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Novel Pharmacological Properties of Transient Potassium Currents in Central Neurons Revealed by *N*-Bromoacetamide and Other Chemical Modifiers

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SUMMARY

The chemical modifiers *N*-bromoacetamide (NBA), *N*-bromosuccinimide (NBS), and chloramine-T (ChT) are commonly used to remove fast inactivation of sodium currents and transient potassium currents (I_A). In the present study, I examined the effects of these chemical modifiers as well as of others on I_A in neurons dissociated from several brain regions. External application of NBA irreversibly inhibited I_A, with higher NBA concentrations increasing the rate of inhibition. The current kinetics, however, were not altered by external NBA at any concentration. I_A was also inhibited by internal NBA but only at high concentrations, and the rate of inhibition was much slower. The current kinetics were not altered by internal NBA at any concentration. NBA is a nonspecific chemical reagent that can modify a protein at several target amino acids. The NBA-induced irreversible inhibition of I_A was reproduced by external diethylpyrocarbonate, a reagent that specifically modifies histidine residues, and by ChT and cyanogen bromide, reagents that modify methionine residues through distinct mechanisms. However, NBS, a reagent that cleaves the peptide bond at tryptophan residues, had no effect on I_A, nor did chemical modifiers specific for cysteine and tyrosine residues. Taken together, these results suggest that the conserved, functionally important methionine and/or histidine residues are the likely targets for NBA modifications. These novel pharmacological properties are in sharp contrast to those known previously, despite their similarity in both kinetics and 4-AP sensitivities. Therefore, the pharmacological treatments presented in the present study should be useful for characterizing other I_A.

I_A activate rapidly and transiently in the presence of maintained membrane depolarization. These currents are generally involved in the setting of action potential duration and the firing frequency of cells (1). Because the inactivation rate of this current determines how long the current will remain activated, it is a potential target for regulating I_A activity and, thus, cellular activity. An example is the activation of thrombocytes in association with nearly complete removal of I_A inactivation (2). NBA, a widely used chemical modifier, also removes the fast inactivation of I_A in thrombocytes (2) and in GH_3 cells (3–6). This chemical has also been used on a widespread basis to remove fast inactivation of sodium currents (7–9). Because NBA is membrane permeable, the slowing of the fast inactivation of I_A may be achieved by applying NBA from outside (3–6) or from inside (2, 4, 6) the cell. Although the action sites of NBA are commonly believed to be located at the intracellular surface of the membrane, a separate, extracellular target for NBA has also been suggested (4).

I recently identified an I_A in suprachiasmatic nucleus neurons, the resting inactivation of which can be removed by external Zn^{2+} application (10). In an attempt to remove I_A inactivation with NBA, I found that NBA inhibited I_A instead of removing its fast inactivation, as in GH_3 cells and thrombocytes. NBA is a nonspecific chemical modifier that oxidizes the sulfur-containing amino acids, such as cysteine and methionine, and breaks the peptide bonds at the nitrogen-containing amino acids, such as histidine, tryptophan, and tyrosine (7). To elucidate the mechanisms underlying the NBA-induced inhibition, I examined the effects on I_A of chemical modifiers specific for these potential NBA targets. Experiments were performed using whole cell recording techniques with neurons dissociated from the suprachiasmatic nucleus and from other brain regions. The results of the present study indicate that in these neurons, NBA inhibits the I_A without altering its kinetics, including the inactivation rate. Furthermore, the NBA effect is mimicked by modifiers specific for histidine and methionine residues but not by

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ABBREVIATIONS: NBA, *N*-bromoacetamide; NBS, *N*-bromosuccinimide; ChT, chloramine-T; NAI, *N*-acetylimidazole; I_A, voltage-dependent transient potassium currents; SCN, suprachiasmatic nucleus; RCA, retrochiasmatic area; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); CNBr, cyanogen bromide; DEP, diethylpyrocarbonate; 4-AP, 4-aminopyridine; PCMPS, *p*-chloromercuriphenylsulfonic acid.

modifiers specific for cysteine, tryptophan, or tyrosine residues. These novel pharmacological properties reveal that the I_A in these neurons is remarkably different from those in GH_3 cells and thrombocytes, and the methodology presented in the study should therefore provide an additional pharmacological tool for studying other I_A.

