has been proposed in other types of channels such as Ca^{2+} channels (Hille, 1992), is also not indicated, judging from the lack of influence by GDP β S. Thus, most likely interpretation of the current inhibition in the present study is an effect resulting from interaction of capsaicin with the channel protein itself or some component tightly coupled with the channels.

The inhibition by capsaicin of the ACh-activated current did not exhibit voltage-dependence (Figure 5), suggesting that

the compound does not affect the properties of nicotinic receptor channels relating to membrane potential such as mechanisms underlying ion-permeation which are affected by positively charged pore blockers of these channels (Ascher *et al.*, 1978; 1979). In contrast, the current inhibition by capsaicin showed a dependence on the concentration of ACh (Figure 4): the inhibition was partially overcome by increasing the concentration of ACh. The dependence on agonist concentration can be explained by reduction in the affinity of the agonist for its binding-site (Ascher *et al.*, 1978; 1979). Capsaicin may inhibit the association of ACh with nicotinic receptors by either competitive antagonism or an allosteric effect. Further investigation is necessary to decide the mode of antagonism by capsaicin of nicotinic receptor channels.

Capsaicin suppressed dopamine release evoked by ACh (Figure 1). Judging from the decreased Ca^{2+} concentration (Figure 2), combined with previous results that demonstrated the dependence of the nicotinic responses on extracellular Ca^{2+} (Ritchie, 1979; Inoue & Kenimer, 1988), the suppression may arise from inhibition of ACh-stimulated Ca^{2+} -influx upon the ACh-stimulation. In PC12 cells, it is generally believed that Ca^{2+} -influx through the voltage-gated Ca^{2+} channels, which are secondarily activated by depolarization, is primarily responsible for the increase in intracellular Ca^{2+} concentration upon nicotinic stimulation (Ritchie, 1979; Inoue & Kenimer, 1988). Although capsaicin suppressed the dopamine release evoked by 80 mM KCl (Figure 1c), inhibition of the Ca^{2+} channel-mediated mechanism may not essentially contribute to the antagonism to the ACh-evoked dopamine release because generation of the depolarization itself is hampered by the block of the nicotinic receptor channels. The smaller magnitude of the suppression of the dopamine release evoked by 80 mM KCl (Figure 1c), compared with that of the ACh-evoked release (Figure 1a), may also exclude major contribution of the Ca^{2+} channel-mediated mechanism.

The ATP-evoked current was enhanced by high concentrations of capsaicin (Figure 6a,b). The enhancement of the current may account for the potentiation by capsaicin of the ATP-evoked dopamine release (Figure 6c) because the dopamine release is directly triggered by Ca^{2+} -permeation through ATP-activated channels in PC12 cells (Nakazawa & Inoue, 1992).

The inflammatory effects of capsaicin appear to result from excitation of a subset of sensory neurones (Bevan & Szolcsanyi, 1990). The blockade of nicotinic receptor channels in the present study may not be directly related to the inflammatory effects because the nicotinic receptor-channels generally serve excitatory neurotransmission, and the blockade of the channels will produce inhibitory effects on neurones. The absence of any effect of resiniferatoxin, which is known to be a highly potent capsaicin-like agonist (Bevan & Szolcsanyi, 1990), suggests that there is not a mechanistic relationship between the excitatory effects of capsaicin on sensory neurones and the observed inhibition of acetylcholine receptor channels. The enhancement by capsaicin of the ATP-activated current may not significantly contribute to the inflammation either because the enhancement required a high concentration (30 μ M) of capsaicin (Figure 6). However, the block by capsaicin of nicotinic receptor channels may promote undesired responses in pharmacological experiments where capsaicin is used as a tool for depletion of neuropeptides from nerve terminals. In other words, capsaicin-sensitive neurogenic response does not necessarily indicate involvement of peptidergic neurotransmission when nicotinic neurotransmission is not negligible. The concentration of capsaicin that maximally activates non-specific cation channels in rat sensory neurones is $> 1 \mu$ M (Bevan & Szolcsanyi, 1990), and this concentration accords with that utilized for the neuropeptide depletion in isolated tissues (e.g. Saito *et al.*, 1987; Ellis & Burnstock, 1989). Saito *et al.* (1987) reported that capsaicin reversibly inhibited part of the neurogenic contraction at 0.1 μ M, but the depletion of the

neurotransmitter responsible for this inhibition (presumably, calcitonin-gene-related peptide) required 1 to 10 μM of capsaicin in smooth muscle of rat vas deferens. Judging from the concentration-dependence of the inhibition of the ACh-activated current (Figure 3b), it is quite likely that the capsaicin-treatment for the neuropeptide depletion simultaneously affects other functions that involve nicotinic responses. It is, therefore, concluded that the possible contribution of a

nicotinic mechanism must be carefully examined when capsaicin is used as a pharmacological tool.

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