Hypaque, stained with biotinylated MAb YTH 616 to human CD2 (*13*) and rat IgG2a MAb KT3 to mouse CD3 [K. Tomonari, *Immunogenetics* **28**, 455 (1988)], and detected by streptavidin R–phycoerythrin (SeraLab) and FITC-conjugated MAb MARG2A to rat IgG2a (Serotec, Oxford, United Kingdom), respectively. Labeled cells were analyzed on a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Oxford, United Kingdom).

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Carboxyl Methylation of Ras-Related Proteins During Signal Transduction in Neutrophils

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In human neutrophils, as in other cell types, Ras-related guanosine triphosphate–binding proteins are directed toward their regulatory targets in membranes by a series of posttranslational modifications that include methyl esterification of a carboxyl-terminal prenylcysteine residue. In intact cells and in a reconstituted in vitro system, the amount of carboxyl methylation of Ras-related proteins increased in response to the chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). Activation of Ras-related proteins by guanosine-5′-*O*-(3-thiotriphosphate) had a similar effect and induced translocation of $p22^{rac2}$ from cytosol to plasma membrane. Inhibitors of prenylcysteine carboxyl methylation effectively blocked neutrophil responses to FMLP. These findings suggest a direct link between receptor-mediated signal transduction and the carboxyl methylation of Ras-related proteins.

The Ras-related guanosine triphosphate (GTP)–binding proteins regulate a wide variety of cellular processes, including signal transduction (*1*). The biological activity of Ras-related proteins depends on the ability of these proteins to associate with membranes. These intrinsically hydrophilic proteins become associated with membranes as a result of a series of posttranslational modifications of their COOH-termini (*2*). The Ras-related proteins of the Ras and Rho subfamilies are among a class of proteins that end with the COOH-terminal consensus sequence CAAX, in which C is cysteine, A is usually an aliphatic amino acid, and X is another amino acid. Several cytosolic prenyl transferases recognize these sequences and catalyze the attachment of a polyisoprene chain (15-carbon farnesyl or 20-carbon geranylgeranyl) by a thioether linkage to the cysteine. The prenylated COOH-terminus is recognized by a membrane-associated protease that removes the AAX amino acids. The new COOH-terminal prenylcysteine subsequently becomes a substrate for a membrane-associated carboxyl methyltransferase that methyl esterifies the α carboxyl group. Thus, prenylation and AAX proteolysis are prerequisites for carboxyl methylation.

Unlike prenylation and proteolysis, carboxyl methylation is reversible under physiologic conditions (*3*). Genetic studies in yeast (*4*) and in vitro analysis of $p21^{K\text{-}ras(B)}$ (*5*) indicate that carboxyl methylation augments the membrane association of processed CAAX-containing proteins, presumably by neutralizing the negative charge at the COOH-terminus. It is unclear whether the COOH-terminal prenylcysteine methyl ester inserts nonspecifically into lipid bilayers or binds to specific membrane targets. The reversibility of carboxyl methylation and the function of Ras-related proteins in signaling suggest that carboxyl methylation may regulate signal transduction.

Ras-mediated signaling has been studied in tissue culture, generally in the context of growth factor–mediated proliferation and differentiation. Unlike tissue culture cells, neutrophils are terminally differentiated, short-lived cells that have no proliferative capacity. Neutrophils have relatively well characterized pathways of signal transduction in which stimulus-response coupling is accomplished within minutes and does not require protein synthesis (*6*). We therefore used human neutrophils as a system in which to test the hypothesis that carboxyl methylation of Ras-related proteins is involved in signal transduction.

We first determined whether circulating neutrophils retain the capacity to process Ras-related proteins. Metabolic labeling of human neutrophils with [^{3}H-methyl]methionine [the precursor of the methyl donor S-adenosyl-L-methionine (AdoMet)] followed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and alkaline hydrolysis of protein methyl esters showed that the peak of carboxyl methylation was in the 20- to 24-kD region of the gel, consistent with the molecular mass of $p21^{ras}$ and its homologs (Fig. 1A). Carboxyl methylation of these proteins was unaffected by cycloheximide and therefore independent of protein synthesis. An inhibitor of $p21^{ras}$ methylation (*7*), *N*-Acetyl-*S*-*trans*,*trans*-farnesyl-L-cysteine (AFC), inhibited carboxyl