Materials and Methods

Cell preparation. Central neurons were dissociated from three different brain regions: SCN, RCA, and the cortex. The neurons from the SCN and the RCA were acutely dissociated from postnatal (after P10) and adult albino rats (Sprague-Dawley) as previously described (10, 11) with some modifications. Briefly, the anterior hypothalamic slices (750 μm) containing SCN and RCA, respectively, were first preincubated in an enzyme-free solution at 37° for 2–8 hr as described (12). The incubation solution contained (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 25 glucose (pH 7.4 when continuously bubbled with 95% O_2/5% CO_2). Enzyme digestion was then carried out in the incubation solution containing 0.05–0.25% trypsin at 37° for 5–45 min. After enzyme digestion, the slices were transferred back to the enzyme-free incubation solution. When needed, the SCN or RCA was dissected from the hypothalamic slices, dissociated into isolated neurons, and plated onto coverslips coated with concanavalin-A (Sigma Chemical Co., St. Louis, MO). The isolated neurons were stored in incubation solution under an atmosphere of 95% O_2/5% CO_2.

Only undifferentiated cortical neurons from E18 rats were used in the study. The cortical neurons were acutely dissociated from the fetal cortex, which was dissected from the fetus after the fetus was removed from the pregnant rat. The isolated neurons were stored as described.

Recordings. Membrane currents were recorded with the whole cell, patch clamp technique (13) at room temperature (20–22°) as described previously (10, 11). The perfusing bath solution contained (in mM): 110 choline Cl, 30 TEA Cl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 0.2 $CdCl_2$, 10 TMA-HEPES, and 10 glucose (pH 7.4). TEA (30 mM) was added to block most of the delayed rectifier. Cd^{2+} (200 μM) was added to block the calcium currents and to remove the resting inactivation of I_A to allow its activation when stepped from a holding potential of −60 mV (10). The bath solution was admitted via a 1-mm-diameter capillary positioned a few millimeters from the recorded cell. Solution change around the cell was complete within a few seconds. The patch electrodes contained (in mM): 120 KCl, 10 K-HEPES, 10 K_2EGTA, and 1 $CaCl_2$ (pH 7.3). The liquid junction potential was ~7 mV and was not corrected. In some experiments (Fig. 1), 20 mM Na^+ substituting for the same concentration of K^+ was used to study outward sodium currents. Signals were low pass filtered at 2–2.5 KHz (eight-pole Bessel) and digitized on-line at 5 KHz (or 20 kHz for sodium current) via a 12-bit analog-digital digitizing board (Data Translation DT2821F-DI) with my program written in C Language. Linear leakage and capacitive currents were subtracted using a P/2 pulse protocol (14). Data were analyzed and plotted with custom-made programs. The current traces shown are leak subtracted.

Chemicals. The chemical reagents NBA, NBS, DTNB, PCMPS, DEP, ChT, CNBr, and NAI were purchased from Sigma Chemical Co. All chemicals were freshly made before the experiments, and some of the solution bottles were wrapped with aluminum foil to protect the solution from light. All solutions were adjusted to pH 7.4 with HEPES.

Results

NBA inhibits I_A. A 200-msec step depolarization to +30 mV activated an outward current in neurons dissociated from the SCN and RCA. At this potential, most SCN and RCA neurons have peak outward current amplitudes in the range of 200-1000 and 1000–2000 pA, respectively. The outward currents contain both transient components (I_A) and sustained components, the latter of which are probably the residual delayed rectifiers not blocked by TEA. In contrast, the outward current in the fetal cortical neurons has only the transient component (I_A), with peak amplitude in the range of 100–300 pA at +60 mV. Although these I_A differ in size, their kinetics were similar (see later). Bath application of 100 μM NBA rapidly inhibited I_A (Fig. 1, A through C), leaving mostly the sustained components (Fig. 1, B and C). The inhibition appears to be irreversible, because no recovery of I_A amplitude was observed after NBA was washed out for as long as 10 min. The specific inhibition of I_A by NBA is also apparent from its lack of effect on I_{Na} within minutes of application (Fig. 1D). At a longer exposure time, however, the inactivation rate of I_{Na} appeared to slow slightly. The rates of I_A inhibition increased with greater concentrations of NBA. Fig. 1E (left) is a plot of the inhibitory time courses of I_A recorded from four different SCN neurons in response to NBA at four different concentrations: 10, 30, 100, and 1000 μM. At 1 mM, NBA also inhibited I_{Na} and the residual delayed rectifiers (not shown). However, no slowing of I_A inactivation rate was observed at any concentration. Because NBA is sensitive to light and temperature, the rate of inhibition also decreased with aging of the NBA solution (Fig. 1E, right). Taken together, these results indicate that the NBA compound irreversibly inhibits I_A.

