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Phosphatidylinositol hydrolysis is involved in production of Ca^{2+}-dependent currents, but not non-selective cation currents, by muscarine in chromaffin cells

Masumi Inoue *, Yasuzi Sakamoto, Issei Imanaga

Department of Physiology, School of Medicine, Fukuoka University, Fukuoka 814-01, Japan

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Abstract

Whether phosphatidylinositol hydrolysis and a subsequent Ca^{2+} mobilization are responsible for muscarine-induced transient outward currents (I_O) and non-selective cation currents (I_{NS}) in the guinea-pig chromaffin cell was investigated using the perforated patch method. I_O, but not I_{NS}, failed to be reproduced in Ca^{2+}-free solution and was markedly reduced by prior exposure to caffeine under Ca^{2+}-free conditions or by addition to normal solution of cyclopiazonic acid (CPA), a Ca^{2+} ATPase inhibitor. Application of CPA in Ca^{2+}-free solution, however, suppressed I_{NS} by about 50% in 73% of the cells tested. Bath application of 1.5 mM neomycin, a phospholipase C inhibitor, induced the time-dependent decline of I_O with near abolition at 20 min or less, whereas it produced a time-independent decrease of I_{NS} and an inwardly rectifying K^+ current. I_{NS} in the presence or absence of neomycin was well fitted to rectangular hyperbolas with the same ED_{50} of 2.17 μM, but with a 33% smaller maximum amplitude in the former, indicating a non-competitive inhibition by neomycin. We conclude that, while phosphatidylinositol hydrolysis mediates the production of I_O, it does not mediate that of I_{NS} by muscarine.

Keywords: Chromaffin cell; Muscarinic receptor; Phosphatidylinositol hydrolysis; Ca^{2+}, intracellular; Cation channel, non-selective; (Guinea-pig)

1. Introduction

Activation of muscarinic receptors in guinea-pig adrenal chromaffin cells induces outward and inward currents, at negative membrane potentials. The former is in major part due to Ca^{2+}-dependent K^+ currents ($I_{K(Ca)}$), whereas the latter is due to a non-selective cation current (I_{NS}) (Inoue and Kuriyama, 1990, Inoue and Kuriyama, 1991). Muscarinic receptor agonists such as muscarine, but not oxotremorine, markedly enhance phosphatidylinositol turnover in the bovine adrenal medulla as well as in nerve ending preparations from the rat and guinea-pig cerebral cortex (Fisher et al., 1983). The enhancement of phosphatidylinositol hydrolysis may trigger either inositol 1,4,5-trisphosphate (IP_3)-Ca^{2+} mobilization or diacylglycerol-protein kinase C cascade, or both (Nishizuka, 1992). This finding raises the possibility that phosphatidylinositol turnover is involved in generation of $I_{K(Ca)}$ and I_{NS}, as was suggested for inhibition of the M current and production of $I_{K(Ca)}$ by bradykinin in NG 108-15 cells (Higashida and Brown, 1986; Brown and Higashida, 1988). This hypothesis may apply to generation of $I_{K(Ca)}$, as muscarine, but not oxotremorine, induced the outward current in a concentration-dependent manner (Inoue and Imanaga, 1995), but this might not be the case for the production of I_{NS}. The muscarine-induced I_{NS} was reversibly suppressed by the general kinase inhibitor, H-7, and the calmodulin inhibitors calmidazolium and trifluoperazine, and a phosphorylation process was suggested to be responsible for the activation (Inoue and Imanaga, 1993a). However, bath application of the protein kinase C activator, phorbol 12,13-dibutyrate, did not mimic muscarinic stimulation (Inoue and Kuriyama, 1991). These results suggest that calmodulin-dependent kinase II (calmodulin kinase II), a putative mediator for an intracellular Ca^{2+} signal (Cohen, 1992), mediates the production of I_{NS}. In a

* Corresponding author. Fax 81-92-865-6032.