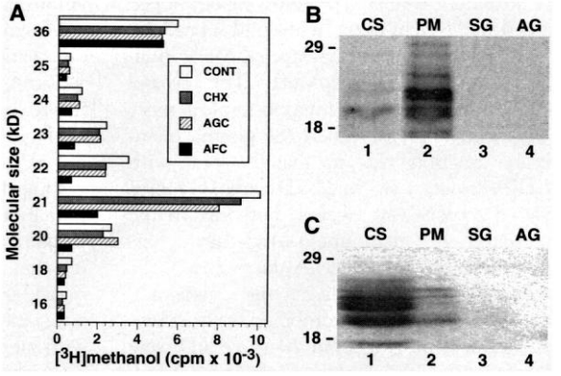

Fig. 1. Metabolic labeling of neutrophil Ras-related proteins. Human neutrophils were incubated with either [^{3}H-methyl]methionine (350 to 500 μCi/ml in Hepes-buffered salt solution for 1 hour) (A and B) or [5-^{3}H]mevalonolactone (115 μCi/ml in Hepes-buffered Dulbecco's modified Eagle's medium with 25 μM lovastatin) (C). (**A**) Trichloroacetic acid (TCA) precipitates of cells labeled with [^{3}H-methyl]methionine in the presence or absence (Cont., control) of either cycloheximide (CHX) (5 μg/ml), AGC (100 μM), or AFC (100 μM) were analyzed for carboxyl methylation by SDS-PAGE followed by alkaline hydrolysis of excised bands and quantitation of volatilized [^{3}H]methanol as described (*22*). As reported previously (*7*), the AFC-insensitive 36-kD carboxyl-methylated protein was not prenylated and is not associated with CAAX processing. Cells labeled with [^{3}H-methyl]methionine (**B**) or [5-^{3}H]mevalonolactone (**C**) were fractionated into cytosolic (CS), plasma membrane (PM), specific granule (SG), and azurophilic granule (AG) compartments as described (*23*) and analyzed by SDS-PAGE and fluorography. The 20- to 24-kD region of the gel shown in (B) was analyzed for carboxyl methylation as in (A). Of the total alkaline-labile counts recovered, 62% were from PM, 23% from CS, 12% from SG, and 3% from AG.

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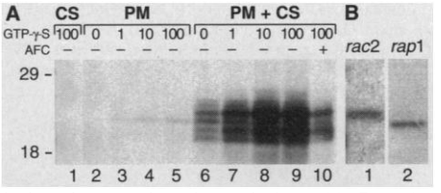

Fig. 2. Cell-free carboxyl methylation: stimulation by GTP-γ-S. (**A**) Neutrophil cytosol (100 μg, lane 1) or plasma membrane (25 μg) in the absence (lanes 2 to 5) or presence (lanes 6 to 10) of cytosol (100 μg) with the indicated concentration of GTP-γ-S (μM) were incubated for 60 min with [^{3}H]AdoMet (1.2 μM; 73 Ci/mmol) in 0.1 M NaH_2PO_4-Na_2HPO_4 (pH 6.8) containing 1 mM EDTA. AFC (100 μM) was added to the reaction shown in lane 10. Reaction products were analyzed by 12% tris-glycine SDS-PAGE and fluorography (1-week exposure). We confirmed carboxyl methylation of all labeled proteins by excising bands and quantitating alkaline-volatilized counts as in Fig. 1A. Molecular size markers are shown at left (in kilodaltons). (**B**) Neutrophil proteins labeled with [^{3}H]AdoMet as above (conditions identical to A, lane 9) were denatured and immunoprecipitated with antiserum sc-96 against p22^{rac2} (lane 1) or MAb 142-24E5 against p21^{rap1} (lane 2).

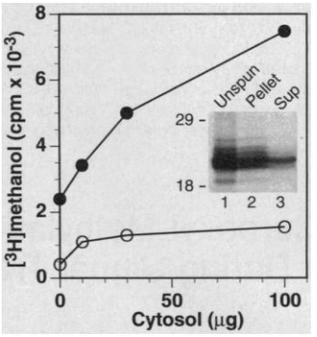

Fig. 3. GTP-γ-S–stimulated carboxyl methylation of cytosolic Ras-related proteins leads to membrane association. Neutrophil plasma membranes (35 μg) in the absence (○) or presence (●) of 50 μM GTP-γ-S were incubated with [^{3}H]AdoMet as in Fig. 2 and with varying amounts of cytosol as indicated along the abscissa. Reaction products (p20–24) were analyzed for carboxyl methylation as in Fig. 1A. The ordinate gives the cpm of an experiment representative of three. (**Inset**) The reaction products of [^{3}H]AdoMet labeling of plasma membrane (35 μg) and cytosol (100 μg) were applied directly (lane 1) or after separation into 100,000*g* pellet (lane 2) and supernatant (lane 3) to SDS-PAGE and visualized by fluorography.