NBA does not alter I_A kinetics. The inhibitory effect of NBA on I_A in these central neurons is in sharp contrast to its action on slowing the inactivation rate of I_A in both GH_3 cells (3–6) and newt thrombocytes (2). To better determine whether NBA alters I_A kinetics, the outward currents of Fig. 1 (A through C) are plotted again in Fig. 2. On the left are shown superimposed outward currents elicited during NBA application. As clearly indicated in the figure, although the peak amplitude is dramatically reduced, there is no obvious change in the current kinetics. Also notable is the persistent presence of the residual delayed rectifiers in both SCN and RCA neurons. Better comparison of the NBA-sensitive I_A in these neurons can be achieved when the NBA-insensitive sustained currents are subtracted from the total outward currents (right). The similarity between the NBA-sensitive I_A in the three different central neurons is apparent. After proper scalings, the I_A current traces (right) can be almost perfectly superimposed (insets). The results clearly indicate that NBA does not alter the I_A kinetics.

NBA acts at or near extracellular sites of I_A channels. NBA is membrane permeable and has been shown to remove I_A fast inactivation when applied at either side of the membrane (2–6). The question arises of whether internal NBA, in contrast to external NBA, can remove inactivation of I_A. In all of the experiments with low concentrations (10–100 μM) of internal NBA (n = 10; 10–30 min in the whole cell mode), no effect was observed on I_A. Therefore, 1 mM NBA was used in all subsequent experiments. In general, the results for high internal NBA experiments are as follows: in the absence of bath perfusion, on breaking into the whole cell mode, the I_A current amplitude remained fairly constant for the first few minutes and then gradually declined (Fig. 3). However, during the declining phase, bath perfusion abolished the slow inhibition and stabilized the current amplitude in some cells (Fig. 3A; n = 2), as if the NBA molecules,

