Mechanism of activation of nonselective cation channels by putative M₄ muscarinic receptor in guinea-pig chromaffin cells

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1 Mechanisms involved in the generation of nonselective cation currents (I_{NS}) by muscarinic agonists in the chromaffin cell were investigated by the perforated patch method.

2 Bath application of muscarine (0.1–30 μ M) produced an inward I_{NS} with or without a transient outward current at –40 mV, whereas oxotremorine (0.06–60 μ M) induced I_{NS} alone. Rectangular hyperbolas with EC_{50} s of 2.01 and 0.21 μ M were fitted to muscarine- and oxotremorine-induced I_{NS} s, respectively, and the maximal amplitude of the former was about 3.4 times larger than that of the latter.

3 In 36% of the cells exposed to Ca^{2+} -free solution, muscarine I_{NS} was suppressed, being 53% of control 20 min after the perfusion, and in four cells that were incubated with Ca^{2+} -free solution for 2 h or more, the I_{NS} averaged 44% of that induced subsequently in normal solution. In contrast, muscarine I_{NS} was enhanced by about 30% when A-23187 was added to normal solution.

4 W-7 and W-5, calmodulin-related agents, were almost equally potent in inhibiting muscarine I_{NS} , whereas compound 5, a potent inhibitor of calmodulin-dependent kinase II (CaM kinase II), produced no evident inhibition.

5 HA1004, a weak kinase C inhibitor, induced a reversible suppression of muscarine I_{NS} with an IC_{50} of 163 μ M, whereas H-8, another kinase inhibitor, produced an even small degree of inhibition. Administration of phorbol 12, 13-dibutyrate did not mimic muscarinic stimulation of NS channels; rather, it led to a progressive inhibition of I_{NS} and this inhibition was almost complete within 20 min. An inactive phorbol ester had no such effect.

6 The muscarinic antagonists, pirenzepine and AF-DX 116, shifted the dose-response curve for the muscarine I_{NS} to the right in a parallel manner. The K_D s for pirenzepine and AF-DX 116 were estimated to be 13 nM (95% confidence interval, 11–16 nM) and 365 nM (283–470 nM), respectively.

7 These results suggest that muscarine efficiently produces I_{NS} , probably through binding to the M₄ subtype, that intracellular Ca^{2+} has a facilitating, but not an essential role in the generation of I_{NS} , and that neither CaM kinase II nor protein kinase C is involved.

Keywords: M₄ muscarinic receptor; nonselective cation current; muscarine; oxotremorine; PI turnover; intracellular Ca^{2+} ; calmodulin-dependent kinase II; protein kinase C

Introduction

Stimulation of muscarinic receptors with muscarine or oxotremorine induces activation of nonselective cation (NS) channels through a GTP-binding protein in the guinea-pig chromaffin cell (Inoue & Kuriyama, 1991; Inoue & Yoshii, 1992). This channel has a substantial permeability to Ca^{2+} and the current-voltage relationship exhibits a negative slope below –20 to –30 mV. NS channels with similar properties have also been found in smooth muscle cells in the rabbit jejunum (Benham *et al.*, 1985) and in the guinea-pig ileum (Inoue & Isenberg, 1990a,b). In a previous study (Inoue & Imanaga, 1993), we obtained evidence that phosphorylation may be responsible for activation of the channel. The general kinase inhibitor, H-7 (Hidaka *et al.*, 1984) reversibly suppressed the muscarine-induced nonselective cation current (I_{NS}), as did the calmodulin antagonists, trifluoperazine (Levin & Weiss, 1977) and calmidazolium (Belle, 1981). Muscarine, but not oxotremorine, markedly enhanced phosphatidylinositol (PI) turnover in bovine adrenal chromaffin cells as well as in nerve ending preparations from the rat and guinea-pig cerebral cortex (Fisher *et al.*, 1983). This enhancement of PI turnover may result in generation of the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DG) (Nishizuka, 1992). Thus, IP_3 might mediate the production of I_{NS} through mobilizing intracellular Ca^{2+} (Berridge, 1993) and subsequently stimulating calmodulin-dependent kinase II (CaM kinase II) (Shenolikar, 1988). Alternatively, DG might generate I_{NS} through activating protein kinase C

