N-Ethylmaleimide Uncouples Muscarinic Receptors from Acetylcholine-sensitive Potassium Channels in Bullfrog Atrium

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ABSTRACT   The effect of N-ethylmaleimide (NEM), a sulphydryl alkylating agent, on the acetylcholine-activated K^+ current, $I_{K(ACh)}$, has been studied in single cells from bullfrog atrium using a tight-seal, whole-cell voltage clamp technique. Addition of NEM (5×10^{-5} M) produced a time-dependent complete block of $I_{K(ACh)}$. Dialysis of guanosine-5'-O-(3-thiotriphosphate) (GTPγS, 5–10×10^{-4} M), a nonhydrolyzable GTP analogue, into the myoplasm from the recording pipette gradually activated $I_{K(ACh)}$ even in the absence of acetylcholine. This effect is thought to be due to a GTPγS-induced dissociation of GTP-binding proteins (G_i and/or G_o) into subunits that can directly activate these K^+ channels. When NEM (5×10^{-5} M) was applied after the GTPγS effect had fully developed, it failed to inhibit the GTPγS-induced K^+ current, indicating that the NEM effect is unlikely to be on the dissociated subunits of the GTP-binding protein(s) or on the K^+ channels. In contrast, pretreatment with NEM before GTPγS application markedly reduced the muscarinic K^+ current, suggesting that NEM can block this K^+ current by inhibition of the dissociation of the GTP-binding proteins into functional subunits. In NEM-treated cells the stimulatory effect of isoproterenol on I_{Ca} was present, but the inhibitory action of ACh on I_{Ca} was completely abolished. These results demonstrated that NEM can preferentially inhibit muscarinic receptor–effector interactions, probably by alkylating the GTP-binding proteins that are essential for these responses.

INTRODUCTION

The transmembrane channels through which ionic currents flow are integral membrane proteins that can be modified by direct actions of certain proteolytic and chemical agents (Armstrong et al., 1973; Stimers et al., 1985; Gonoi and Hille, 1987; Duncan et al., 1988; Hescheler and Trautwein, 1988). Some of these channels can also be regulated by various second messengers or intramembrane processes, which in turn can be indirectly modified by chemical and proteolytic agents.

In atrial cells from both amphibian and mammalian hearts, muscarinic acetylcholine (ACh) receptors are linked to a specific population of K^+ channels ($I_{K(ACh)}$) via

GTP-binding proteins (Breitwieser and Szabo, 1985; Pfaffinger et al., 1985; Kurachi et al., 1986a, b, c; Nakajima et al., 1990). Some of the strongest evidence that G proteins are involved comes from experiments using the islet-activating protein, pertussis toxin (IAP), which ADP-ribosylates α-subunits of GTP-binding proteins (G_i and/or G_o) and selectively blocks this muscarinic response. In cardiac muscle activation of the GTP-binding proteins by agonists (e.g., ACh) or nonhormonal agents (e.g., nonhydrolyzable GTP analogues) results in dissociation of G proteins into subunits (α and $\beta\gamma$), which then can directly activate, for example, K^+ channels (for review see Brown and Birnbaumer, 1988; Neer and Clapham, 1988). However, IAP also blocks muscarinic receptor-mediated inhibition of adenylate cyclase, thereby reversing the anti-β-adrenergic (inhibitory) effect of ACh on the calcium current, I_{Ca} (Hescheler et al., 1986; Nakajima et al., 1990).

N-Ethylmaleimide (NEM), a sulphydryl alkylating agent, can abolish hormone-induced inhibition of adenylate cyclase by alkylating the α-subunits of GTP-binding proteins, e.g., G_i and/or G_o (Aktories et al., 1982; Jakobs et al., 1982; Ukena et al., 1984; Wong et al., 1985; Asano and Ogasawara, 1986; Winslow et al., 1987). Although it is known that NEM can inhibit the electrophysiological effects of ACh in intact atrial preparations (Roberts and Konjovic, 1969; Doods et al., 1986; Braun and Sperelakis, 1988), the mechanisms of this effect remain unclear.

Our main goal was to learn more about the molecular pharmacology of NEM-induced changes in both the direct and indirect effects of ACh in bullfrog atrium. The direct effects were studied by recording the K^+ current activated by muscarinic receptors, $I_{K(ACh)}$. The indirect effects were investigated by measuring the effects of ACh on the calcium current, I_{Ca}, after it had been enhanced by the β-adrenergic agonist, isoproterenol (ISO). The results show that NEM can preferentially block certain muscarinic receptor–effector interactions, probably by alkylating the G proteins (G_i and/or G_o) that are involved in these direct responses. However, NEM has no effect on the G protein (G_s) involved in the indirect muscarinic response, which consists of an inhibition of I_{Ca}.

METHODS

Single cells were isolated from the atria of adult bullfrog hearts by enzymatic dissociation as described previously (Hume and Giles, 1981; Giles and Shibata, 1985; Nakajima et al., 1990a, b). The transmembrane ionic currents were recorded using a whole-cell voltage clamp technique (Hamill et al., 1981; Hume and Giles, 1983). Details of microelectrode fabrication and electronic circuits for suction microelectrode voltage clamp have been described (Giles and Shibata, 1985; Clark and Giles, 1987). Membrane currents were monitored on a storage oscilloscope and stored in a microcomputer (IBM-AT) using an analogue-to-digital conversion board controlled by our acquisition and display software (Robinson and Giles, 1986). The solution used to fill the micropipette contained (in mM): K-aspartate 90, KCl 20, EGTA-KOH 0.77, $MgCl_2$ 1.0, $CaCl_2$ 0.045, Na^{2+}-ATP 3, HEPES 5, buffered to pH 7.2–7.3 with KOH. P_{Ca} of the pipette solution was calculated to be 8.0 (Fabiato and Fabiato, 1979). The cells were superfused with a HEPES-buffered Ringer's solution having the following composition (in mM): NaCl 110, KCl 2.5, $MgCl_2$ 5.0, $CaCl_2$ 2.5, glucose 5, HEPES 5, buffered to pH 7.4 with NaOH. In some experiments GTPγS (guanosine 5'-O-(3-thiotriphosphate) tetralithium salt (Boehringer Mannheim Biochemicals, Indianapolis, IN), or Gpp(NH)p (Sigma Chemical Co., St. Louis, MO) were added to the pipette-filling solution. Acetylcholine chloride, atropine

sulfate, and NEM (all from Sigma Chemical Co.) were added to the superfusing Ringer's solution from freshly prepared stock solutions. All experiments were performed at room temperature (22–24°C).