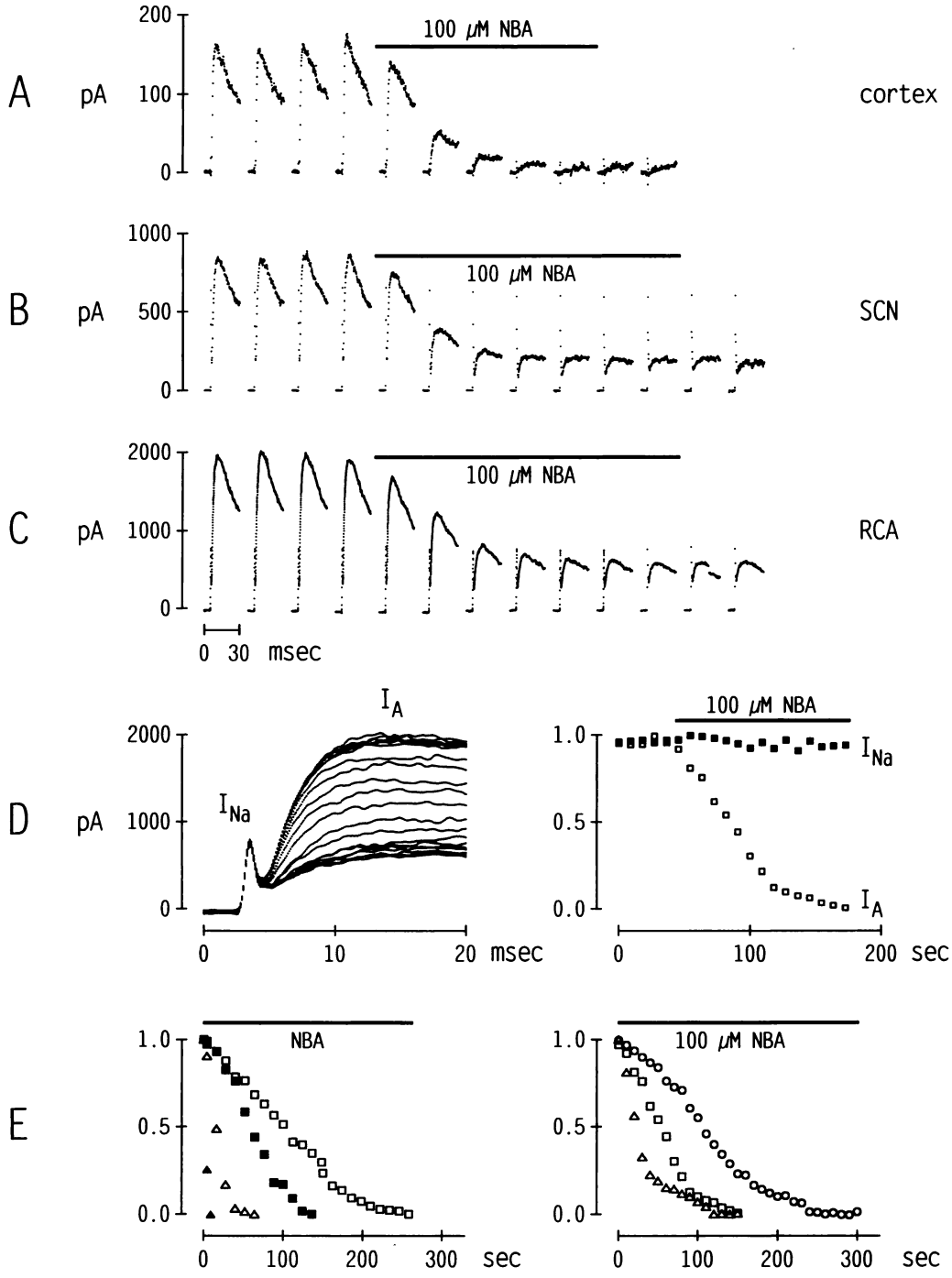

Fig. 1. Inhibition of I_A by external NBA in central neurons. Whole cell transient potassium currents were activated every 10 sec from a holding potential of −60 mV to +60 mV in a cortical neuron (*A*) and to +30 mV in an SCN (*B*) and an RCA (*C*) neuron. NBA inhibited the I_A but not I_{Na} (*D*). Total outward currents I_A and I_{Na} were activated at +30 mV in a RCA neuron (*left*). Time courses of these two currents are plotted (*right*). Dependence is shown of inhibition rate of I_A on NBA concentration (*E*). *Left*, Plots of the response time course of I_A evoked at +30 mV to NBA at 10 μM (□), 30 μM (■), 100 μM (△), and 1000 μM (▲). *Right*, Plots of the time course of I_A (+30 mV) in the presence of 1-hr-old (△), 8-hr-old (□), and 24-hr-old (○) 100 μM NBA. *Straight lines*, time during which NBA was applied. I_A and I_{Na} amplitude were measured at the peak.

diffusing out of the cell to inhibit the I_A from the outside, were washed away by perfusion. Note that the current begins to decline again (dashed lines), with some delay, only after perfusion is halted, possibly indicating the time for NBA to diffuse outside to inhibit the current. However, in other cells, this perfusion effect is less obvious or not present (Fig. 3B; $n = 5$). Even in the continued presence of 1 mM internal NBA, which produced only very slow inhibition, bath application of 100 μM NBA still rapidly reduced I_A (Fig. 3A). This indicates that the rapid inhibition of I_A is mediated by NBA acting on the extracellular targets (see Discussion). Again, no slowing of inactivation rate was observed even with 1 mM internal NBA ($n = 11$). Fetal cortical neurons were not tested because of the small size of the cells and of the I_A amplitude and because of the difficulty involved in doing the experiments. Taken together, these results indicate that unlike in GH_3 cells (3–6) and thrombocytes (2), the kinetics of I_A in SCN and RCA neurons are not altered by internal NBA.

The NBA effect is reproduced by chemical modifiers specific for methionine or histidine residues. NBA is a nonspecific reagent that can modify proteins at cysteine, methionine, tyrosine, tryptophan, and histidine residues (see introductory paragraphs). To determine the potential targets for NBA modification, the effects on I_A of chemical modifiers specific for each of these residues were determined. A 2-min application of DTNB (Fig 4A; $n = 10$), NBS (Fig. 4B; $n = 6$), and NAI (Fig. 4E; $n = 6$) had no effect on I_A. However, the subsequent application of a 10-fold lower concentration of