(PKC) (Nishizuka, 1992). The primary objective of our experiment was to address the question of whether CaM kinase II or PKC is responsible for the generation of I_{NS} . For this purpose, we compared the efficacy of muscarine and oxotremorine in inducing I_{NS} and examined effects of calmodulin- and PKC-related agents. We also attempted to elucidate the muscarinic receptor subtype involved in activation of NS channels. mRNA extracted from the bovine adrenal medulla exclusively hybridized with a M₄ cDNA probe, suggesting the presence of M₄ receptors in adrenal chromaffin cells. Consistent with this notion, (Fernando *et al.*, 1991) molecular biological techniques have shown that the muscarinic receptor subtype in the rat adrenal pheochromocytoma cell line, PC 12, were M₄ (Ashkenazi *et al.*, 1988). Our previous study with muscarinic antagonists and pertussis toxin treatment (Inoue & Kuriyama, 1991) also suggest a possible involvement of the M₄ receptor in the generation of I_{NS} . However, the estimated K_D s for antagonists were not completely compatible with this notion. Thus, this issue was re-examined, using the perforated patch method. With this technique, stable I_{NS} was elicited repetitively by muscarinic stimulation, and reliable K_D s were obtained.

Methods

Preparation

Female guinea-pigs (240–350 g) were killed by a blow to the neck and the adrenal glands were immediately put into ice-

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cooled Ca^{2+} -free balanced salt solution. Adrenal medullae were dissected from the adrenal cortex under a dissecting microscope, then were cut into three to six pieces and incubated for 30 min with 0.25% collagenase dissolved in a nominally Ca^{2+} -free solution. During the initial 10 min of this enzyme treatment, the preparation was gently agitated by bubbling with 99.9% O_2 to facilitate digestion. After the incubation, the tissues were washed three or four times in Ca^{2+} -free solution and gently dissociated with a fire-polished Pasteur pipette. The isolated cells were kept in Ca^{2+} -free solution at room temperature ($24 \pm 1^\circ\text{C}$) for 3–10 h.

Current recording

The whole-cell current was recorded using the perforated patch method (Horn & Marty, 1988). Dispersed chromaffin cells were left in the bath for a few minutes to facilitate attachment to the bottom of the bath, before perfusion with a external solution at a rate of about 1 ml min^{-1} . Currents were recorded with an EPC-7 (List, Germany) patch clamp amplifier. Currents were fed into a pen recorder after filtering at 3 or 5 Hz and into a video cassette recorder after digitizing with an analog-to-digital converter. Series resistance in whole-cell recording was $26.7 \pm 5.8 \text{ M}\Omega$ (mean $\pm$ s.e.mean, $n = 6$).

Solutions

The normal external solution contained (in mM): NaCl 137, KCl 5.4, CaCl_2 1.8, MgCl_2 0.5, NaH_2PO_4 0.53, D-glucose 5, HEPES 5 and NaOH 4. The pH was adjusted to 7.4. In Ca^{2+} -free experiments, Ca^{2+} was replaced with 3.6 mM Mg^{2+} . The internal (pipette) solution contained (in mM): K isethionate 120, KCl 20, NaCl 10, HEPES 10 and KOH 2.6. The pH was 7.2. On the day of the experiment, fresh nystatin was dissolved in dimethyl sulfoxide and was added to the internal solution, at a final concentration of $100 \mu\text{g ml}^{-1}$. This nystatin solution was subjected to vortex-mixing for 5 min. The liquid junction potential between the external solution and nystatin solution was about 3 mV and was not corrected for membrane potential measurements. All the experiments were done at a holding potential of -40 mV and at $24 \pm 1^\circ\text{C}$. Chemicals were added to the perfusate. The results are expressed as means $\pm$ s.e.mean and Student's *t* test was used to determine statistical significance.

Analysis of dose-response curves

The sigmoid curve $I = I_{\text{max}} [A]^n / ([A]^n + K_A^n)$ was fitted by computer using a non-linear least-squares method to the peak current (*I*) induced by the agonist (*A*): I_{max} is a maximum value of *I*, [*A*] is the concentration of agonist, K_A is a

constant equal to the EC_{50} or IC_{50} and *n* is a Hill coefficient. The antagonists, pirenzepine and AF-DX 116, were assumed to act competitively; their K_D s were estimated by Schild analyses (Arunlakshana & Schild, 1959).

Chemicals

Sources of chemicals were as follows: ($\pm$)-muscarine chloride, oxotremorine sesquifumarate, phorbol 12, 13-dibutyrate, 4α -phorbol 12, 13-didecanoate, A-23187, and nystatin (Sigma, U.S.A.); HA1004 [N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride] and H-8[N-(2-(methylamino)ethyl)-5-isoquinolinesulfonamide dihydrochloride] (Seikagaku, Japan); pirenzepine dihydrochloride and AF-DX 116 (11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one) (Boehringer Ingelheim, Germany); W-7[N-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide hydrochloride] (Biomol Research, U.S.A.); W-5 [N-(6-aminoethyl)-1-naphthalenesulfonamide hydrochloride] (Molecular Probes, U.S.A.); compound 5 [N-(2', 5'-dihydroxybenzyl)-3-amino-salicylic acid] (LC services, U.S.A.); collagenase (Yakult, Japan). The phorbol esters were dissolved in dimethyl sulfoxide in such a way that the final concentration of the solvent was always less than 0.1%.