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nominally Ca^{2+}-free solution, however, muscarine consistently elicited I_{NS} without any decrease in 75% of the cells tested, whereas in the remaining 25%, I_{NS} was reduced by about 50% within 20 min (Inoue and Imanaga, 1995). This apparent contradiction might be explained by the highly efficient Ca^{2+} uptake into store sites in the chromaffin cell. Similar recycling of intracellular Ca^{2+} was noted in guinea-pig portal vein smooth muscle (Bond et al., 1984). The objective of the present experiments was to investigate the role of phosphatidylinositol hydrolysis and subsequent mobilization of intracellular Ca^{2+} in the generation of I_{NS} in the adrenal chromaffin cell. For this purpose, we studied the effects of cyclopiazonic acid (CPA), an endoplasmic reticulum Ca^{2+} ATPase inhibitor (Seidler et al., 1989), and neomycin, a phospholipase C inhibitor ((Schacht, 1976; Lipsky and Lietman, 1982).

2. Materials and methods

2.1. Preparation

Female guinea-pigs (240–350 g) were killed by a blow to the neck and the adrenal glands were immediately put into ice-cooled Ca^{2+}-free balanced salt solution. Adrenal medullas 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 3 or 4 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 (23–25°C) for 3–10 h.

2.2. Current recording

The whole-cell current was recorded using the perforated patch method (Horn and 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 external solution at a rate of about 1 ml/min. 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. The series resistance in whole-cell recording was about 25 MΩ.

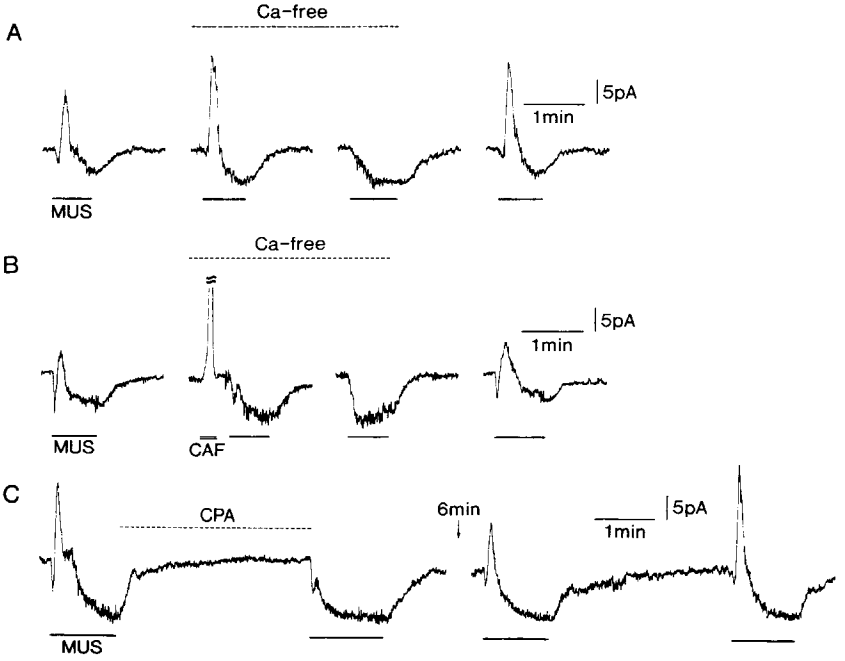

Fig. 1. Role for Ca^{2+} mobilization in muscarine-induced outward and inward currents. Holding potential, −40 mV. (A) Second application of muscarine (MUS) failed to induce an outward current (I_O), but did elicit an inward non-selective cation current (I_{NS}) in the absence of external Ca^{2+} (interrupted line). 6 μM muscarine was added to normal or nominally Ca^{2+}-free solution during the periods indicated by horizontal lines. Each trace was interrupted for 2–3 min. (B) Prior exposure to caffeine (CAF) produced a marked diminution of I_O, but not I_{NS} in response to 6 μM muscarine in the absence of external Ca^{2+} (interrupted line). 5 mM caffeine was applied in Ca^{2+}-free solution during the period (double line). The outward current induced by caffeine is not fully displayed due to saturation of the recorder. Each trace was interrupted for 2–3 min. (C) Addition of 3 μM cyclopiazonic acid (CPA) almost completely abolished I_O in response to subsequent application of 6 μM muscarine. Muscarine or cyclopiazonic acid was added to normal solution during the periods indicated by horizontal or interrupted line, respectively. The traces were interrupted for 6 min. Note that in normal solution, an interval of 3 min was sufficient for reproduction of I_O.