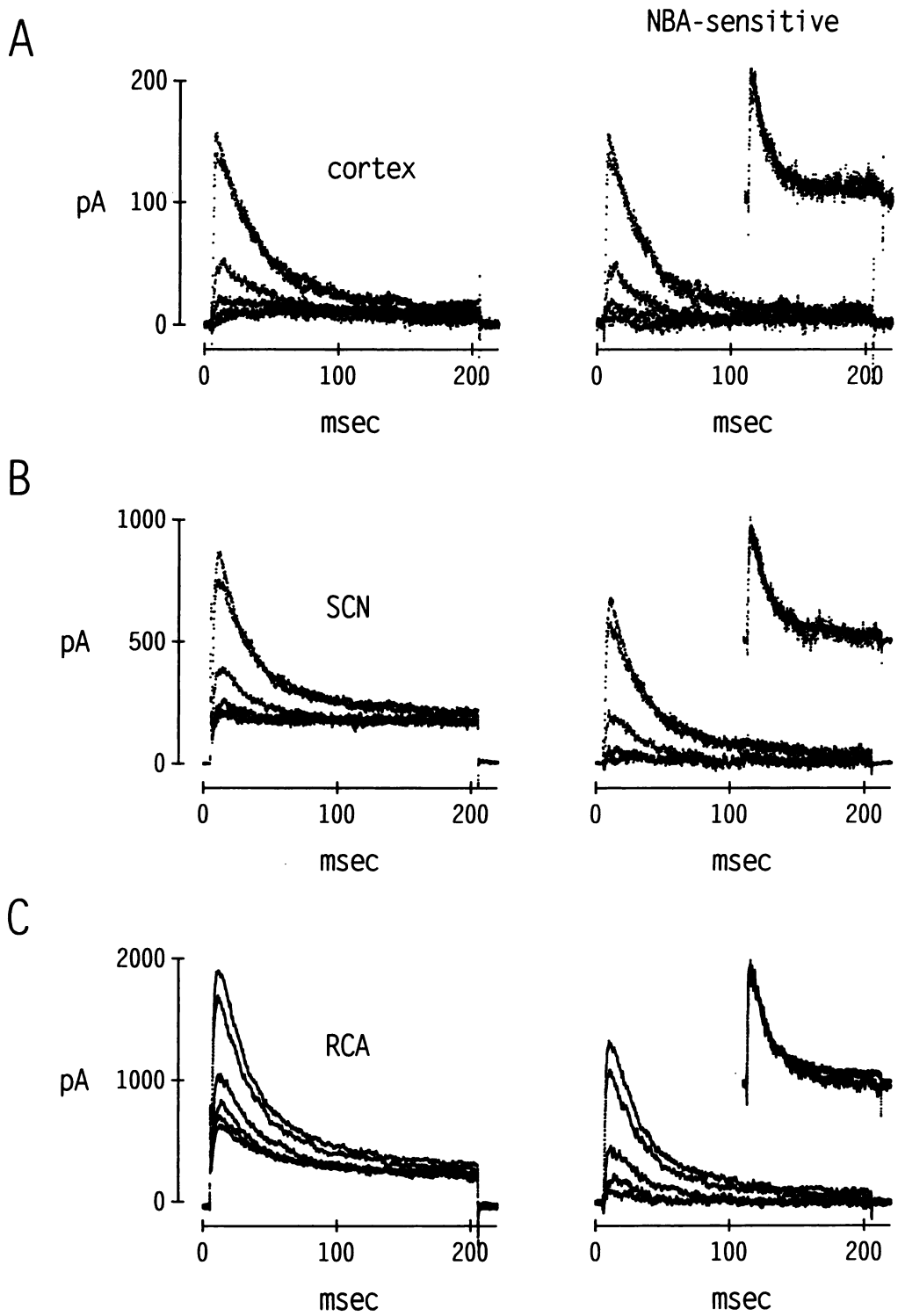

Fig. 2. Lack of effect of NBA on I_A kinetics. The transient components of outward currents decreased proportionally during NBA application (*A* through *C*, *left*). Current traces are from Fig. 1 (see text). Note that NBA inhibited only the transient (I_A) but not the sustained components. The NBA-sensitive currents were obtained after subtracting the NBA-insensitive current from the outward currents (*right*). *Insets*, first three scaled superimposed NBA-sensitive I_A traces.

NBA (100 μM) still rapidly inhibited I_A (Fig. 4, A through C). These chemical modifiers are specific for cysteine, tryptophan, and tyrosine residues, respectively (for reviews, see Refs. 15 and 16). PCMPS, another cysteine-specific modifier, also had no effect on I_A (n = 6). At a longer exposure time, however, NBS appeared to slightly inhibit the I_A (not shown). In contrast, 1 mM DEP, a histidine-specific modifier, irreversibly inhibited I_A (Fig. 4D; n = 10). Nevertheless, the inhibition was incomplete (n = 10). However, 1 mM ChT, a methionine modifier, completely inhibited I_A in an irreversible manner (Fig. 4E). CNBr (1 mM), a reagent that cleaves peptide bonds at methionine residues, also rapidly eliminated I_A (see Fig. 4, *inset*). Taken together, the results reveal that the histidine and methionine residues on the I_A channel protein are the likely targets for NBA modifications.

Discussion

The results of the present study demonstrated an inhibitory effect of NBA on I_A in central neurons dissociated from