methylation of the 20- to 24-kD proteins (p20–24), indicating that the carboxyl methylation was prenylcysteine specific. This effect was not observed with *N*-acetyl-*S*-*trans*-geranyl-L-cysteine (AGC), a prenylcysteine analog that does not inhibit p21^{ras} methylation. Metabolic labeling of neutrophils with a polyisoprene precursor, [5-^{3}H]mevalonolactone, labeled a set of 20- to 24-kD proteins that comigrated with the carboxyl-methylated species. One of these proteins was immunoprecipitated by a monoclonal antibody (MAb) to p21^{rap}. Unlike carboxyl methylation, prenylation of neutrophil 20- to 24-kD proteins was abolished by cycloheximide. Subcellular localization of [^{3}H-methyl]methionine-labeled versus [5-^{3}H]mevalonolactone-labeled p20–24 also revealed a difference. Whereas carboxyl-methylated p20–24 were predominantly found in the plasma membrane fraction (Fig. 1B), the newly synthesized prenylated species were cytosolic (Fig. 1C). These data suggest that nascent, prenylated Ras-related proteins initially enter a cytosolic pool and are subsequently carboxyl methylated upon membrane association.

To further analyze carboxyl methylation of neutrophil Ras-related proteins, we developed a cell-free system using neutrophil subcellular fractions and ^{3}H-methyl–labeled *S*-adenosyl-L-methionine ([^{3}H]AdoMet). The cytosol showed no carboxyl methyltransferase activity (Fig. 2A, lane 1). When the plasma membrane fraction was incubated alone with [^{3}H]AdoMet, a single 21-kD carboxyl-methylated protein was labeled, but only in the presence of guanosine-5-*O*-(3-thiotriphosphate) (GTP-γ-S) (Fig. 2A, lanes 2 to 5). In contrast, when the plasma membrane and cytosol fractions were combined, at least seven proteins ranging from 20 to 24 kD were labeled by [^{3}H]AdoMet. Carboxyl methylation of each of these proteins was GTP-γ-S dependent (Fig. 2A, lanes 6 to 9). This is consistent with previous observations in a murine-macrophage cell line and rabbit brain (*8*) as well as in human platelets (*9*). GTP was much less potent than GTP-γ-S in augmenting carboxyl methylation, and guanosine-5′-*O*-(2-thiodiphosphate) had no effect. AFC inhibited carboxyl methylation of each of these proteins (Fig. 2A, lane 10). Two of the carboxyl-methylated Ras-related proteins were identified by immunoprecipitation as p21^{rap1} and p22^{rac2} (Fig. 2B).

Neutrophil carboxyl methyltransferase activity was further analyzed with AFC as an artificial substrate (*10*). AFC was efficiently methyl esterified in the presence of [^{3}H]AdoMet by neutrophil plasma and specific granule membrane fractions but not cytosol, confirming a membrane localization for carboxyl methyltransferase activity similar to that described in other mammalian tissues and in yeast (*7*, *11*). Unlike carboxyl methylation of endogenous substrates, methyl esterification of AFC was not affected by GTP-γ-S, suggesting that the methyltransferase is not directly modulated by GTP. In contrast to the [^{3}H]AdoMet labeling of endogenous p20–24 substrates, which was cytosol-dependent (Fig. 3), plasma membrane–mediated methylation of AFC was inhibited by neutrophil cytosol in a GTP-dependent fashion. This argues against a cytosol-derived positive regulator of carboxyl methyltransferase and suggests that endogenous, activated cytosolic Ras-related proteins compete with AFC for carboxyl methyltransferase. The cytosolic localization of a GTP-sensitive element was confirmed by the observation that cytosol treated with GTP-γ-S and subsequently dialyzed supported enhanced carboxyl methylation of p20–24. GTP-γ-S is therefore likely to act at the level of the GTP-binding protein substrates, suggesting that in the active, GTP-bound conformation these proteins are more efficiently carboxyl methylated. This raises the possibility that carboxyl methylation functions in signal transduction, perhaps by facilitating the association of activated Ras-related proteins with membrane targets.