2.3. Solutions

The normal external solution contained (in mM): 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.53 NaH_2PO_4, 5 D-glucose, 5 Hepes and 4 NaOH. 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): 120 K isethionate, 20 KCl, 10 NaCl, 10 Hepes and 2.6 KOH. 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 μg/ml. This nystatin solution was subjected to vortex mixing for 5 min. The liquid junction potential between the external solution and the nystatin solution was about 3 mV and was not corrected for membrane potential measurements. Unless otherwise noted, experiments were done at a holding potential of −40 mV and at 23–25°C. Chemicals were added to the perfusate. The results are expressed as means ± S.D. where not specified, and Student's t-test was used to determine statistical significance.

2.4. Analysis of dose-response curves

The rectangular hyperbola $I = I_{MAX}\,[A]/([A] + K_A)$ was fitted by computer using a non-linear least-squares method to the peak current (I) induced by muscarine: I_{MAX} is a maximum value of I, [A] is the concentration of muscarine, and K_A is a constant equal to the EC_{50}. The resulting dose-response curve was evaluated using the correlation coefficient (r).

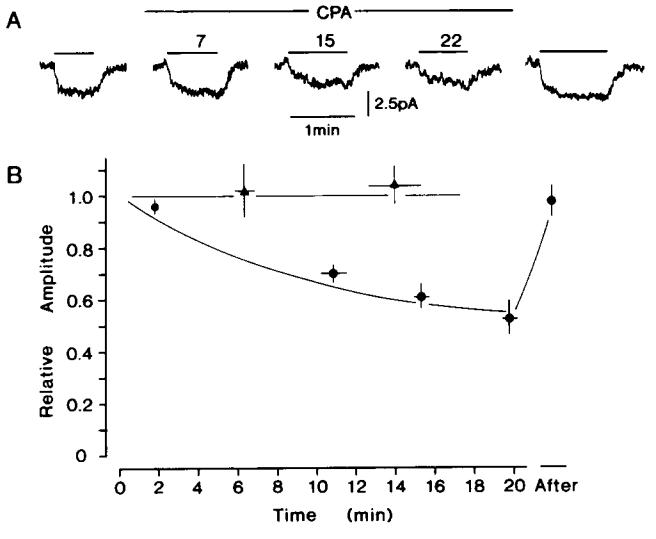

Fig. 2. Effect of cyclopiazonic acid on muscarine-induced I_{NS} in the absence of external Ca^{2+}. (A) Muscarine-induced I_{NS} in normal solution and nominally Ca^{2+}-free solution containing 3 μM cyclopiazonic acid (CPA). The cell was continuously perfused with the CPA solution during the indicated period. 1 μM muscarine was added to normal or CPA solution during the periods indicated by the horizontal line. The numbers above lines represent approximate times of perfusion with CPA solution. (B) Summary of 1 or 3 μM muscarine-induced I_{NS} which was successively decreased (●) or was maintained (▲) in CPA solution. Amplitudes of I_{NS} were expressed relative to that before perfusion with CPA solution in the same cell and plotted against the time of perfusion. ● and ▲ represent means ± S.E.M. for eight and three cells.