Results

Differences in response to muscarine and oxotremorine

Figure 1 shows current responses to muscarine and oxotremorine at a holding potential of -40 mV in the same chromaffin cell. The addition of muscarine at concentrations equal to, or greater than $3 \mu\text{M}$ to the perfusate induced a transient outward current (I_O) with concomitant generation of I_{NS} . The presence of oxotremorine at concentrations up to $60 \mu\text{M}$ never produced I_O but did produce I_{NS} consistently ($n = 9$). In one series of experiments, transient I_O was produced in zero, 50, and 83% of six cells by 1, 3, and $10 \mu\text{M}$ muscarine, respectively and the amplitude of I_O increased according to the concentration. In a previous study (Inoue & Kuriyama, 1990), we suggested that this transient I_O is due to Ca^{2+} mobilized from intracellular stores.

Consistent with this notion, a second application of muscarine in a nominally Ca^{2+} -free solution failed to induce I_O and prior exposure to 5 mM caffeine under Ca^{2+} -free conditions led to a marked reduction of I_O in response to muscarine (not shown). Thus, it is likely that muscarine, but not oxotremorine, can facilitate PI turnover with the subsequent generation of I_O .

The putative difference between muscarine and oxotre-

Figure 1 Differences in current response of a guinea-pig chromaffin cell to muscarine and oxotremorine. Muscarine (Musc) and oxotremorine (Oxo) each at 1, 3 and $10 \mu\text{M}$ were added to the perfusate during the periods indicated by horizontal lines. Drug application was interrupted for 2 to 3 min between records. Holding potential, -40 mV . Upper and lower panels, from the same cell.

morine in inducing PI hydrolysis might relate to that in generating I_{NS} . As the concentration of muscarine was increased from 1 μM , the generation of I_{NS} was markedly enhanced with the concomitant production of I_O ; in contrast 3 μM oxotremorine produced a maximal activation of I_{NS} (Figure 1). Thus, we investigated quantitatively the potency and efficacy of muscarine and oxotremorine in the cells where high concentrations of muscarine induced no apparent I_O (potency and efficacy are used differently here: the former refers to dose-dependent properties and the latter refers to magnitudes of response e.g., Hoyer & Boddeke, 1993). For a comparison among different cells, amplitudes of I_{NS} were expressed relative to that produced by 1 μM muscarine. Figure 2a illustrates I_{NS} in response to various concentrations of muscarine and 1 μM oxotremorine. The amplitude of 1 μM oxotremorine-induced I_{NS} was about 70% ($71 \pm 4\%$, $n = 6$) of that seen with 1 μM muscarine. Oxotremorine-induced I_{NS} (∇ in Figure 2b) were well fitted by a rectangular hyperbola with a K_A of 0.21 μM and an I_{max} of 0.88. These values of the parameters are in fairly good accord with those obtained previously using a conventional patch clamp technique. The muscarine-induced currents were fitted by a rectangular hyperbola with a K_A of 2.01 μM and an I_{max} of 3.00 ($\circ$ in Figure 2b).

Effect of Ca^{2+} removal on I_{NS}

In the ileal smooth muscle, ACh failed to produce a depolarization due to I_{NS} in the absence of Ca^{2+} (Inoue & Isenberg, 1990b), whereas in the chromaffin cell, oxotremorine induced the current in a nominally Ca^{2+} -free solution (Inoue & Kuriyama, 1991). To investigate whether the difference of I_{NS} between the chromaffin cell and the smooth muscle cell can be ascribed to genuine properties of NC channels in the former, chromaffin cells were exposed for 20 min to Ca^{2+} -free solution in the presence or absence of 0.5 mM EGTA. When

perfused with a nominally Ca^{2+} -free solution, amplitudes of I_{NS} in response to 1 or 3 μM muscarine decreased successively in two of eight cells tested. Figure 3a illustrates such a decrease in I_{NS} in one of these two cells. The amplitude of the current was reduced by 37% and 57% 2 min and 10 min after start of the perfusion. This decrease of I_{NS} was completely reversed at 4 min, but not at 1 min, after switching back to normal solution. The addition of 0.5 mM EGTA to Ca^{2+} -free solution produced a successive decrease of the muscarinic I_{NS} in three of six cells tested. Figure 3b summarizes time courses of I_{NS} in the five cells where reduction was observed in the presence or absence of EGTA ($\bullet$) and in the nine cells ($\blacktriangle$) where noticeable decreases did not occur. This suppression of I_{NS} by Ca^{2+} removal was not influenced by concentrations of muscarine. Figure 4a shows I_{NS} induced by various concentrations of muscarine before and after perfusion with Ca^{2+} -free solution in the same cell. I_{NS} in response to 3 μM muscarine, in this cell, was reduced to 50% of control 10 min after perfusion with a nominally Ca^{2+} -free solution and remained at this level for the following 12 min. Muscarine-induced I_{NS} elicited in the absence of external Ca^{2+} still fell approximately on the dose-response curve for the control I_{NS} , obtained in Ca^{2+} -containing medium (Figure 4b).