RESULTS

Effect of NEM on the Acetylcholine-activated K^+ Current

Fig. 1 shows the effects of NEM on the K^+ current ($I_{K(ACh)}$) activated by muscarinic receptors in single atrial cells. In control conditions (panel *A*, part *a*), a TTX-resistant inward current, I_{Ca}, is recorded positive to −30 mV. I_{Ca} is followed by slower activation of I_K, the delayed rectifier K^+ current. Upon repolarization to the holding potential, I_K deactivates and corresponding tail currents are recorded. An inwardly rectifying background K^+ current, I_{K1}, can be elicited by hyperpolarizing pulses. After the application of ACh (10^{-6} M; panel *A*, part *b*), both the holding current at −40 mV and the currents during the depolarizing pulses increase in the outward direction, and concomitantly the inward current during the hyperpolarizing pulses negative to −90 mV also increases. Fig. 1 *B* shows the corresponding current–voltage relationships, obtained by measuring the currents at the end of the voltage clamp pulses. The ACh-induced currents, defined as the difference between the currents recorded before and after the application of ACh, exhibited inward rectification, reversed near −85 mV, and showed time-dependent relaxations in response to the hyperpolarizing pulses. These ACh effects were blocked completely by atropine (10^{-6} M), demonstrating that they are mediated by muscarinic receptors (results not shown). These results confirm that these ACh-induced current changes are due to the activation of $I_{K(ACh)}$ (Momose et al., 1984; Simmons and Hartzell, 1987; Breitwieser and Szabo, 1988; Clark et al., 1990; Nakajima et al., 1990).

After the application of NEM (5×10^{-5} M) for ~3 min, the muscarinic effects of ACh were almost completely abolished (Fig. 1 *A*, part *c*). In contrast, neither I_{K1} nor the I_K tails were changed significantly, indicating that these ACh-independent K^+ currents were not affected by NEM. These results demonstrate that NEM selectively inhibits $I_{K(ACh)}$.

To obtain additional information concerning the time course of development of the inhibitory actions of NEM on $I_{K(ACh)}$, NEM (5×10^{-5} M) and ACh (10^{-5} M) were applied simultaneously (Fig. 2). At first, the holding current at −40 mV increased in the outward direction. At the same time, the inward current activated by the command pulse to −110 mV also increased. Later $I_{K(ACh)}$ gradually decreased, in spite of the continuous application of ACh, and 5 min after the recording began $I_{K(ACh)}$ had declined to near zero. Complete inhibition of $I_{K(ACh)}$ was obtained 3–6 min after the application of 5×10^{-5} M NEM ($n = 4$). Note that this time-dependent inhibition of $I_{K(ACh)}$ by NEM is much different than the time course of the inhibition of $I_{K(ACh)}$ by atropine. When atropine (10^{-6} M) and ACh (10^{-5} M) are applied simultaneously, $I_{K(ACh)}$ failed to develop. The marked differences in these time courses of the inhibition of $I_{K(ACh)}$ suggest that NEM does not block $I_{K(ACh)}$ by a simple ligand–receptor interaction, as atropine does.
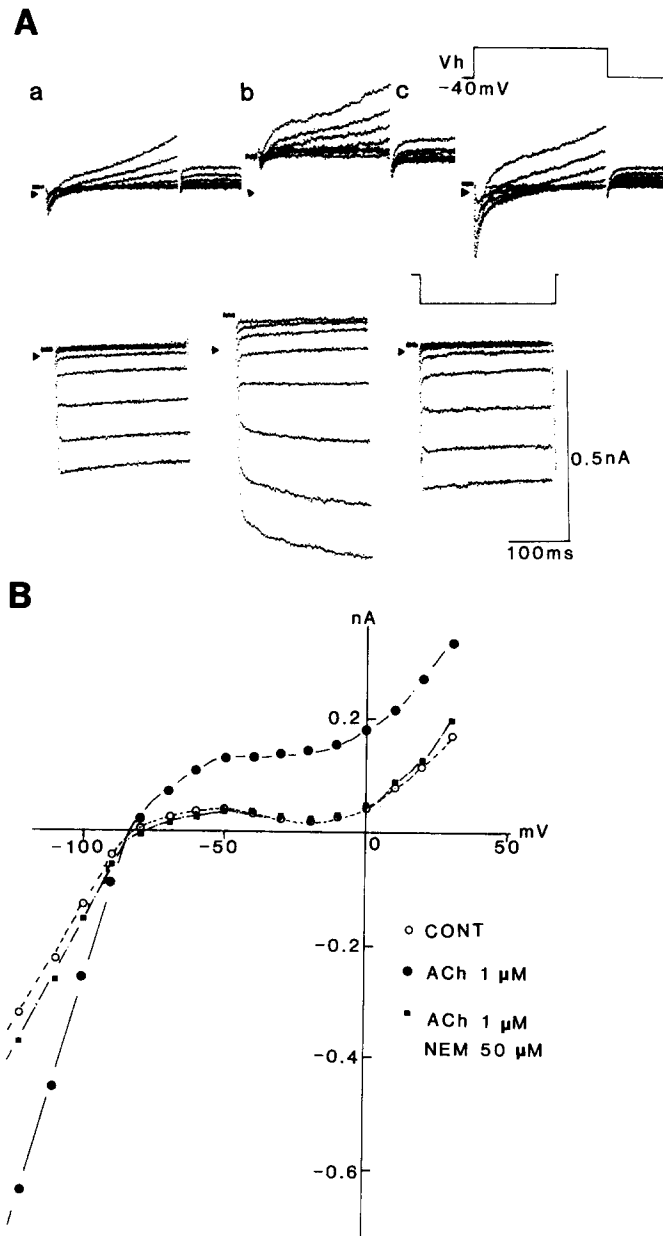
A

FIGURE 1. NEM-induced inhibition of the effects of ACh on muscarinic receptor-activated K^+ current, $I_{K(ACh)}$. The membrane potential was held at -40 mV and 200-ms command pulses to various membrane potentials were applied at 0.2 Hz. The current traces shown in A were recorded in control (a), in 10^{-6} M ACh (b), and in 10^{-6} M ACh plus 5×10^{-5} M NEM (c). The zero current level is indicated by the solid triangle. The current traces during the depolarizing $(-30$ to $+30$ mV$)$ and hyperpolarizing pulses $(-50$ to -120 mV$)$ are shown in the upper and lower parts, respectively. B shows current–voltage relationships measured at the end of the pulses (control $[O]$, 10^{-6} M ACh $[\bullet]$, and 10^{-6} M ACh plus 5×10^{-5} M NEM $[\blacksquare]$).