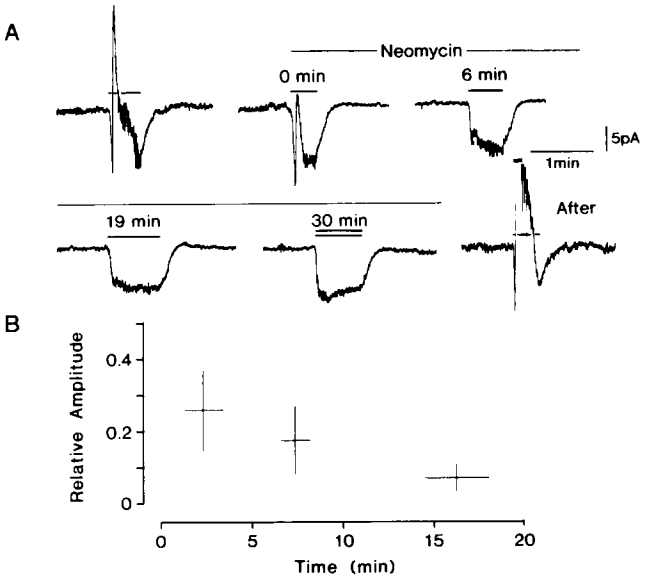

Fig. 3. Time-dependent inhibition of muscarine-induced I_O by neomycin. (A) I_{NS} with or without I_O in response to 10 μM (single horizontal line) and 30 μM muscarine (double horizontal line) in the absence and in the presence of 1.5 mM neomycin. Neomycin was added to normal solution during the indicated period. Time shown above each trace represents the approximate time after switching to neomycin solution. Last I_{NS} with I_O (After) was produced 8 min after washout of neomycin. (B) Summary of I_O in response to 6 or 10 μM muscarine in the presence of 1.5 mM neomycin. Amplitudes were measured conventionally from the current level observed at the end of application, expressed relative to that before application of neomycin, and plotted against the time of treatment. Data are means ± S.E.M. for four cells.

2.5. Chemicals

Sources of chemicals were as follows: (±)-tetrahydro-4β-hydroxy-N,N,N,5α-tetramethyl-2α-furan-methanaminium chloride((±)-muscarine chloride), cyclopiazonic acid, neomycin sulfate, and nystatin (Sigma, USA); collagenase (microbial collagenase, EC 3.4.24.3) (Yakult, Japan); 1,3,7-trimethylxanthine (caffeine) (Ishizu, Japan).

3. Results

3.1. Role for mobilization of intracellular Ca^{2+} in generation of I_O and I_{NS}

To elucidate the role of Ca^{2+} mobilization in the production of I_O and I_{NS}, Ca^{2+} store sites were depleted by various means. Fig. 1A shows that a second

application of 6 μM muscarine in a nominally Ca^{2+}-free solution failed to induce I_O. In contrast, the second I_{NS} in the absence of Ca^{2+} was enhanced, due to abolition of I_O. Prior exposure to 5 mM caffeine under Ca^{2+}-free conditions also led to a marked reduction of I_O in response to 6 μM muscarine and the I_{NS} was apparently enhanced (Fig. 1B). When the maximum amplitude of I_O was measured conventionally from the current level observed at the end of application (this procedure can result in underestimation of the amplitude because of the concomitant generation of I_{NS} in normal solution), it was reduced to 31 ± 20% (n = 3) of the control after caffeine application. For exact measurement of the effects of caffeine on I_{NS}, 1 μM muscarine was applied as this concentration of muscarine did not generate I_O. The second I_{NS} induced by 1 μM in Ca^{2+}-free solution was 82 ± 17% (n = 3) of that before exposure to 10 mM caffeine, which consistently generated a transient I_O. Thus, the application of caffeine did not noticeably alter the subsequent I_{NS} under Ca^{2+}-free conditions. These results suggest that muscarine-induced I_O depends totally on Ca^{2+} mobilized from intracellular store sites, whereas I_{NS} apparently does not. To obtain further support for this notion, the effects of CPA, a specific inhibitor of