The finding that I_{NS} decreased successively under Ca^{2+} -free conditions in 5/14 of the cells tested suggests the presence of two groups of I_{NS} in the chromaffin cell, only one of which is partially dependent on extracellular Ca^{2+} . Alternatively, the difference might be accounted for by the rate of decrease in intracellular $[\text{Ca}^{2+}]$ after Ca^{2+} removal. To explore this possibility, we investigated effects of a long incubation of cells in Ca^{2+} -free solution. After over 2 h incubation in a nominally Ca^{2+} -free solution, cells were exposed to 3 μM muscarine; then, the response to the same concentration of muscarine was determined in the presence of Ca^{2+} . The amplitude of I_{NS} in the absence of external Ca^{2+} was $44 \pm 6\%$ ($n = 4$) of the

Figure 2 Dose-response relationship for muscarine and oxotremorine-induced nonselective cation currents. (a) Nonselective cation currents (I_{NS}) in response to muscarine and oxotremorine: (i) I_{NS} induced by 0.1, 0.3, 0.6, and 1 μM muscarine (Musc) and 1 μM oxotremorine (Oxo); (ii) I_{NS} induced by 1, 3, 6, and 30 μM muscarine and 1 μM oxotremorine; (i) and (ii) from different cells. (b) Muscarine ($\circ$)- and oxotremorine (∇)-induced I_{NS} s were plotted against their concentrations. Amplitudes of both I_{NS} s were expressed relative to that of 1 μM muscarine-induced I_{NS} measured in the same cells. Data represent mean $\pm$ s.e. mean of 3 to 26 and 2 to 8 observations at each concentration of muscarine and oxotremorine, respectively. Rectangular hyperbolas (lines) with K_A of 2.01 μM and I_{max} of 3.00 (r , 0.963) and with K_A of 0.21 μM and I_{max} of 0.88 (r , 0.952) were fitted to muscarine- and oxotremorine-induced I_{NS} s.

Figure 3 Effects of Ca^{2+} removal on muscarine-induced I_{NS} . (a) Muscarine-induced I_{NS} in the presence and absence of external Ca^{2+} : 3 μM muscarine was added to normal or nominally Ca^{2+} -free solution during the periods indicated by horizontal lines. The numbers above lines represent approximate times after perfusion with Ca^{2+} -free or normal solution. (b) Summary of 1 or 3 μM muscarine-induced I_{NS} which was successively decreased ($\bullet$) or was maintained ($\blacktriangle$) in Ca^{2+} -free solution. Amplitudes of I_{NS} elicited in Ca^{2+} -free and after switching back to normal (After) were expressed relative to that before perfusion with Ca^{2+} -free in the same cell and plotted against the time of perfusion; ($\bullet$) and ($\blacktriangle$) represent mean $\pm$ s.e. mean of 5 and 9 cells (see text). Straight line and curve were drawn through ($\bullet$) and ($\blacktriangle$) by eye.

Figure 4 Reduction of muscarine-induced I_{NS} by Ca^{2+} removal did not depend on the concentration. (a) I_{NS} s in response to muscarine (Musc) before (i) and after (ii) removal of external Ca^{2+} : 1 to 10 μM muscarine was added to normal (i) and to Ca^{2+} -free solution (ii) during the indicated period. About 10 min after start of perfusion with Ca^{2+} -free solution, I_{NS} in response to 3 μM was reduced to 50% of I_{NS} elicited in normal solution. (b) I_{NS} obtained after reduction was plotted against concentrations of muscarine. Data represent mean $\pm$ s.e. mean of 2 to 11 observations at each concentration. The curve was the same as that for muscarine-induced I_{NS} in normal Ca^{2+} medium shown in Figure 2.