B

○ CONT

● ACh 1 µM

■ ACh 1 µM
NEM 50 µM

Effect of NEM on the GTPγS-enhanced K^+ Current

Intracellular dialysis from the recording pipette with a nonhydrolyzable GTP analogue, GTPγS $(5 \times 10^{-5}$ M$)$, gradually increased $I_{K(ACh)}$ even in the absence of ACh (Fig. 3). Time-dependent relaxations of $I_{K(ACh)}$ during applied hyperpolarizations and after return to -40 mV were also observed (Fig. 3 A, part b) after GTPγS was dialyzed into the cell. These current changes could not be blocked by atropine

$(10^{-6}$ M) (Fig. 3 *A*, part *c*) and were probably due to direct activation of GTP-binding proteins (Breitwieser and Szabo, 1985; Kurachi et al., 1986*a, b, c*; Nakajima et al., 1990). Fig. 3, *A* and *B* shows that this GTPγS-enhanced K^+ current could not be inhibited by NEM when NEM was applied after GTPγS. Furthermore, as illustrated in Fig. 3 *C*, the activation of $I_{K(ACh)}$ by GTPγS was enhanced and accelerated by
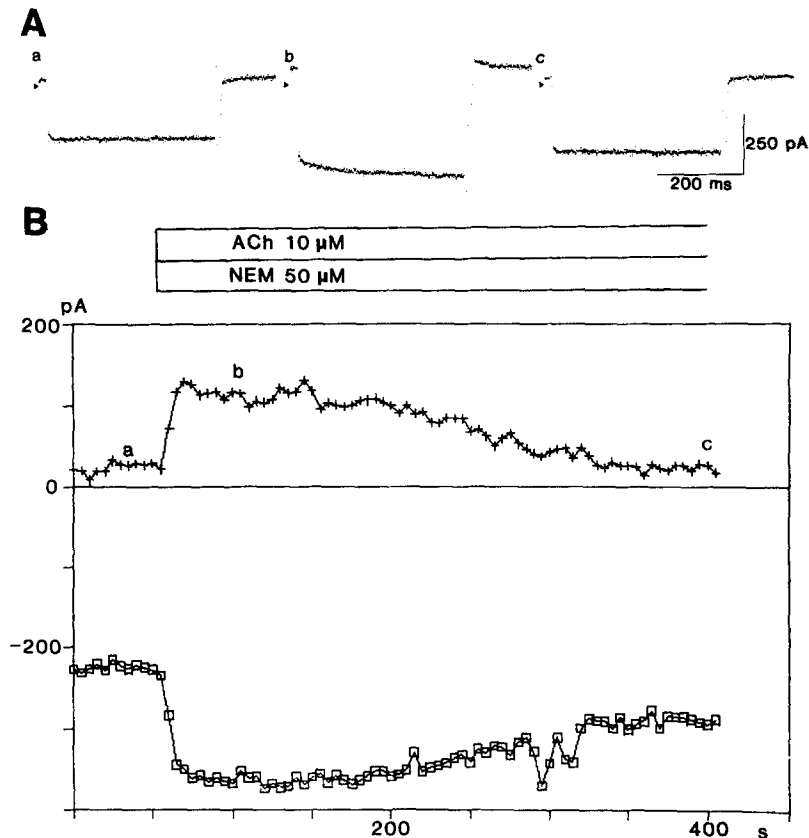

FIGURE 2. Time-dependent inhibition by NEM of the ACh-activated K^+ current, $I_{K(ACh)}$. 400-ms hyperpolarizing pulses to −110 mV from a holding potential of −40 mV were applied at 0.2 Hz. ACh $(10^{-5}$ M) and NEM $(5 \times 10^{-5}$ M) were both applied simultaneously. The current traces shown in *A* were recorded in control (*a*), or in ACh $(10^{-5}$ M) plus NEM $(5 \times 10^{-5}$ M) (*b, c*) at the times indicated by the corresponding letters in *B. B* shows the time course of the changes in the holding current (+, top trace) and the inward current measured at the end of the command pulse of −110 mV (□, bottom trace). The current changes were measured from the zero current level, indicated by the solid triangles.

simultaneous superfusion with ACh $(10^{-6}$ M). After the increase in $I_{K(ACh)}$ had reached a steady state, atropine $(10^{-6}$ M) failed to inhibit it, indicating that the K^+ channels were uncoupled from the muscarinic receptors and had been activated irreversibly by GTPγS. Fig. 3 *C* also shows that NEM $(5 \times 10^{-5}$ M) failed to inhibit the GTPγS-enhanced K^+ current. These results suggest that NEM does not affect
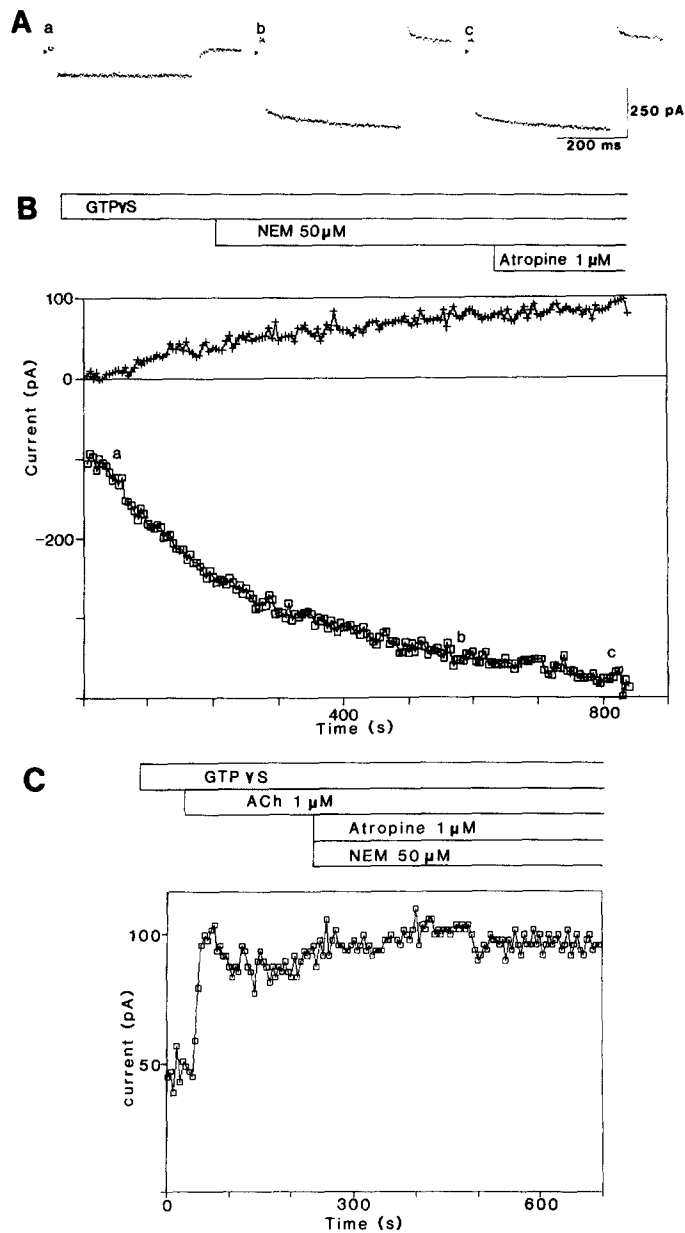
A
a