Although the majority of substrates appeared to be derived from the cytosol, the bulk of the carboxyl-methylated p20–24 sedimented with membranes (Fig. 3, inset), suggesting translocation of these proteins followed by their carboxyl methylation and stable membrane association. We obtained direct evidence of an activation-dependent translocation to the plasma membrane of p22^{rac2}. This Ras-related protein has been isolated from neutrophil cytosol in a complex with RhoGDI [guanosine diphosphate (GDP)–dissociation inhibitors] and shown to activate the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that assembles at the plasma membrane (*12*). GTP-γ-S induced the translocation of p22^{rac2} but not RhoGDI to the plasma membrane (Fig. 4). These results suggest that activation of cytosolic Ras-related proteins occurs before their translocation

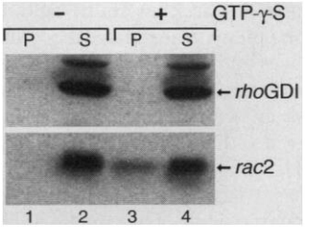

Fig. 4. GTP-dependent translocation of p22^{rac2}. Neutrophil plasma membranes (25 μg) and cytosol (100 μg) were incubated without (lanes 1 and 2) or with (lanes 3 and 4) GTP-γ-S (100 μM) and then separated into 100,000*g* membrane pellet (P) (lanes 1 and 3) and supernatant (S) (lanes 2 and 4). Samples were subjected to immunoblot analysis with peptide antisera 5066 (top panel) that recognizes RhoGDI (D. Rotrosen, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland) and sc-96 (bottom panel) directed against p22^{rac2}. Results are representative of five experiments.

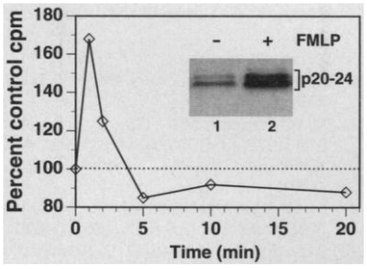

Fig. 5. Carboxyl methylation is stimulated in whole cells and in a cell-free system by FMLP. Neutrophils were metabolically labeled with [^{3}H-methyl]methionine as in Fig. 1 and then incubated with or without 100 nM FMLP for the times indicated along the abscissa before TCA precipitation. Carboxyl methylation of 20- to 24-kD proteins was quantitated as in Fig. 1A. Results are expressed as the percentage of counts detected in FMLP-stimulated cells relative to control cells (2740 versus 1630 cpm at 1 min) and are representative of four independent experiments. (**Inset**) Neutrophil plasma membranes (35 μg) and cytosol (50 μg) were incubated with [^{3}H]AdoMet as in Fig. 2 (without GTP-γ-S) in the absence (lane 1) or presence (lane 2) of 100 nM FMLP for 1 min. Reactions were stopped with SDS sample buffer, and products were analyzed by 15% tricine SDS-PAGE and fluorography to more tightly focus p20–24. Analysis of carboxyl methylation as in Fig. 1A revealed a 2.4-fold increase in the presence of FMLP. Results shown are representative of three experiments.

to membranes and subsequent carboxyl methylation.

To determine whether activation of neutrophils would induce carboxyl methylation of Ras-related proteins, we stimulated cells with the chemoattractant FMLP and assayed the carboxyl methylation of p20–24 (Fig. 5). FMLP induced a transient increase in the carboxyl methylation of p20–24 with a peak of 153 ± 9% (mean ± SEM, n = 4) of control at 1 min and a return to baseline or below by 5 min. These data are similar to those obtained by O'Dea *et al.* (*13*), who analyzed carboxyl methylation of total cellular proteins from FMLP-stimulated rabbit neutrophils. Moreover, the kinetics of p20–24 carboxyl methylation are consistent with those of second messengers in neutrophils such as cytosolic calcium and diacylglycerol (6, *14*). The effect of FMLP on carboxyl methylation in vivo was also observed in vitro. Neutrophil plasma membranes and cytosol were labeled with [^{3}H]AdoMet in the presence or absence of FMLP (Fig. 5, inset). After a 1-min incubation there was a marked increase in carboxyl methylation of all labeled p20–24. From these data we conclude that activation of neutrophils by formyl peptides leads to carboxyl methylation of multiple Ras-related proteins, most likely by initiation of GTP-GDP exchange.