Figure 5 Potentiation of muscarine-induced I_{NS} by A-23187. (a) Muscarine-induced I_{NS} in the presence and absence of 0.5 μM A-23187: 3 μM muscarine (Musc) was added to normal solution during the periods indicated by horizontal lines in the presence (interrupted line) or absence of A-23187. (b) Summary of muscarine-induced I_{NS} in the presence or absence of A-23187 under normal (open column, $n = 6$) or Ca^{2+} -free conditions (stippled column, $n = 4$). Amplitudes of I_{NS} were expressed relative to that of I_{NS} induced by the first application of muscarine. Before administration of 0.5 or 1 μM A-23187, I_{NS} was generated consecutively three times by application of muscarine. Data are mean $\pm$ s.e. mean. There is no significant change among these three I_{NS} s. *Statistical significance ($P < 0.05$).

response in the presence of normal external Ca^{2+} . This value of I_{NS} did not differ from the $53 \pm 6\%$ ($n = 5$) seen about 20 min after perfusion with Ca^{2+} -free solution. Thus, we conclude that the apparent heterogeneity of I_{NS} with respect to Ca^{2+} removal is due to a difference in the rate of decrease in intracellular $[\text{Ca}^{2+}]$.

Effects of A-23187 on I_{NS}

The foregoing results suggest that the activity of NS channels in the chromaffin cell also depends partially on intracellular Ca^{2+} . To elucidate further this putative Ca^{2+} -dependent property, the effects of A-23187, a Ca^{2+} ionophore, were examined. When 0.5 or 1 μM A-23187 was added to normal solution, outward currents were transiently generated in three of six cells (Figure 5a). After decay of this outward current, the level of holding current reverted to control, with no noticeable generation of an inward current. Subsequently, the application of 3 μM muscarine produced a larger I_{NS} (30% increase). This enhancement of the muscarine-induced I_{NS} was not due to a spontaneous variation of current since I_{NS} of a constant amplitude was generated three consecutive times before the addition of the Ca^{2+} ionophore and the enhancement did not occur in the absence of external Ca^{2+} (Figure 5b). Thus, the enhancing effect of A-23187 is likely to be due to an increase in intracellular $[\text{Ca}^{2+}]$.

Effects of calmodulin-related agents

The results obtained with Ca^{2+} removal and A-23187 indicate that Ca^{2+} mobilization is not a prerequisite for the generation of I_{NS} ; however, this observation does not exclude the involvement of CaM kinase II. We then investigated the possible involvement of this kinase using the inhibitors W-5 and W-7. If calmodulin was present in the signal pathway,

Figure 6 Dose-response curves for W-7 and W-5-induced inhibition of I_{NS} . (a) and (b) Dose-dependent inhibition by W-7 and W-5, respectively. Upper, actual traces of 1 μM muscarine-induced I_{NS} in the presence and absence of W-7 or W-5. Muscarine was added to normal solution during the period indicated by a horizontal line, and 1 and 10 μM W-7 or W-5 were further added to muscarine-containing perfusate during the indicated period. Lower, amplitudes of I_{NS} in the presence of W-7 or W-5 were expressed relative to that in the absence. Rectangular hyperbolas with IC_{50} s of 7.6 ($r = 0.970$) and 4.6 μM ($r = 0.964$) were fitted to the inhibitions by W-7 and W-5, respectively. The same symbols in each figure represent the same cells.

Figure 7 Effects of HA1004 and H-8 on muscarine-induced I_{NS} . (a) HA1004 (i) or H-8 (ii) induced reversible inhibition of 3 μM muscarine-induced I_{NS} in a dose-dependent manner; 100, 150, or 300 μM HA1004 or H-8 was added to muscarine-containing solution (double line) during the indicated period. (b) Relative amplitudes of muscarine I_{NS} in the presence of HA1004 ($\bullet$) or H-8 ($\blacktriangle$) were plotted against concentrations. I_{NS} in the presence of inhibitor was expressed relative to that in the absence. Sigmoid curve with I_{max} of 1, K_A of 163 μM , and Hill coefficient of -1.5 ($r = 0.945$) was fitted to data for HA1004 inhibition (see Methods). The curve for H-8 inhibition was drawn by eye. Data for HA1004 and H-8 represent mean $\pm$ s.e. mean of 3 to 7 observations at each concentration and of 5 to 6, respectively.

W-7 should induce a more potent inhibition than W-5 (Hidaka *et al.*, 1981). When W-5 or W-7 was added to normal solution, the holding current level at -40 mV was not significantly altered. On the other hand, the addition of either agent to 1 μM muscarine-containing perfusate led to the inhibition of I_{NS} in a dose-dependent manner (upper part of Figure 6). This inhibition did not require pretreatment, and withdrawal of W-5 or W-7 readily restored I_{NS} to the control level. The lower panels of Figure 6 represent summaries of the dose-dependent inhibition. Rectangular hyperbolas with IC_{50} s of 4.6 and 7.6 μM fit well to data points for W-5 and W-7, respectively. Next, we investigated effects of compound 5 since this agent has been reported to be a potent inhibitor ($\text{IC}_{50} = 0.2$ μM) of CaM kinase II (O'Dell *et al.*, 1991). Simultaneous application of 10 μM compound 5 and 1 μM muscarine for at least 10 min induced no change in I_{NS} . The amplitude of muscarine-induced I_{NS} was $102.3 \pm 2.7\%$ ($n = 3$) of I_{NS} prior to the application.