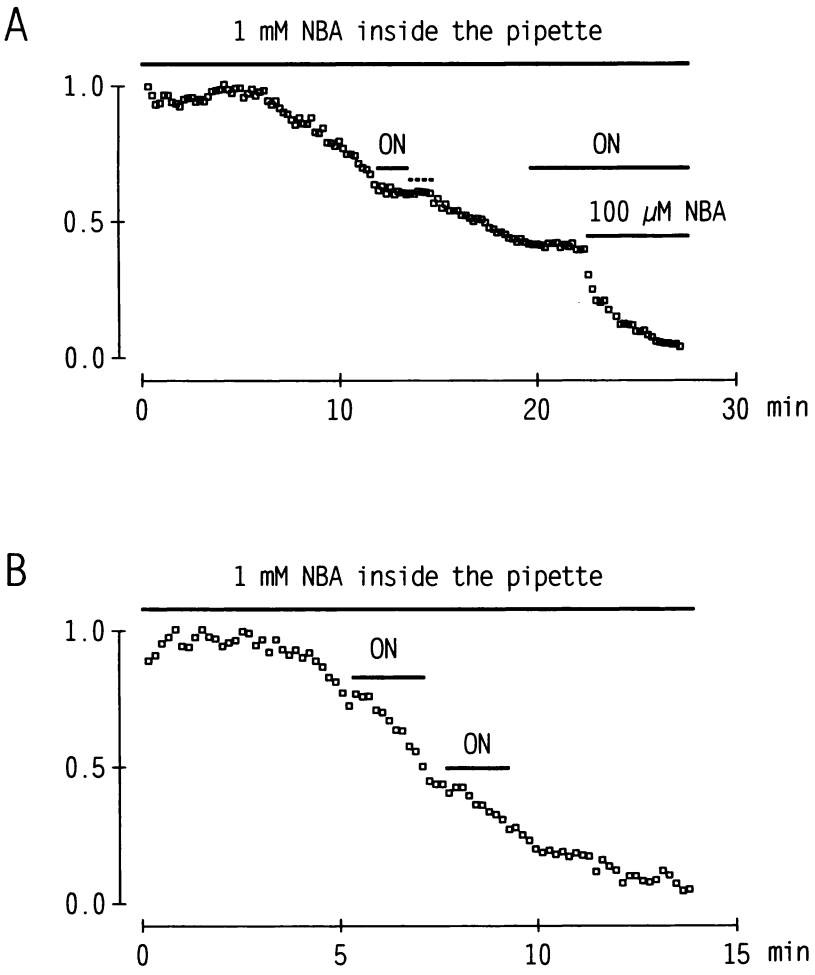

Fig. 3. Slow inhibition of I_A by high internal NBA. NBA (1 mM) was present in the pipette during the recordings. I_A was evoked by a depolarizing pulse to +30 mV. Inhibition began with some delay and proceeded slowly. *A*, Turning on the perfusion rapidly stabilized the current amplitude. Note that the current only began to decline again with some delay (*dashed lines*) after turning off the perfusion (see text). A 10-fold lower concentration of external NBA rapidly reduced I_A, as opposed to the slow onset and slow inhibition by internal NBA. *B*, In this cell, perfusion did not appear to have clear effect on I_A.

three different brain regions. The inhibition is rather specific, because at concentrations of <100 μM, as typically used in the present study, NBA has no effect on the sodium currents and delayed rectifiers. Furthermore, no alterations of I_A kinetics (including the inactivation rate) are observed with external and/or internal NBA. This observation is remarkably different from the well known effects of NBA in removing fast inactivation of I_A in GH_3 cells and thrombocytes.

It could be argued that the inability to detect an alteration of the inactivation rate can be attributed to the rapid inhibition by NBA, which then masks the slow removal of the inactivation rate. This may not be the case, however, because the slowing of the inactivation rate by external NBA in GH_3 cells is fast (1–100 μM NBA slows the inactivation rate in 1–30 sec [4]). During this period, the I_A in central neurons is only partially inhibited by NBA (inhibition complete in 1–4 min at 10–100 μM NBA; Fig. 1E). If slowing of inactivation did occur, I would have observed the reduction of inactivation rate along with the reduction of peak amplitude. This never happened in these central neurons. Even with internal NBA, the reported values for slowing I_A inactivation rate are 1–10 min (2, 4). However, in the present study, even with 1 mM internal NBA for more than 10–30 min, no slowing of inactivation was observed. The only reasonable conclusion is that these I_A are different, probably reflecting distinct molecular identities.

The rapid inhibition of I_A is apparently mediated by NBA acting on its targets at or near the outside of the membrane. This conclusion comes from the observation that in the presence of 1 mM internal NBA, which produced only very slow inhibition, a 10-fold lower concentration of external NBA still rapidly inhibits I_A. If external NBA had to cross the membrane to inhibit I_A from inside the cell, then 1 mM internal NBA should have totally and very rapidly eliminated the current, as did 1 mM external NBA (Fig. 1E). Nevertheless, this does not rule out the possibility of additional intracellular NBA targets.