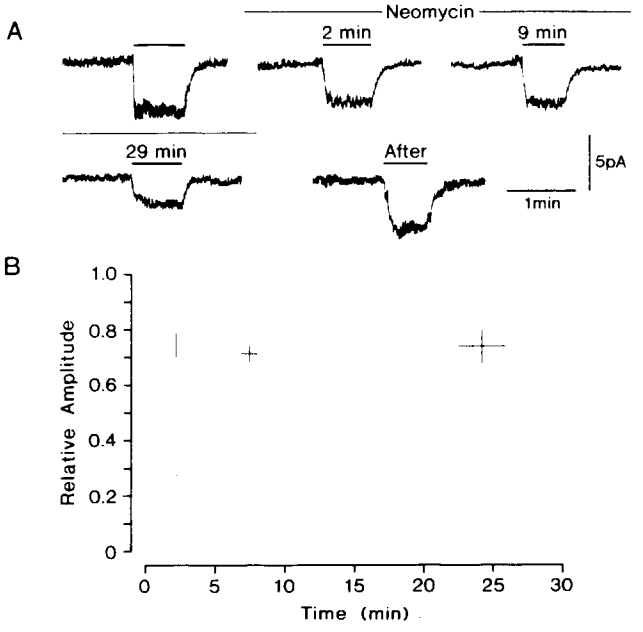

Fig. 4. Time-independent suppression of muscarine-induced I_{NS} by neomycin. (A) I_{NS} in response to 1 μM muscarine (single horizontal line) in the absence and in the presence of 1.5 mM neomycin. Neomycin was added to normal solution during the indicated period. Time shown above each trace represents the approximate time after switching to neomycin solution. Last I_{NS} (After) was elicited 4 min after washout of neomycin. (B) Summary of 1 μM muscarine-induced I_{NS} in the presence of 1.5 mM neomycin. Amplitudes were expressed and plotted in the same manner as described for Fig. 3. Data are means ± S.E.M. for five cells.

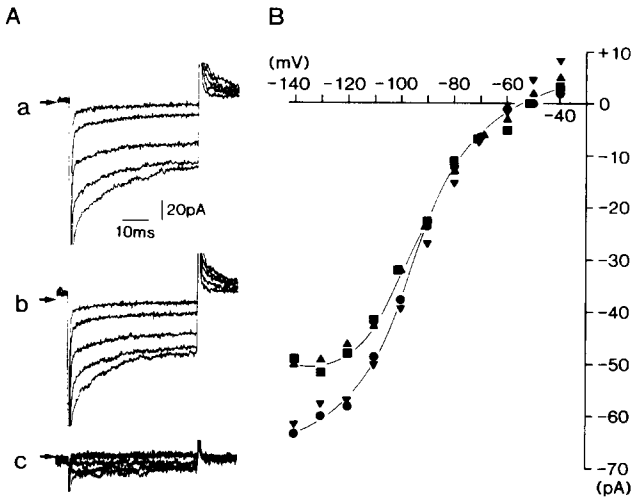

Fig. 5. Time-independent inhibition of inwardly rectifying K^+ current by neomycin. (Aa and b) Currents induced by test pulses applied to −60, −80, −100, 120, and −140 mV from −40 mV before and 5 min after application of 1.5 mM neomycin. (Ac) Difference currents from a to b. Arrows represent zero current level. (B) Current-voltage relationships before (●), 1 min (■) and 5 min (▲) after application of neomycin solution, and 2 min after washout (▼). a and b in A correspond to ● and ▲, respectively.

endoplasmic reticulum Ca^{2+}ATPase, on 6 μM muscarine-induced I_O were investigated. When 3 μM CPA was added to normal solution for 2–3 min after the termination of muscarine application, the subsequent exposure to muscarine consistently failed to produce a full I_O without any apparent effect on I_{NS} (n = 3; Fig. 1C), an effect readily eliminated by washout. This result is consistent with our thesis. However, there is the possibility that recycling of a small amount of Ca^{2+}, insufficient for generation of I_O, may be responsible for repetitive generation of I_{NS} under Ca^{2+}-free conditions. To explore this possibility, 3 μM CPA was added to a nominally Ca^{2+}-free solution (Fig. 2). In 8 of 11 cells tested with 1 or 3 μM CPA, I_{NS} was successively diminished to 53 ± 20% (n = 8) of the control in the presence of CPA, but in the remaining 3 cells, it was not noticeably altered. This incidence (73%) of I_{NS} decrease was higher than that (25%) seen in Ca^{2+}-free solution without CPA, but the extent (47%) of inhibition did not differ from the previous value (47%) (Inoue and Imanaga, 1995).