Effects of protein kinase C-related agents

Another possible mediator of the muscarinic signal is PKC. The reversible suppression of I_{NS} by H-7, a general kinase inhibitor, is compatible with the involvement of this kinase, although the IC_{50} for the inhibition was about 50 times larger than a K_i against PKC (Inoue & Imanaga, 1993). Thus we investigated effects of HA1004 and H-8 on 3 μM muscarine-induced I_{NS} since these agents are 6.7 and 2.5 times less potent than H-7, respectively (Hidaka *et al.*, 1984). When HA1004 or H-8 was added to normal solution, the holding current level of -40 mV was not significantly altered. On the other hand, the addition of either agent to the 3 μM muscarine-containing perfusate resulted in the dose-dependent inhibition of I_{NS} . Figure 7a shows that suppression by the inhibitors did not require pretreatment and termination of the application readily restored the current to the control level. With the addition of 300 μM HA1004 or H-8, I_{NS} was

reduced to $25 \pm 3\%$ ($n = 8$) or $72 \pm 5\%$ ($n = 5$) of control, values smaller or larger than that ($46 \pm 3\%$) in the presence of H-7, respectively. Figure 7b summarizes the dose-dependent inhibitions. Data points for HA1004 fell on a sigmoid curve with a Hill coefficient of -1.5 and an IC_{50} of $163 \mu\text{M}$. This value of IC_{50} was smaller than that ($254 \mu\text{M}$) for the H-7 inhibition. These results suggest that PKC may not be involved in production of the current.

The effects of phorbol esters on muscarine I_{NS} were then examined (Figure 8). When $1 \mu\text{M}$ phorbol 12, 13-dibutyrate was added to the perfusate, the holding current level was not noticeably altered. On the contrary, I_{NS} in response to $3 \mu\text{M}$ muscarine was successively diminished during the application (●). About 5 min after start of the treatment, I_{NS} induced by muscarine (added to normal solution without the phorbol ester) was suppressed by $67 \pm 14\%$ ($n = 4$) and 20 min later the response disappeared almost completely. On the other hand, 4α -phorbol 12, 13-didecanoate, inactive in stimulating PKC (Castagna *et al.*, 1982), was without effect (▲). When one cell was subjected to treatment with the inactive phorbol ester and then with the active phorbol ester for 20 min each, I_{NS} was completely suppressed. Thus, the inhibition by phorbol 12, 13-dibutyrate is probably linked to the activation of PKC.

Properties of the receptor responsible for muscarine I_{NS}

Previous K_{D} s for pirenzepine and AF-DX 116 found in oxotremorine-induced I_{NS} may have been distorted by run-down of the current under conditions of conventional whole-cell recording, even though care had been taken to lessen the distortion. In the present experiment, most of this kind of error could be avoided by use of the nystatin method. Figure 9a shows I_{NS} s induced by various concentrations of muscarine in the presence and absence of 60 nM pirenzepine. As the full development of inhibition required at least 5 min of pretreatment, the effect of pirenzepine was investigated five min after application. This inhibition was reversed readily by washout of the antagonist. It is evident that the pirenzepine shifts the dose-response curve for muscarine-induced I_{NS} to the right by sixfold (Figure 9b). A similar comparison of the

dose-response curve in presence and absence of 10 , 30 , 60 , or 100 nM pirenzepine was done in the same cells, and the shift of dose-response curve was measured with respect to the amplitude of control I_{NS} induced by $1 \mu\text{M}$ muscarine. Schild analysis of these shifts gave an apparent K_{D} of 13 nM (95% confidence interval, 11 to 16 nM) for pirenzepine (Figure 9c).

In contrast to pirenzepine, the inhibition by AF-DX 116 developed fully with pretreatment for 2 min. This inhibition was also readily reversed after washout. Figure 10a shows I_{NS} s induced by various concentrations of muscarine in the presence and absence of $3 \mu\text{M}$ AF-DX 116. This inhibitor at $3 \mu\text{M}$ shifted the dose-response curve to the right by 8.8fold (Figure 10b). Schild analysis of the shifts in the presence of 0.3 , 1 , and $3 \mu\text{M}$ AF-DX 116 gave an apparent K_{D} of 365 nM (95% confidence interval, 283 to 470 nM) for AF-DX 116 (Figure 10c).