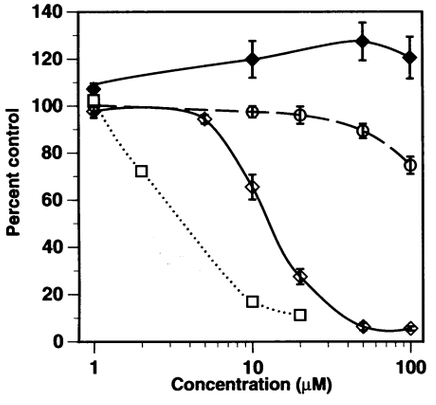

Fig. 6. The effects of prenylcysteine compounds on neutrophil superoxide anion production. Neutrophils were incubated in Hepes-buffered salt solution with or without the indicated concentrations of AFC (◇, ◆), AGC (○), or AGGC (□) for 10 min and then assayed for FMLP- (open symbols) or PMA- (◆) induced O_2^- generation as the superoxide dismutase-inhibitable reduction of cytochrome c (*24*). Results are expressed as the percentage of O_2^- produced by cells incubated in buffer alone and are given as the mean ± SEM for AGC and AFC (n = 9) and a representative experiment for AGGC (n = 2).

To further investigate the role of carboxyl methylation, we studied the effects of prenylcysteine analogs that were previously shown to enter cells and inhibit carboxyl methylation of p21^{ras} (*7*) on FMLP-induced stimulus-response coupling (Fig. 6). AFC inhibited FMLP-induced superoxide anion (O_2^-) generation in a dose-dependent fashion with a 50% inhibitory concentration (IC_{50}) of 15 μM. This value is similar to the Michaelis constant (K_m) (20 μM) of AFC for carboxyl methyltransferase (*10*). *N*-Acetyl-*S-all trans*-geranylgeranylcysteine (AGGC), an analog of the geranylgeranylated COOH-termini of the predominant Ras-related proteins found in neutrophils (*15*), was a more effective inhibitor of FMLP-induced O_2^- generation (IC_{50} = 4 μM). This correlated with the lower K_m (7 μM) of AGGC for carboxyl methyltransferase (*16*). AGC, an ineffective inhibitor of p21^{ras} carboxyl methylation, had little effect on O_2^- generation. Doses of AFC up to 100 μM caused no leakage of the cytosolic marker enzyme lactate dehydrogenase from neutrophils. AFC did not inhibit phorbol myristate acetate (PMA)–induced neutrophil O_2^- production, which confirms both the viability of neutrophils incubated with AFC and the stimulus dependence of the inhibition.

Because PMA bypasses surface receptors and directly activates protein kinase C, our data suggest that prenylcysteine methylation inhibitors act upstream of protein kinase C in receptor–G protein–mediated signal transduction. Recent studies have implicated the neutrophil Ras-related proteins p20^{rap1A} (*17*), p21^{rac1}, and p21^{rac2} (*12*) as candidates for the factor (or factors) conveying GTP dependence on the cell-free NADPH oxidase system. This raises the possibility that AFC directly inhibits activation of the oxidase. However, AFC also inhibited FMLP-induced neutrophil homotypic aggregation and secretion from primary granules, supporting a proximal site of action in stimulus-response coupling as was suggested by the lack of inhibition of PMA-induced O_2^- generation. These data suggest that carboxyl methylation of one or more Ras-related proteins is an essential reaction in the pathway of chemoattractant-mediated signal transduction.

We have demonstrated that neutrophil Ras-related proteins are carboxyl methylated in a GTP-γ-S– and FMLP-dependent fashion. Our data support the following model. Inactive, GDP-bound cytosolic Ras-related proteins remain soluble by association with GDI through their prenylated COOH-termini (*18*). This association is regulated by GDP-GTP exchange, as has been shown for p20^{rhoB} and RhoGDI (*19*). GDP-GTP exchange on cytosolic Ras-related proteins is influenced either directly or indirectly by heterotrimeric G protein signaling. Activation and release from GDI renders the COOH-termini accessible to carboxyl methyltransferase. Carboxyl methylation may promote nonspecific membrane association or facilitate the interaction of Ras-related proteins with specific membrane targets.

The discovery over a decade ago that bacterial chemotaxis is regulated by carboxyl methylation at glutamate residues of membrane receptor-transducer proteins (*20*) prompted speculation that, like protein phosphorylation, reversible carboxyl methylation may represent a general mechanism of covalently modifying regulatory proteins. Indeed, Pike *et al.* (*21*) reported that AdoMet-mediated methylation was required for chemotaxis of human monocytes, and recent results suggest that carboxyl methylation at prenylcysteine residues may be required for chemotaxis of murine macrophages (*7*). The results presented here demonstrate a link between carboxyl methylation of Ras-related proteins and signal transduction in human neutrophils.