b

c

250 pA

200 ms

B

GTPγS

NEM 50µM

Atropine 1 µM

100

0

Current (pA)

a

-200

b

c

400
Time (s)

800

C

GTP γS

ACh 1 µM

Atropine 1 µM

NEM 50 µM

100

current (pA)

50

0

300

600

Time (s)

FIGURE 3. Lack of effect of NEM on the GTPγS-induced increase in K^+ current. GTPγS $(5 \times 10^{-4}$ M) was included in the recording pipette. The membrane potential was held at -40 mV and hyperpolarizing pulses to -110 mV were applied at 0.2 Hz. The time course of the changes in the holding current (+) and the current measured at the end of the clamp pulses (□) are plotted in B, beginning immediately after the rupture of the membrane. The current traces shown in A were recorded (a) in control (i.e., immediately after the rupture of the membrane), (b) after the application of NEM $(5 \times 10^{-5}$ M), and (c) after NEM $(5 \times 10^{-5}$ M) plus atropine $(10^{-6}$ M) treatment. Zero current levels are shown by solid triangles. The holding current change was measured from zero current level. C shows data from a different cell. ACh $(10^{-6}$ M) was added after the rupture of the membrane, the time course of the changes in the holding current (□) was plotted, and GTPγS was applied intracellularly from the recording pipette. Finally, ACh $(10^{-6}$ M) and atropine $(10^{-6}$ M) plus NEM $(5 \times 10^{-5}$ M) were added to the bath at the times indicated in the top part of C. The holding current change was measured from zero current level.

either the K^+ channels or the dissociated subunits of the GTP-binding proteins which are essential for activation of $I_{K(ACh)}$ (Brown and Birnbaumer, 1988; Neer and Clapham, 1988). In addition, NEM has no direct effects on the other types of K^+ channels (I_{K1} and I_K) present in bullfrog atrium (shown in Fig. 1). Thus, NEM appears to act on ACh-sensitive K^+ channels at sites located either on the muscarinic receptors or on the GTP-binding proteins (G_i and/or G_o) that are coupled to these K^+ channels.

Effect of GTPγS on Cells Pretreated with NEM

To obtain additional evidence concerning whether the site of NEM action involves GTP-binding protein(s), atrial cells were treated with NEM before the application of GTPγS. To ensure that the effects of NEM on $I_{K(ACh)}$ had developed fully, NEM (5×10^{-5} M) was added to the superfusate ~10 min before the dialysis of GTPγS was started (Fig. 4), i.e., before the rupture of the membrane. After this pretreatment with NEM, intracellular application of GTPγS (10^{-3} M) by dialysis from the recording pipette failed to activate $I_{K(ACh)}$, in contrast to the results shown in Fig. 3. This result was also obtained in cells treated with NEM only; thus, it was not related to the presence or absence of ACh, to the concentration of GTPγS ($5-10 \times 10^{-4}$ M), or to the choice of the nonhydrolyzable GTP analogue, since experiments in which Gpp(NH)p (2 mM) was used also consistently yielded negative results (not shown). In combination, these findings show that in cells pretreated with NEM (5×10^{-5} M) the activation of $I_{K(ACh)}$ by GTPγS is reduced significantly, and they suggest that NEM can inhibit the function of the GTP-binding proteins, e.g., their dissociation, without affecting the properties of the active dissociated subunits themselves.

Effect of NEM on the Inhibitory Action of ACh on I_{Ca}

To test whether the actions of NEM on the GTP-binding proteins (G_i/G_o) were selective, the effects of NEM on I_{Ca} were also examined. Fig. 5 shows I_{Ca} traces recorded before and after the application of NEM (5×10^{-5} M). The corresponding peak current–voltage relationships are shown below. The membrane potential was held at -40 mV and command pulses were applied at 0.2 Hz. NEM (5×10^{-5} M) increased I_{Ca} markedly with no significant change in its voltage dependence. Once again, I_{K1} was not changed by NEM. Fig. 5 *B* shows that NEM (5×10^{-5} M) very gradually increased I_{Ca}; the steady-state effect was recorded ~3 min after its application. Subsequent addition of ISO (10^{-6} M) further increased I_{Ca}, indicating that the stimulatory GTP-binding protein(s) (G_s) remained functional after NEM treatment.

The histograms in Fig. 6 summarize the observed effects of NEM on the indirect inhibitory actions of ACh on I_{Ca}. As shown in the previous paper (Nakajima et al., 1990), ACh (10^{-5} M) can completely inhibit the effects of ISO (10^{-6} M) on I_{Ca} in bullfrog atrium (Fig. 6, *left*). However, this inhibitory effect of ACh (10^{-5} M) on I_{Ca} was completely abolished (Fig. 6, *right*) by NEM, even though NEM by itself increased I_{Ca}, and application of ISO (10^{-6} M) after NEM further increased I_{Ca}.