3.2. Effects of neomycin on I_O and I_{NS}

The results concerning Ca^{2+} depletion suggest that phosphatidylinositol hydrolysis is involved in the generation of I_O, but not of I_{NS}. To address this issue more directly, the effects of neomycin, an inhibitor of phospholipase C activity, were investigated. Fig. 3A shows a time-dependent suppression of I_O by 1.5 mM neomycin. I_O in response to 10 μM muscarine was

suppressed by the concomitant application of neomycin. The inhibition progressed in a time-dependent manner, and complete inhibition was seen at 19 min. This abolition of I_O was readily overcome by washout. In four of five cells tested, I_O was completely inhibited within 20 min of treatment, and in one cell, it was reduced to 13% of the control at 18 min. Fig. 3B summarizes the data for five cells. On the other hand, bath application of 1.5 mM neomycin suppressed 1 μM muscarine-induced I_{NS} by about 30%, but in a time-independent manner (Fig. 4). A similar time-independent suppression was noted on an inwardly rectifying K^+ current (I_{IR}) (Fig. 5). The inward-going rectification of the current-voltage relationship, a property which is mediated by I_{IR} (Inoue and Imanaga, 1993b), was diminished to a similar extent 1 and 5 min after the addition of 1.5 mM neomycin to the perfusate (slope conductance between -90 and -110 mV was reduced by 26% and 22%, respectively). This inhibition was readily reverted by washout. In three of four cells tested with 1.5 mM neomycin, the slope conductance was suppressed by 20 ± 5% over 10 min, and in one cell, no significant suppression was seen.

3.3. Dose-response curve for I_{NS} in the presence of neomycin

The foregoing results suggest that phosphatidylinositol turnover-related events were almost completely suppressed by neomycin. If I_{NS} generation is related to phosphatidylinositol hydrolysis, then the dose-dependent property of muscarine-induced I_{NS} would be altered by this inhibitor. Fig. 6Aa and b show I_{NS} in response to various concentrations of muscarine in the absence and presence of 1.5 mM neomycin. In the absence of the inhibitor, I_{NS} with a transient I_O was generated on application of 6 and 10 μM, whereas in the presence of the inhibitor, I_{NS} alone was produced in a dose-dependent manner between 1 and 30 μM. Fig. 6B summarizes I_{NS} in the presence and in the absence of neomycin. To facilitate comparison of I_{NS} among different cells, the amplitude of I_{NS} was expressed relative to that of the control I_{NS} induced by 1 μM muscarine in the same cells. Relative amplitudes of I_{NS} in the presence of neomycin were fitted using a least-squares method to a rectangular hyperbola with I_{MAX} of 2.17 and K_A of 2.19 μM ($r = 0.954$). The I_{NS}