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Altered Prevalence of Gating Modes in Neurotransmitter Inhibition of N-Type Calcium Channels

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G protein–mediated inhibition of voltage-activated calcium channels by neurotransmitters has important consequences for the control of synaptic strength. Single-channel recordings of N-type calcium channels in frog sympathetic neurons reveal at least three distinct patterns of gating, designated low-P_o, medium-P_o, and high-P_o modes according to their probability of being open (P_o) at −10 millivolts. The high-P_o mode is responsible for the bulk of divalent cation entry in the absence of neurotransmitter. Norepinephrine greatly decreased the prevalence of high-P_o gating and increased the proportion of time a channel exhibited low-P_o behavior or no activity at all, which thereby reduced the overall current. Directly observed patterns of transition between the various modes suggest that activated G protein alters the balance between modal behaviors that freely interconvert even in the absence of modulatory signaling.

A wide variety of neurotransmitters inhibit high voltage–activated Ca^{2+} channels in vertebrate neurons (*1*–*6*). At nerve terminals, this diminishes transmitter release and thereby contributes to presynaptic inhibition (*1*) or autoinhibition (*5*). Inhibitory modulation is exemplified by norepinephrine (NE) acting by means of α_2-adrenergic receptors to inhibit N-type channels (*1*, *4*, *5*, *7*). This modulation involves G proteins (*7*–*10*) acting by a fast, membrane-delimited pathway (*5*, *6*), but how this alters the activity of individual Ca^{2+} channels is unclear. On the basis of whole-cell recordings, several hypothetical mechanisms of down-modulation have been proposed: (i) channel block with no change in time- or voltage-dependent kinetics (*1*); (ii) modulation by the addition of an extra nonconducting state with kinetically slow connections to normal gating states (*9*, *10*); and (iii) modulation by shifts in the mode of gating, from a "willing" mode, where the channel can be opened by relatively weak depolarizations, to a "reluctant" mode, in which much stronger depolarizations are required (*4*, *11*, *12*). To test these hypotheses, we examined neurotransmitter modulation of unitary N-type Ca^{2+} channel activity (*5*, *13*).

In the absence of neurotransmitters, N-type Ca^{2+} channels in frog sympathetic neurons exhibit three prominent modes of gating (*13*). When evoked by pulses to a convenient test potential (−10 mV), the gating modes differ widely in their probability of being open (P_o) and are thus classified as high-, medium-, and low-P_o modes (*13*) (Fig. 1, A and B). In the high-P_o mode (Fig. 1A), channel openings are relatively long lasting and closings are short-lived, and P_o at −10 mV is typically ~0.5 or greater. In the medium-P_o mode (Fig. 1B), the openings are somewhat briefer and the closings longer. In the low-P_o mode, the openings are even briefer and the closings even more prolonged.

Figure 1 compares data collected from ten patches with no transmitter present and from ten patches where the patch pipette contained 100 μM NE, a concentration that produces nearly saturating inhibition with little desensitization (*5*). In the presence of NE, the mean patch current was less than half that in the control patches (Fig. 1, insets). Norepinephrine markedly affected the prevalence of high-P_o gating. In the control patches, high-P_o sweeps are represented by a cluster of data points, centered around a mean open duration ($\bar{t}_o$) of ~3 ms and a mean closed time ($\bar{t}_c$) of ~2 ms ($P_o \geq 0.45$). Under the influence of NE, high-P_o gating was largely absent (Fig. 1D).

The near-abolition of high-P_o gating conforms to the proposed NE-induced shift from willing to reluctant gating (*4*, *11*). However, even with high-P_o behavior set aside, the gating behavior that remained with NE was far from homogeneous, contrary to predictions of the willing-reluctant hypothesis for maximal neurotransmitter inhibition. The $\bar{t}_o$-$\bar{t}_c$ plot with NE (Fig. 1D) shows a significant negative correlation between $\bar{t}_o$ and $\bar{t}_c$ ($P < 0.001$), which is indicative of more than just one pattern of gating (*14*).

The dominant patterns of gating in the presence of NE are illustrated by the set of consecutive current records in Fig. 2A. Spontaneous switching between two kinds of gating is evident. One pattern, exemplified by sweeps 1 and 2 and 4 to 7, is qualitatively similar to the medium-P_o mode in control recordings (Fig. 1B). Another pattern, typified by sweeps 3 and 8 to 11, resembles low-P_o gating in the control.

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