In bullfrog atrial cells, the effects of ACh on both I_{Ca} and $I_{K(ACh)}$ are mediated by a

biochemical pathway involving both muscarinic receptors and GTP-binding proteins (G_i and/or G_o) (Nakajima et al., 1990). Our results suggest that NEM can block this pathway. To determine whether NEM selectivity affects G_i or G_o rather than G_s, GTPγS (5×10^{-4} M) was applied to a cell that had been pretreated with NEM

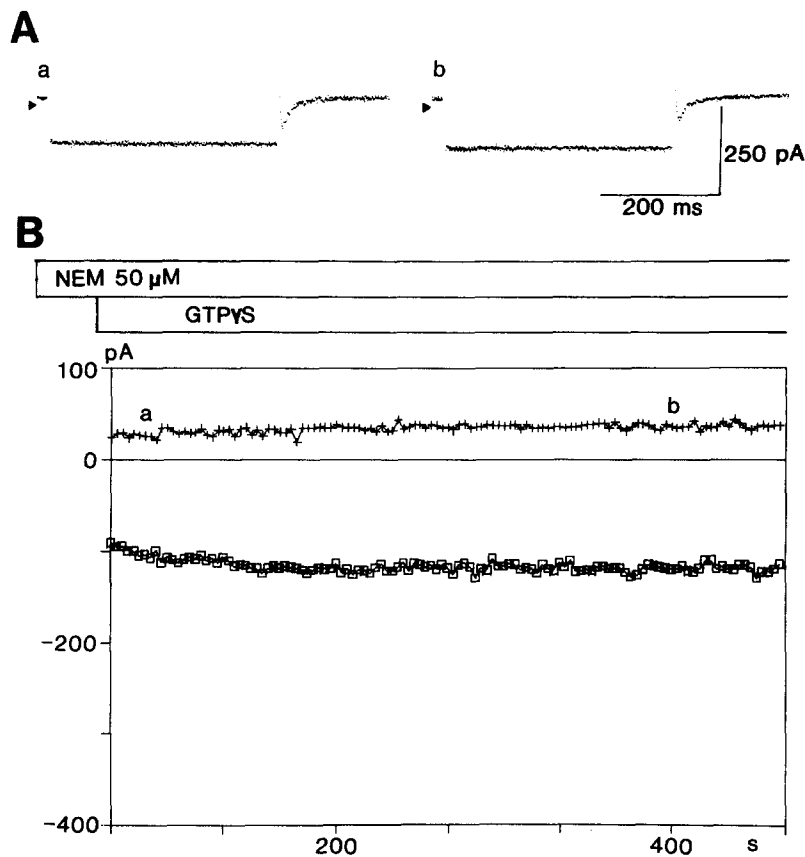FIGURE 4. Block of the effect of GTPγS on $I_{K(ACh)}$ after pretreatment with NEM. GTPγS (10^{-3} M) was added intracellularly after making an impalement with a patch pipette. 400-ms command pulses to -110 mV from a holding potential of -40 mV were applied at 0.2 Hz beginning immediately after the rupture of the membrane. The current traces (a, b) shown in A were recorded at times indicated by the corresponding letters in B. B shows the time courses of the changes in the holding current ($+$, top trace) and the inward current measured at the end of the command pulse of -110 mV ($\square$, bottom trace). Zero current levels are shown by solid triangles.

(5×10^{-5} M) for ~10 min (as shown in Fig. 7). In response to GTPγS dialysis into the cell, I_{Ca} gradually increased, probably due to the direct activation of the GTP-binding proteins (G_s). ISO (10^{-6} M) further increased I_{Ca}, suggesting that the activation of G_s by GTPγS is enhanced by ISO. Thus, after NEM treatment the
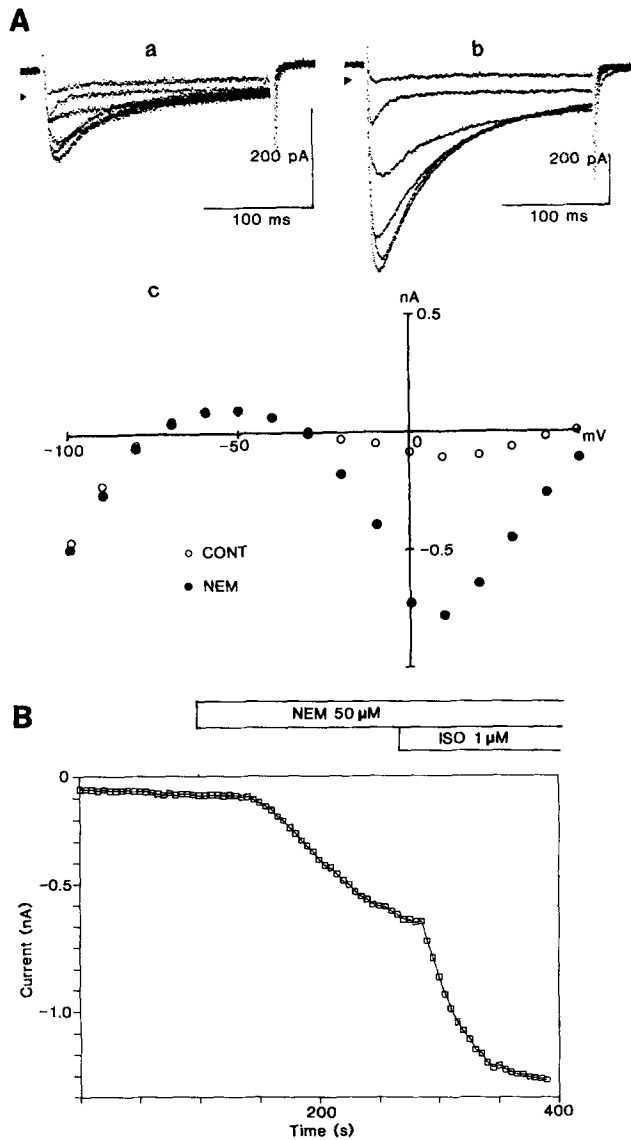
A

FIGURE 5. Effects of NEM on the Ca^{2+} current in single bullfrog atrial cells. The membrane potential was held at -40 mV and command pulses were applied at 0.2 Hz. The original current traces obtained by depolarizing pulses between -30 mV and $+20$ mV are illustrated (a) in control and (b) after NEM (5×10^{-5} M) treatment. Zero current levels are shown by solid triangles. The initial (peak) current–voltage relationships before (O) and after (●) the application of NEM (5×10^{-5} M) are shown in c. B illustrates the time course of the changes in the Ca^{2+} current. NEM (5×10^{-5} M) and ISO (10^{-6} M) were added at the times indicated. These data were obtained from a different cell than those in A.

functions of G_s remain intact. The sites of NEM action therefore appear to be located on the GTP-binding proteins (G_i and/or G_o).