The slow inhibition of I_A induced by high concentrations of internal NBA suggests three possibilities. First, NBA molecules may simply diffuse out of the cell to inhibit I_A from outside. Second, the slow rate of I_A inhibition may be a result of a very different nature of internal NBA targets, as opposed to its external targets. Third, high internal NBA may slowly damage the cell and cause irreversible decline of the current. The first possibility is supported by the repeatable observation that perfusion abolishes the slow inhibition and stabilizes the current (Fig. 3A). Perfusion totally eliminates any further inhibition, suggesting that the inhibition is caused by NBA molecules that diffuse out of the cell and are then washed away by perfusion and arguing against the second possibility. Nevertheless, in more than half of the experiments, this perfusion effect is less obvious or not present. It is tempting to suggest that in these cases, the cells are deteriorating. This is not unlikely, because external NBA at

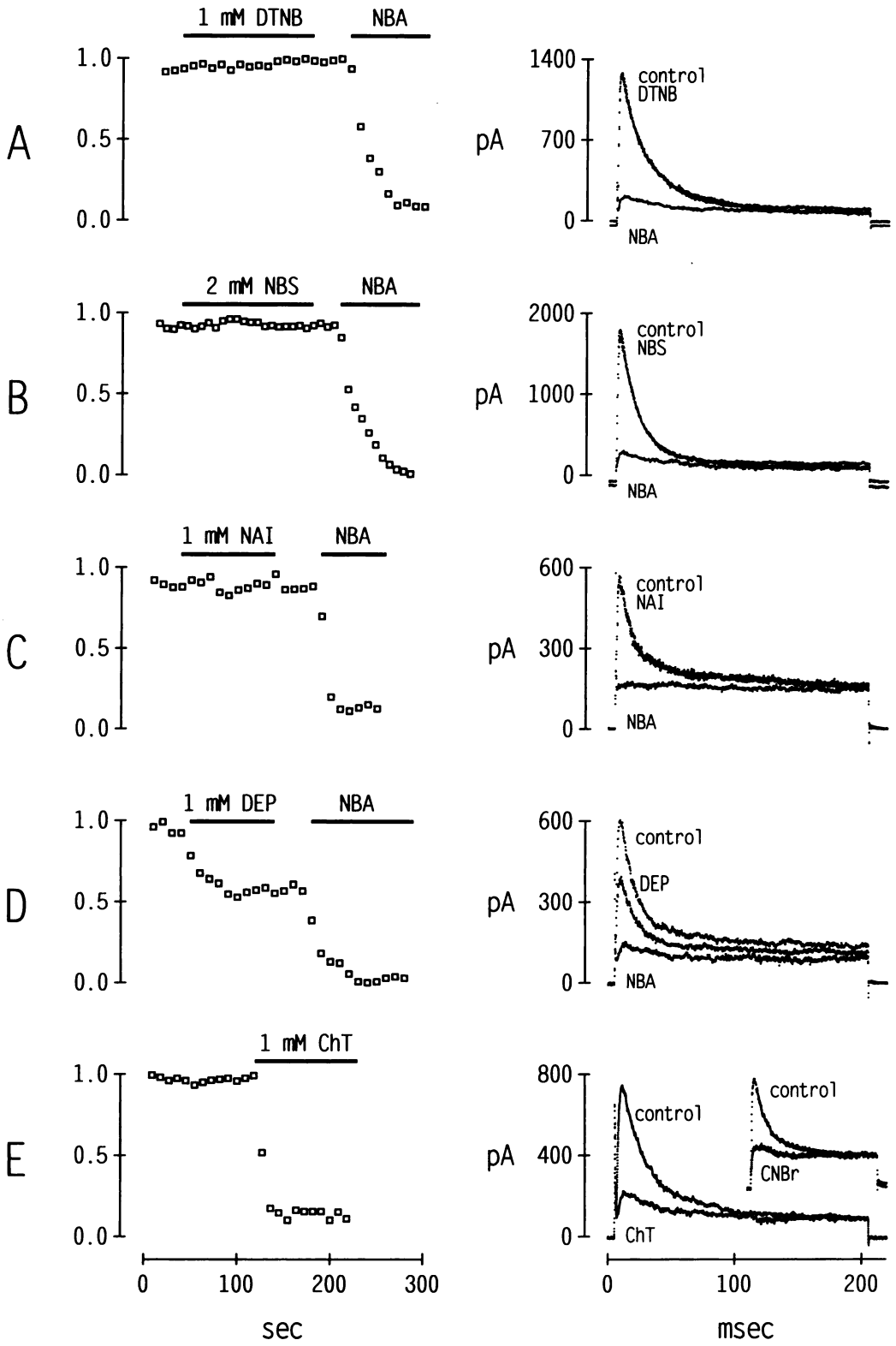

Fig. 4. Chemical modifications of I_A by reagents specific for cysteine, tryptophan, tyrosine, histidine, and methionine residues. *Straight lines*, duration of application of chemical modifiers DTNB, NBS, NAI, DEP, and ChT. *Left*, Time courses of I_A, evoked by a depolarizing pulse to +30 mV. *Right*, Representative current traces. DTNB (*A*), NBS (*B*), and NAI (*C*) had no effect on I_A and did not prevent NBA from inhibiting I_A. Only modifications of histidine (*D*) and methionine (*E*) residues were able to reproduce NBA effects. Note that DEP at 1 mM only partially inhibited I_A. *Inset*, Inhibition of I_A by CNBr.