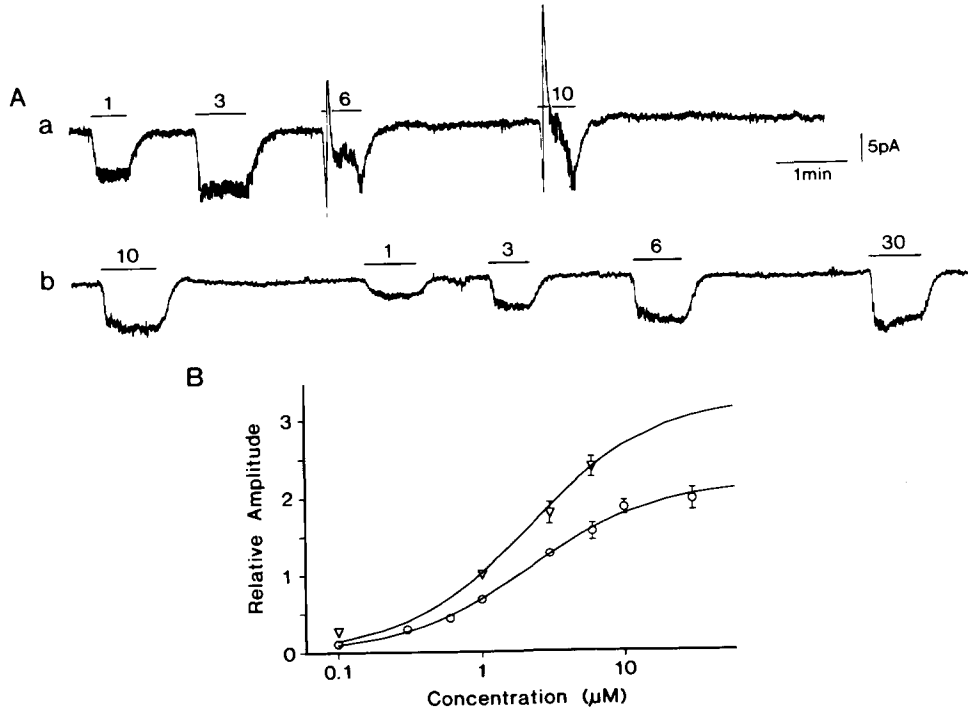

Fig. 6. Dose-response curves for muscarine-induced I_{NS} in the absence and in the presence of neomycin. (Aa and b) I_{NS} with and without I_O in response to muscarine at various concentrations in the absence and presence of 1.5 mM neomycin. Muscarine was added to the perfusate during the period indicated by horizontal lines. Numbers shown above the lines are concentrations (μM) of muscarine. (Aa and b) The same cell. (B) Dose-response relationships for muscarine I_{NS} in the absence (∇) and in the presence ($\circ$) of 1.5 mM neomycin. Amplitudes of I_{NS} without I_O were measured from the holding current level and expressed relative to that of control 1 μM muscarine-induced I_{NS} in the same cell. Data at each concentration are means $\pm$ S.E.M. of 5–11 cells ($\circ$) and of 2–5 cells (∇). Curves are rectangular hyperbolas with I_{MAX} of 3.24 and K_A of 2.19 and with I_{MAX} of 2.17 and K_A of 2.19 (see text).

induced by 1 μM muscarine in the presence of neomycin was 67 ± 7% ($n = 12$) of the control. Thus, I_{MAX} of control I_{NS} was estimated to be 3.24 (i.e. 2.17/0.67). When the control I_{NS} was fitted to a rectangular hyperbola with I_{MAX} of 3.24 and K_A of 2.17 μM, a similar correlation ($r = 0.972$) was obtained. These parameters of the control dose-response curve are in good agreement with those ($I_{MAX} = 3.00$, $K_A = 2.01$) obtained for muscarine-induced I_{NS} without apparent generation of a transient I_O (Inoue and Imanaga, 1995).

4. Discussion

The aminoglycoside antibiotic, neomycin, has been shown to inhibit phospholipase C activity, probably through binding to polyphosphoinositides (Schacht, 1976). This action of the antibiotic was noted in sea-urchin eggs only on application to the cytosolic side of the plasma membrane (Whitaker and Aitchison, 1985). Similarly, neomycin did not suppress bradykinin-induced phosphatidylinositol hydrolysis in non-permeabilized porcine endothelial cells (Kaya et al., 1989). In bovine adrenal chromaffin cells (Negishi et al., 1990) and hamster fibroblasts (Carney et al., 1985), however, agonist-stimulated phosphatidylinositol hydrolysis was suppressed when neomycin was included in extracellular solutions. This difference in effectiveness, when the antibiotic was administered extracellularly, may be related to readiness with which the chemical penetrates the plasma membrane involved. In the guinea-pig chromaffin cell, addition of neomycin to the perfusate suppressed muscarine-induced I_O in a time-dependent manner. This I_O was suggested to be due to Ca^{2+} mobilized from intracellular store sites (Inoue and Kuriyama, 1990). This notion was further supported by the present results. Under Ca^{2+}-free conditions, a transient I_O was not elicited by a second application of muscarine and was markedly suppressed by prior exposure to caffeine. Addition of CPA, an endoplasmic reticulum Ca^{2+} ATPase inhibitor, to normal solution also markedly diminished this current. These results indicate that the Ca^{2+} store sites involved resemble IP_3-sensitive ones found in cultured bovine adrenal chromaffin cells (Stauderman et al., 1991) and guinea-pig smooth muscle (Iino et al., 1988). Thus, in guinea-pig chromaffin cells, muscarine may facilitate phosphatidylinositol hydrolysis with subsequent generation of Ca^{2+}-dependent I_O. This involvement of phosphatidylinositol hydrolysis in muscarine-induced I_O may account for the time-dependent abolition of the current by neomycin. Consistent with this notion, extracellular application of neomycin induced a time-dependent inhibition of phosphatidylinositol hydrolysis-related events in rat chromaffin cells (Malgaroli et al., 1990).