Effect of NEM on Ca^{2+} Current Enhanced by ISO and Cyclic AMP

NEM by itself can increase I_{Ca} substantially, from approximately -100 pA to -750 pA (mean value, $n = 4$; Figs. 1, 5, and 6). It is likely that one of the underlying mechanisms of NEM actions on I_{Ca} involves inactivation of G_i, which could increase basal levels of cyclic AMP (cAMP). We therefore examined the effects of NEM on I_{Ca}

after increasing I_{Ca} with the β-agonist, ISO, or with intracellular cAMP. As shown in Fig. 8 A, NEM enhanced I_{Ca} even after maximal effects of ISO (10^{-6} M) had developed. $LaCl_3$ (5×10^{-5} M), a potent blocker of I_{Ca}, completely blocked the effects of both ISO and NEM on I_{Ca}. In addition, in experiments in which intracellular application of cAMP (2×10^{-4} M) had increased I_{Ca} maximally, and
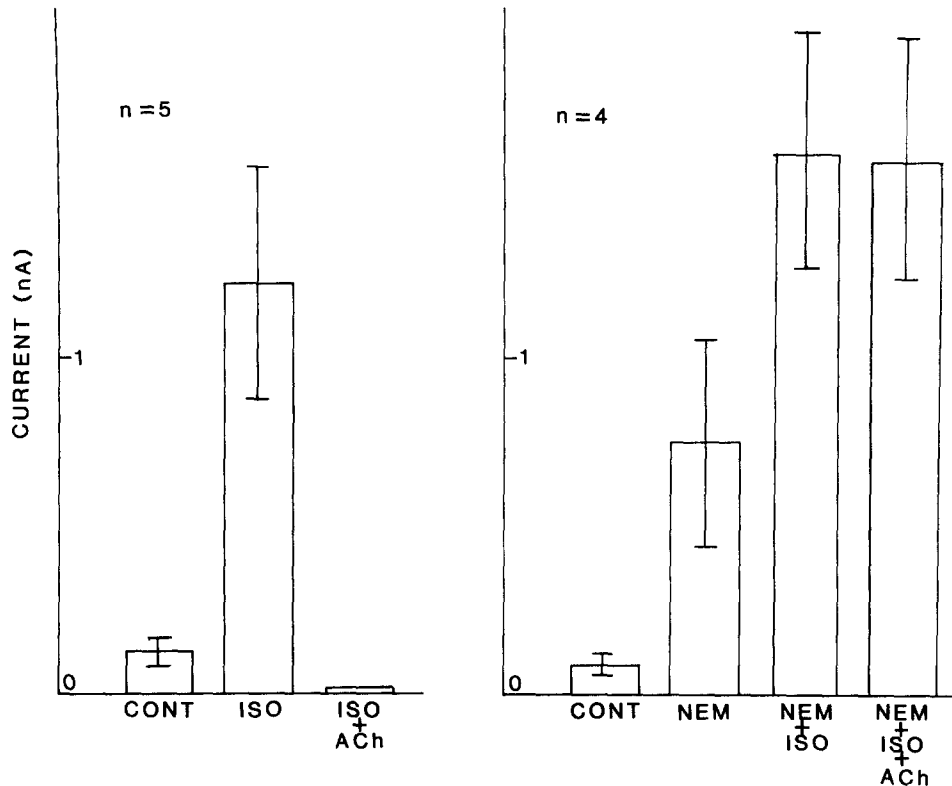

FIGURE 6.   Summary of the NEM-induced inhibition of ACh effects on the calcium current, I_{Ca}. 200-ms depolarizing pulses to 0 mV from a holding potential of -40 mV were applied at 0.2 Hz. Peak I_{Ca} was measured from zero current level. n = number of cells tested. Mean values and standard deviations are illustrated by the open columns and bars, respectively.

subsequent application of ISO (10^{-6} M) failed to increase I_{Ca} any further, NEM (5×10^{-5} M) consistently increased I_{Ca}. These results suggest that NEM may affect the Ca^{2+} channel directly, in addition to inactivating GTP-binding proteins.

DISCUSSION

Our results show that NEM can inhibit the muscarinic receptor-activated K^+ current, $I_{K(ACh)}$, as well as block the inhibitory effect of ACh on the calcium current, I_{Ca}, in single myocytes from bullfrog atrium. These inhibitory actions of this sulphydryl alkylating agent are consistent with previous findings showing that NEM

attenuates the negative chronotropic effects of muscarinic agonists in rat and guinea pig hearts (Robert and Konjovic, 1969; Doods et al., 1986; Braun and Sperelakis, 1988).

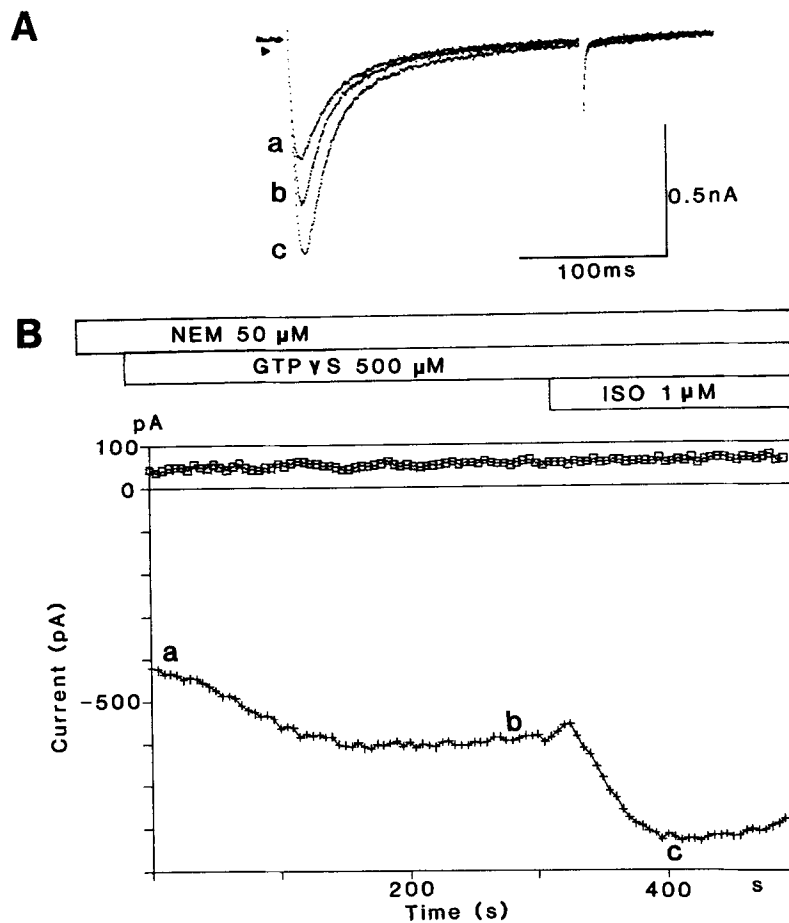To determine whether GTP-binding proteins are involved in these actions of