Discussion

Bath application of muscarine, but not oxotremorine, induced a transient I_{O} , probably due to activation of Ca^{2+} -dependent K^{+} channels (Inoue & Kuriyama, 1990). This current was not induced by a second application of muscarine and was markedly diminished by prior exposure to caffeine under Ca^{2+} -free conditions. These results suggest that transient I_{O} is due to Ca^{2+} mobilized from intracellular store sites, which are at least in part caffeine-sensitive. Thus, muscarine is probably capable of stimulating PI hydrolysis in some chromaffin cells. This notion is consistent with the biochemical results that muscarine, but not oxotremorine, conspicuously enhanced PI hydrolysis in nerve ending preparations of the rat and guinea-pig cerebral cortex and in bovine chromaffin cells (Fisher *et al.*, 1983). However, the stimulation of PI turnover by muscarine may not account for its high efficiency in generating I_{NS} since neither the IP_3 - Ca^{2+} -CaM Kinase II nor the DG-PKC pathway was involved in muscarinic stimulation of NS channels. The reasons for this conclusion are given below.

Figure 8 Inhibition of muscarine-induced I_{NS} by phorbol 12, 13-dibutyrate. (a) $3 \mu\text{M}$ muscarine induced I_{NS} before (Con) and 6, 15, and 20 min after application of $1 \mu\text{M}$ phorbol 12, 13-dibutyrate. Muscarine was added to normal solution, without the phorbol ester, for the indicated period. (b) Summary of $3 \mu\text{M}$ muscarine induced I_{NS} before and after application of $1 \mu\text{M}$ phorbol 12, 13-dibutyrate (●) or $1 \mu\text{M}$ 4α -phorbol 12, 13-didecanoate (▲). Data are mean $\pm$ s.e. mean of 4 and 3 cells for the former and the latter, respectively.

Figure 9 Antagonism of muscarine-induced I_{NS} with pirenzepine. (a) Muscarine-induced I_{NS} in the absence (i) and presence (ii) of 60 nM pirenzepine. In (i) 0.6, 1, 3, or 10 μM muscarine was added to normal solution during the indicated period; in (ii) 3, 6, or 10 μM was applied with pirenzepine, 5 min after pretreatment (see text). (b) Dose-response curves for muscarine I_{NS} in the absence (●) and presence (▲) of 60 nM pirenzepine. The amplitude of I_{NS} was plotted against concentrations of muscarine, and curves were drawn through data points by eye. Arrow indicates 6-fold shift of the dose-response curve. (a) and (b) the same cell. (c) Schild plot. Concentrations of muscarine responsible for the amplitude of 1 μM muscarine-induced I_{NS} were measured in the presence of 10, 30, 60, and 100 nM, as shown in (b). The ratios of these concentrations to 1 μM (control) were represented by R . Logarithms of $R-1$ were plotted against $-\log$ of pirenzepine concentrations. $y = 7.88 - x$ was fitted to data points with a least-squares method (r , 0.935). K_D for pirenzepine is obtained with $y = 0$ and is 13 nM (95% confidence interval, 11 to 16 nM).

Figure 10 Antagonism of muscarine-induced I_{NS} with AF-DX 116. (a) Muscarine-induced I_{NS} in the absence (i) and presence (ii) of 3 μM AF-DX 116. In (i) 0.3, 0.6, 1 or 3 μM muscarine was added to normal solution during the indicated period; in (ii) 1, 3, 6, or 10 μM was applied with the antagonist, 2 min after pretreatment (see text). (b) Dose-response curves for muscarine I_{NS} in the absence (●) and presence (▲) of 3 μM AF-DX 116. The amplitude of I_{NS} was plotted against muscarine concentrations. Arrow indicates 8.8-fold shift of the dose-response curve. (c) Schild plot. Concentrations of muscarine responsible for the amplitude of 1 μM muscarine-induced I_{NS} were measured in the presence of 0.3, 1 and 3 μM AF-DX 116. R has the same meaning as for Figure 9. Logarithms of $R-1$ were plotted against $-\log$ of AF-DX 116 concentrations. $y = 6.44 - x$ was fitted to data points with a least-squares method (r , 0.873). K_D for AF-DX 116 is obtained with $y = 0$ and is 365 nM (95% confidence interval, 283 to 470 nM).