1 mM also slowly increases the leak conductance of SCN neurons. The increase in the leak conductance could indicate deterioration of the cell condition. A similar increase in leak current in response to prolonged exposure to NBA has also been reported (7). Although the presence of internal NBA targets are not certain at this point, the rapid, irreversible inhibition of I_A caused by NBA is clearly mediated by acting on targets located at or near the extracellular surface.

NBA has several potential targets, but only modifiers of methionine and histidine residues are able to reproduce the NBA effects, suggesting that NBA exerts its effect by modifying these two residues on the I_A channel proteins. Note that

although both CNBr and ChT covalently alter methionine residues, their actions are distinct (15, 16). CNBr acts by cleaving the peptide bond, and ChT, similar to NBA, acts by oxidizing it. Likewise, the mechanisms of modification of the histidine residues by DEP and NBA are different. NBA acts by breaking the peptide bond at histidine residues, whereas DEP acts by ethylcarboxylating the nitrogen at position 1 or 3 of the imidazole ring of histidine (15, 16). However, unlike NBA, DEP at 1 mM only partially inhibited I_A. It is possible that higher concentrations might cause complete inhibition. The observation that modification of these two residues by different methods yields the same results suggests that methionine and/or histidine residues are important in the normal function of I_A channels. Because the I_A in each of these central neurons respond similarly to these chemicals, these residues are probably conserved, at least among the channels in these neurons.

On the contrary, chemical modifiers for cysteine (DTNB and PCMPS), tryptophan (NBS), and tyrosine (NAI) have virtually no effect on I_A, and at the same time they do not prevent NBA from eliminating I_A. A simple explanation is that these residues are not responsible for the NBA effects. However, it is possible that these chemical modifiers simply do not reach the NBA targets. It should be noted that 200-fold higher concentrations of NBS (2 mM NBS versus 10 μM NBA) still cannot reproduce the NBA effect. This is unlike previous results showing that NBS mimics, although less effectively, the effects of NBA in removing the fast inactivation of I_A and sodium currents (4, 7, 17).

I_A are characterized conventionally by rapid activation and inactivation and by sensitivity to 4-AP (18, 19), although native I_A channels in different tissues vary widely in their kinetics and pharmacological profiles (1). For comparison, I_A in both SCN neurons and GH_3 cells have rather similar inactivation time constants (~20–30 msec between +10 and +60 mV in SCN neurons [10]; 21 msec between −10 and +90 mV in GH_3 cells [4]) and 4-AP sensitivities (complete inhibition by 5 mM 4-AP in SCN neurons [10]; complete inhibition by 3.5 mM 4-AP in GH_3 cells [4]). However, their pharmacological responses to NBA and other chemical modifiers are remarkably different. In thrombocytes and GH_3 cells, NBA, NBS, and ChT remove fast inactivation of I_A and increase I_A amplitude (2–6; ChT effect is noted in Ref. 20). Similar effects of these chemicals are observed on sodium channels (7–9, 21, 22). In contrast, in the central neurons described in the present study, ChT and NBA irreversibly inhibited I_A without altering its kinetics, and NBS was without effect. This indicates that although the conventional characterization of I_A reveals similarities, the responses of these currents to chemical modifiers are very different. Therefore, the pharmacological treatments discussed in the present study should be useful in characterizing other I_A.

The mechanisms underlying these different pharmacological properties for central neurons and GH_3 cells are not clear. To further elucidate these differences, a certain degree of knowledge about the molecular identities of the proteins involved is probably required. Recent advances in molecular cloning and biophysical studies of I_A channels have indicated that the remarkable diversity of I_A channels might be due to a large number of subunits of K^+ channels along with their ability to form heteromultimeric channels, with kinetics and pharmacological characteristics that are different from those of homomultimeric channels (for review, see Ref. 23). In vertebrates, one member in each of the subfamilies Kv1.4 and Kv3.4 has been found to express a rapidly inactivating, A-type K^+ channel. Although the molecular identity of the subunit or subunits composing the I_A channel in the central neurons is not known, the I_A in the GH_3 cells has been suggested to be mediated by homomultimeric or heteromultimeric channels containing Kv1.4 subunits (23). It is possible that the differences lie in one or more of the subunits. Similarly, it is not unlikely to find other I_A channels that have a combination of properties of the I_A channels in GH_3 cells and in central neurons. Along that line of thinking, it is worth mentioning that in expressed noninactivating mouse Kv1.1 channels, *shaker* B peptide and human Kv3.4 peptide both inactivate the channels, but only the channels with human Kv3.4 have their inactivation rate slowed by NBA and ChT (24).

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