In contrast to the suppression of I_O, the bath application of neomycin induced inhibition of I_{NS} and I_{IR} in a time-independent manner. These actions are similar to suppression of voltage-dependent Ca^{2+} channels in *Paramecium* (Gustin and Hennessey, 1988) and the guinea-pig papillary muscle (Hino et al., 1982) by neomycin and gentamicin, another aminoglycoside. The suppression in both preparations occurred rapidly with the addition to external solutions. In the papillary muscle, gentamicin inhibited delayed rectifier K^+ channels as well. Thus, the neomycin suppression of I_{NS} may be attributed to a direct action on the channels, rather than inhibition of phospholipase C activity. This thesis was further supported by the non-competitive inhibition of I_{NS} by the antibiotic.

The apparent threshold for muscarine to produce a transient I_O was between 3 and 6 μM, and the amplitude of I_O became greater with an increase in concentration (Inoue and Kuriyama, 1990). This dose dependence is roughly in accord with that of phosphatidylinositol hydrolysis induced in nerve ending preparations from the guinea-pig and rat cerebral cortex (Fisher et al., 1983) or in embryonic chick heart cells (Brown and Brown, 1984) by full muscarinic receptor agonists, such as carbamylcholine and muscarine. Thus, if phosphatidylinositol hydrolysis contributes to production of I_{NS}, then I_{NS} induced by muscarine at 6 μM or more should have been significantly suppressed by neomycin. However, the dose-response relationship for I_{NS} in the presence of neomycin was well expressed by a rectangular hyperbola in a wide range of concentrations between 0.1 and 30 μM. Furthermore, its K_A value was in good agreement with that noted for I_{NS} without an apparent generation of I_O, thereby indicating a non-competitive inhibition by neomycin. This mode of inhibition suggests that neomycin directly suppressed the NS channel. Thus, we conclude that phosphatidylinositol hydrolysis is not a prerequisite for generation of I_{NS}. This conclusion is compatible with the previous findings (Inoue and Imanaga, 1995) that neither the IP_3-Ca^{2+}-calmodulin kinase II nor the diacylglycerol-protein kinase C pathway was involved in muscarinic receptor stimulation of NS channels.

Addition of CPA to Ca^{2+}-free solution, however, significantly increased the incidence of I_{NS} reduction during a 20 min incubation, compared with that noted in Ca^{2+}-free solution without CPA. Intracellular Ca^{2+} was previously found to have a facilitating action on muscarine-induced I_{NS}, irrespective of concentrations of the agonist (Inoue and Imanaga, 1995). Thus, there is a possibility that I_{NS} is mediated by a small amount of Ca^{2+} mobilized from IP_3-sensitive, caffeine-non-sensitive store sites. This possibility, however, is not compatible with the non-competitive inhibition of muscarine-induced I_{NS} by neomycin. Furthermore, the decline of I_{NS} in the presence of CPA was not more than

47% in the responsive cells, a value which did not differ from that seen in 25% of the cells exposed to Ca^{2+}-free solution without CPA. In addition, even if this had been the case, the involvement of calmodulin kinase II, a strong candidate mediator for a Ca^{2+} signal would not be feasible (Inoue and Imanaga, 1995). The effects of CPA may well be due to the disruption of resting Ca^{2+} movement between the endoplasmic reticulum and the cytosol. The non-selective cation channel or its regulator may be closely associated with endoplasmic reticulum, and Ca^{2+} near the channel complex may be moving back and forth between the cytosol and endoplasmic reticulum even under Ca^{2+}-free conditions. This movement may maintain the Ca^{2+} concentration at a sufficiently high level to sustain the current. When the addition of CPA suppresses the uptake by Ca^{2+} ATPase, this sustaining mechanism of Ca^{2+} is expected to be disrupted; thus it would eventually lower a Ca^{2+} concentration near the channel complex with a subsequent decrease in I_{NS}. In fact, addition of thapsigargin, another Ca^{2+} pump inhibitor, to Ca^{2+}-free solution facilitated the depletion of intracellular Ca^{2+} store sites in bovine adrenal chromaffin cells (Cheek et al., 1989).

Acknowledgements

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