FIGURE 7.   Inhibition of the effects of GTPγS on $I_{K(ACh)}$ and I_{Ca} after pretreatment with NEM (5 × 10⁻⁵ M) for ~10 min. GTPγS (5 × 10⁻⁴ M) was applied intracellularly from the patch pipette. 200-ms command pulses to 0 mV from a holding potential of −40 mV were applied at 0.2 Hz, beginning immediately after the rupture of the membrane. The current traces shown in *A* were recorded at the times indicated by the corresponding letters in *B*. *B* shows the time courses of the changes in the holding current (□, top trace) and the Ca^{2+} current (+, bottom trace). The zero current levels are illustrated by solid triangles.

NEM, GTPγS was used to activate $I_{K(ACh)}$. In cells pretreated with NEM, GTPγS effects were blocked (Figs. 4 and 9), indicating that NEM eliminated the function of GTP-binding proteins, perhaps by inhibiting the binding of GTPγS to GTP-binding proteins or preventing their dissociation into active subunits. However, NEM was
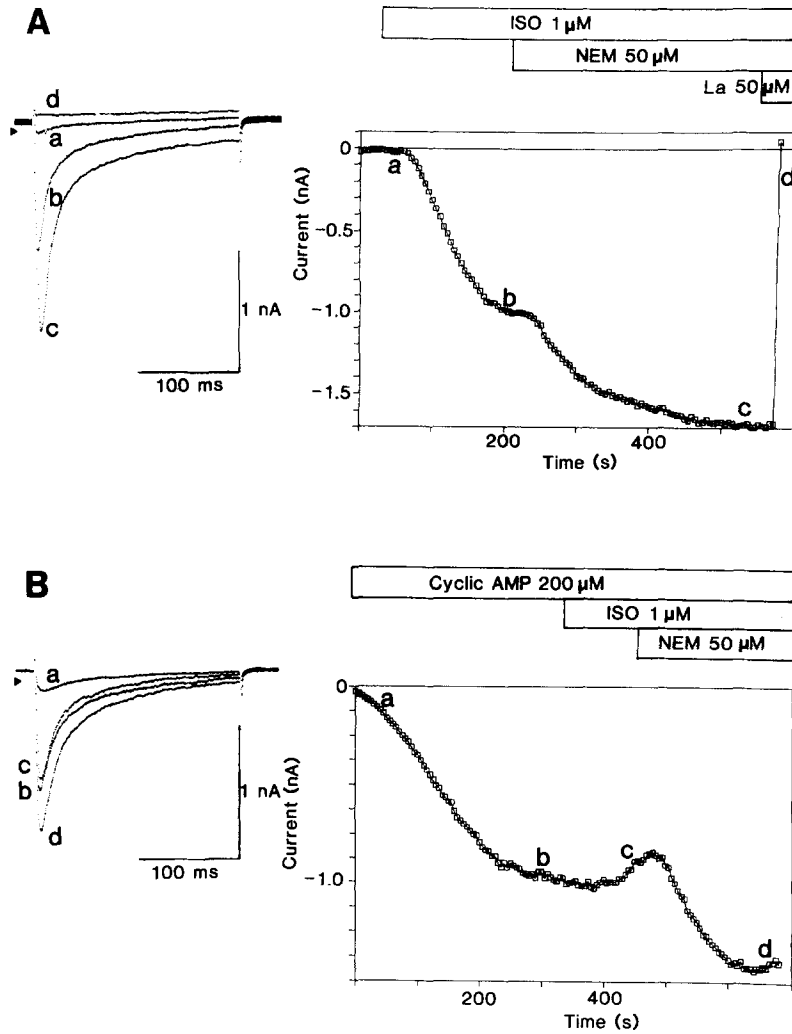
FIGURE 8. Effect of NEM on I_{Ca} after ISO or cAMP had been applied. Each cell was held at −40 mV and the depolarizing pulses to 0 mV were applied at 0.2 Hz. The changes in the amplitude of I_{Ca} measured from the zero current level are plotted on the right side of A and B. A, Effect of NEM on ISO-enhanced I_{Ca}. The current traces shown in A were recorded in (a) control, (b) ISO (10^{-6} M), (c) ISO (10^{-6} M) plus NEM (5×10^{-5} M), and (d) after the addition of LaCl$_3$ (5×10^{-5} M) to c. B shows the effects of NEM on cAMP-enhanced I_{Ca}. cAMP (2×10^{-4} M) was applied intracellularly from the patch pipette. The current traces shown in B were recorded (a) immediately after the rupture of the membrane, (b) before, and (c) after the application of ISO (10^{-6} M), and (d) NEM (5×10^{-5} M) was added. The diagrams at the right sides of both panels show peak I_{Ca} measured at the times indicated by the corresponding letters on the raw data. Zero current levels are shown by solid triangles.

not effective when applied after GTPγS and activated $I_{K(ACh)}$ (Figs. 3 and 9). These results indicate that the site of action of NEM is not on the K$^+$ channel itself or on the dissociated subunits of the GTP-binding proteins.