An increase in intracellular $[Ca^{2+}]$ was not a prerequisite for the generation of I_{NS} . Under Ca^{2+} -free conditions, a second application of muscarine always failed to evoke a transient I_O , probably due to the depletion of intracellular Ca^{2+} . In contrast, I_{NS} was suppressed only in about one third of cells exposed to Ca^{2+} -free solution. Thus, this successive decrease in I_{NS} in some cells may not be attributed to a diminished mobilization of intracellular Ca^{2+} , but rather to a slow decrease in the resting $[Ca^{2+}]$. In support of this notion, the amplitude of the I_{NS} measured in the cells incubated in Ca^{2+} -free solution for more than 2 h was 44% of the current elicited subsequently in normal solution. Furthermore, the muscarine-induced I_{NS} was enhanced by application of the Ca^{2+} ionophore A-23187, but only in the presence of external Ca^{2+} . Thus, intracellular Ca^{2+} apparently plays a facilitating but not an essential role in generating I_{NS} and CaM kinase II probably does not activate NS channels. This idea was further supported by failure of the CaM kinase II inhibitors KN-62 (Inoue & Imanaga, 1993) and compound 5 to suppress the muscarine-induced I_{NS} . In addition, W-5 and W-7 were found to be equally potent as inhibitors of the response. If calmodulin is involved in the transduction process, W-7 should be more potent than W-5. Other calmodulin-dependent processes may not be involved.

The Ca^{2+} dependent PKCs, which are classified as classical PKCs (cPKC) (Nishizuka, 1992), are also unlikely to mediate the generation of I_{NS} . The rank order of isoquinolinesulfonamides in inhibiting I_{NS} was HA1004 > H-7 > H-8, being different from that (H-7 > H-8 > HA1004) in suppressing cPKCs (Hidaka *et al.*, 1984). Furthermore, bath application of phorbol 12, 13-dibutyrate did not mimic muscarinic stimulation of the current, rather it resulted in a successive suppression of I_{NS} . The time course of this suppression was roughly compatible with that of internalization of muscarinic receptors induced by phorbol ester in NIE-115 neuroblastoma cells (Liles *et al.*, 1986), where the predominant subtype is of the M_4 (Ashkenazi *et al.*, 1989). In addition, the failure of phorbol ester to produce I_{NS} excludes a possible involvement of another group of PKCs termed new PKC, which is insensitive to Ca^{2+} but is activated by phosphatidylserine and DG or phorbol esters. Whether the other group of PKCs termed atypical PKC is responsible for generation of I_{NS} remains an open question. So-called calmodulin antagonists, such as calmidazolium or W-5, were potent in inhibiting the muscarinic I_{NS} . These antagonists are known to inhibit not only calmodulin, but also PKCs, probably through binding a

hydrophobic site of the enzymes (Hidaka & Hagiwara, 1987).

The dose-response curves for muscarine- and oxotremorine-induced I_{NS} s (Figure 2) show that muscarine is more efficient but less potent than oxotremorine. This difference between the two might be accounted for by the muscarinic subtypes involved. The results with pirenzepine and AF-DX 116, however, suggest that the same subtype is responsible for both currents. The K_D value of the muscarine-stimulated receptor for pirenzepine was estimated to be 13 nM, being about one fourth of that (50 nM) in case of oxotremorine stimulation (Inoue & Kuriyama, 1991). In light of the present findings, this difference may relate to an insufficient development of inhibition in the previous experiment, where cells were pretreated with the antagonist for only 2 min. On the contrary, the K_D value for AF-DX 116 was 365 nM, being 5.2 times larger than that (70 nM) found for the oxotremorine-stimulated receptor. This previous value is extremely small and indeed is smaller than that for the M_2 expressed in mammalian cell lines (Bonner *et al.*, 1988; Buckley *et al.*, 1989) or oocytes (Fukuda *et al.*, 1987), the subtype which has the highest affinity for the antagonist among the five subtypes (Bonner, 1989). Thus, the previous value might have been erroneously estimated, possibly because of run-down of I_{NS} under conventional whole-cell conditions. The K_D s of muscarine-stimulated receptors for pirenzepine and AF-DX 116 agree with those of the M_4 endogenously present in NG 108-15 cells (Michel *et al.*, 1989) and that expressed in Chinese hamster ovary cells (CHO-K1) (Buckley *et al.*, 1989; Dörje *et al.*, 1991). Although these K_D s alone would not exclude a possible involvement of M_1 , the presence of only $m4$ transcript in the bovine adrenal medulla (Fernando *et al.*, 1991) and the rat pheochromocytoma PC 12 cell line (Ashkenazi *et al.*, 1988) suggests that the M_4 receptor is involved in the generation of I_{NS} induced by muscarine and probably by oxotremorine.

We propose that muscarine efficiently stimulates the M_4 muscarinic receptor subtype to produce I_{NS} and that the second messengers resulting from PI hydrolysis, Ca^{2+} and PKC, do not transduce this response.

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