Previous work has shown that NEM can selectively abolish hormone- or transmitter-induced inhibition of adenylate cyclase in various preparations, including heart cells (Aktories et al., 1982; Jakobs et al., 1982, 1983; Ukena et al., 1984).
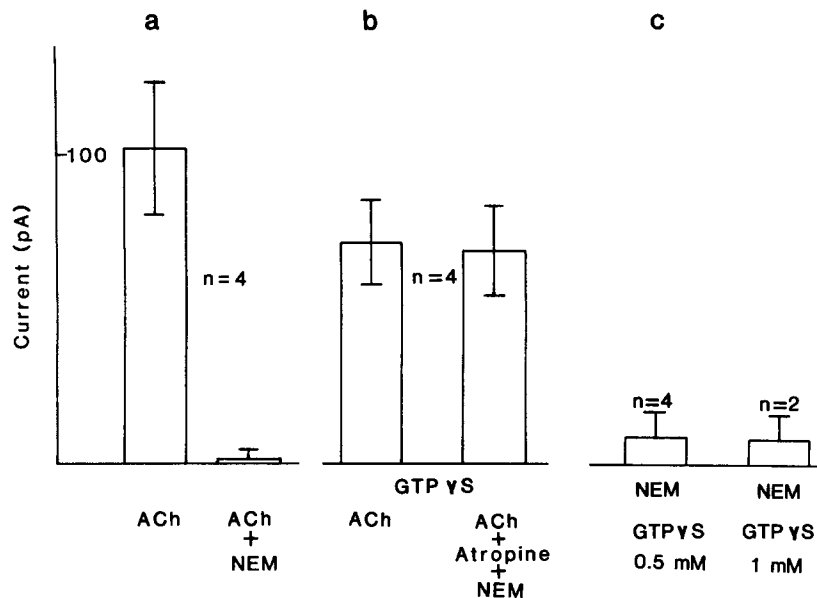
FIGURE 9.   Effect of NEM on muscarinic receptor-regulated K$^+$ current. *a*, The current changes at a holding potential of -40 mV after the application of ACh (10^{-5} M) (*left*) and 3–5 min after the additional application of NEM (5×10^{-5} M) (*right*) were measured. n = number of cells tested. *b*, The effect of NEM on GTPγS-activated K$^+$ current. GTPγS (5×10^{-4} M) was applied intracellularly from the patch pipette. In these experiments, after the rupture of the membrane ACh (10^{-6} M) was added to ensure that GTPγS activated the GTP-binding proteins maximally. The left bar shows the current changes measured when the ACh-induced current reached a steady state, compared with the control value immediately after the rupture of the membrane. The right bar corresponds to the current changes measured at 5–7 min after the addition of atropine (10^{-6} M) and NEM (5×10^{-5} M). *c*, The effect of GTPγS in cells pretreated with NEM; i.e., after 7–10 min of NEM treatment, GTPγS (5–10×10^{-4} M) was applied intracellularly from the recording pipettes. The current changes were measured 5–7 min after the rupture of the membrane, and were compared with the holding current immediately after the rupture of the membrane. In all panels the mean value and the standard deviation are shown in open columns and bars, respectively.

Our electrophysiological data indicate that the observed effects of NEM on $I_{K(ACh)}$ are quite specific. Thus, two other K$^+$ currents that are present in these myocytes, I_{K1} and I_K, are not significantly changed by NEM (5×10^{-5} M) treatment. The specificity of NEM effects may arise from selective actions on different GTP-binding proteins.

G proteins are heterotrimers composed of α-, β-, and γ-subunits (for review see Gilman, 1987). Recently, Winslow et al. (1987) demonstrated that NEM alkylates only specific sites on α-subunits of G_i and/or G_o; the $\beta\gamma$-subunits of these G proteins are not affected by NEM (Asano and Ogasawara, 1986). It is possible, therefore, that the NEM effects we have observed are due to alkylation of sulphydryl groups only on the α-subunits of G_i and/or G_o.

Previous biochemical studies have shown that NEM can affect several enzymatic responses, even in a particular cell type. For example, Aktories et al. (1982) observed that treatment of intact adipocytes from hamsters with NEM abolished both the receptor-mediated inhibition of adenylate cyclase and the inhibition of adenylate cyclase activity by GTP or nonhydrolyzable GTP analogues (GTPγS and GMP-P(NH)P). In human platelets NEM not only abolished the α_2-induced inhibition of the adenylate cyclase, but also caused an increase in the basal, prostaglandin E_1–stimulated activity of this enzyme (Jakobs et al., 1982). Our results are consistent with these biochemical data, since in myocytes from bullfrog atrium NEM removed the inhibitory effect of ACh on I_{Ca} (Fig. 7), as well as inhibiting $I_{K(ACh)}$.

IAP uncouples muscarinic receptors from the GTP-binding proteins (G_i and/or G_o) (Katada and Ui, 1982; Katada et al., 1986; for review, see Gilman, 1987). We observed that receptor-independent, GTPγS-induced activation of these G proteins and their dissociation into subunits remained unchanged after IAP treatment (Cote et al., 1984; Katada et al., 1986). Previous electrophysiological studies (Logothetis et al., 1987; Nakajima et al., 1990) have also shown that GTPγS can activate $I_{K(ACh)}$ in cells that have been pretreated with IAP. These results suggest that the ability of the GTP-binding proteins to couple to receptors is selectively blocked by IAP. In contrast with this action of IAP, our data suggest that NEM prevents the GTPγS-induced dissociation of the GTP-binding proteins. Thus, it is likely that different sites on the GTP-binding proteins are modified by NEM and IAP (cf. Neer and Clapham, 1988).

Winslow et al. (1987) have reported that NEM alkylates three cysteine residues on the GTP-binding proteins (G_o and G_i). One of these sites is the one that ADP-ribosylated by IAP and is involved in the coupling of the receptors to G proteins. This effect of NEM can explain previous findings showing that this agent reduces the affinity of the receptor for its agonist, an effect that mimics one of the actions of GTP analogues (Wei and Sulakhe, 1980; Harden et al., 1982; Korn et al., 1983; Flynn and Potter, 1985). The other sites are involved in GTP binding, but at these sites the action of NEM is different from that of IAP. After $I_{K(ACh)}$ was activated by GTPγS, NEM failed to inhibit it, which may suggest that the binding of GTPγS can prevent the effect of NEM. Thus, NEM seems to be able to act on several sites on GTP-binding proteins.

One implication of the effects of NEM that we have observed is that sulphydryl groups on the sarcolemma of atrial cells can modulate certain ionic channels. One of the mechanisms underlying the effect of NEM on I_{Ca} may involve the inactivation of G_i, and a resultant increase in basal levels of cAMP (Jakobs et al., 1982). However, the effect of NEM on I_{Ca} cannot be explained only by the inactivation of G_i.

In summary, NEM inhibits the effect of ACh on $I_{K(ACh)}$ and inhibitory action of ACh on I_{Ca}, probably by alkylating the GTP-binding proteins (G_i and/or G_o) that are

essential for these responses. Our results provide the first direct evidence that sulphydryl groups on GTP-binding proteins are essential for these responses, and suggest that NEM could be a very useful tool for studying the molecular pharmacology of certain G protein–linked responses.

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