Early regulation of membrane excitability by ras oncogene proteins

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ABSTRACT Two electrode voltage clamp conditions were used to study the early effects on ionic membrane channels of the intracellularly injected proto-oncogenic form of c-Ha-ras (c-ras) and its oncogenic counterpart v-Ha-ras (v-ras). These experiments were conducted on isolated somata of identified fully differentiated neurons of the sea snail *Hermissenda*. 20 min after c-ras, and 10 min after v-ras intracellular injections into type B medial photoreceptors of *Hermissenda*, the peak amplitude of two outward potassium currents (I_A and I_C), across the isolated Type B soma membrane begin to decrease. These two currents have been previously isolated by differences in activation and inactivation kinetics and their response to pharmacological blockers. c- or v-ras injections did not have any effect on a voltage-dependent inward calcium current. Reduction of I_A preceded that of I_C. Current reductions due to c-ras, but not to v-ras injection reversed spontaneously after 40 min. The voltage dependence of the steady state inactivation of I_A shifted toward more negative potentials with ras injections. Ras-mediated cell transformations therefore, could involve, perhaps as initial events, prolonged modification of membrane currents.

INTRODUCTION

Ras gene products are a large family of 21 Kd proteins, widely distributed across species that have an essential, but as yet poorly characterized, cellular function (1–3). Because of their binding of guanine nucleotides and GTPase activity, they have been classified as G proteins (4). A cytosolic co-factor modulates ras activation and inactivation upon interaction with unknown plasma membrane target(s)(5). Mutating forms of ras have reduced GTPase activity and resistance to modulation by a cytoplasmic co-factor, the GTPase activating protein (GAP). Such forms may cause irreversible activation of biochemical pathways that control cell growth and development and that eventually lead to oncogenic transformation. Microinjections of ras have been shown to induce cell growth (6), pinocytosis, and membrane ruffling (7). The earliest effect reported so far of ras injection is a rapid increase in pH within 10 min of v-ras but not c-ras injections. This effect was attributed to an increase in the Na/H antiporter (8). Phosphoinositide metabolism was initially postulated as a ras target (3), but recent studies (9–11) have suggested nonspecific changes secondary to all oncogenic transformations.

We have previously isolated and characterized a 20-Kd G protein (12) which undergoes increased protein kinase C-mediated phosphorylation during long-term storage of an associative memory. This G protein also regulates axonal transport (13) as well as reduces K^+ currents. It, therefore, could be responsible for profound memory-specific cellular transformations such as the recently observed structural changes of neuronal terminal branches (14). The present report was undertaken to examine the possibility that ras proteins regulate potassium channels in a manner similar to that recently demonstrated for cp20.

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METHODS

Ras protein preparation

H-ras soluble proteins were isolated from *Escherichia coli* cells essentially as described previously (15). The prokaryotic vector (pJCL-30) places the *ras* mutants under the control of the temperature-inducible lambda pL promoter. 75% purity was first obtained on a AcA54 (molecular sizing) column of the expressed proteins and 90% purity after a FPLC mono Q column. After dialysis into 50% glycerol, they were stored at −20° C. Before use, proteins were solubilized in 10 mM tris, pH 8.0, 5 mM MgCl2, and 1 mM EDTA.

Cell isolation and recording

All the experiments were performed on the axotomized type B medial photoreceptors as previously described (12, 16, 17). Axotomized photoreceptors were impaled with two intracellular microelectrodes of ~15–20 MΩ and connected to a 7100 Small-cell Voltage-clamp amplifier (Pelagic Electronics, Falmouth, MA). The voltage electrode was filled with either v- or c-ras solution containing (in millimolar) Tris, 10; $MgCl_2$, 5; EDTA, 1; KCl, 20. 1 M KAc was used as a charge carrier. The current passing electrode was filled with 3 M KCl, and the

preparation was bathed in 100 mM TEA- artificial sea water, containing (in millimolar): NaCl, 330; KCl, 10; $CaCl_2$ 10; $MgCl_2$ 50; tris 10 with osmolarity 990 mOsm at pH 7.4. A high potassium artificial sea water solution was also employed for calcium current measurements, and contained (in mM): KCl, 300; NaCl, 35 $MgCl_2$ 50; $CaCl_2$ 10; Tris, 10; TEA, 100; 4-AP, 5. High external potassium in the bathing medium made it possible to measure voltage-sensitive calcium currents (I_{Ca}^{2+}) at the new equilibrium potential for potassium flux, 0 mV. Command steps to this level allow maximal activation of the calcium channel, in the absence of any net potassium flux. Pulse protocols, data acquisition, and analysis was performed with a computer system (pClamp; Axon Instruments, Burlingame, CA).

Immediately after impalement, cells were voltage-clamped to −60 mV, and dark adapted for 10 min. Current was sampled at 2 kHz and leak subtracted on-line using a P/8 protocol. A family of voltage-activated currents was elicited every 5 min with a series of 10 mV, 1 s depolarizing steps from −40 to 10 mV, with and without a 500-ms hyperpolarizing conditioning prepulse to −90 mV. Currents obtained were in agreement with previous reports for this preparation (12, 16, 17). A rapidly activating and inactivating outward voltage-dependent current peaking at ~15 ms and blocked by 4-AP (I_A), and a later outward current, measured at 250 ms and previously characterized as a calcium-dependent potassium current I_C were initially measured. All the experiments were carried out in darkness.

Intracellular injections

After stable baseline outward currents were obtained, the recording condition was switched from the voltage-clamp to the current-clamp mode and iontophoretic injection of ras was accomplished by applying a −2 nA constant negative current for 2 min through the voltage recording electrode. Compensation for the hyperpolarizing effects of the injection was provided by injecting current of opposite polarity through the current electrode. Electrodes were loaded with 70 μg/ml of v-ras or 150 μg/ml of c-ras. The electrode volume was <10 μl, and we estimate that the electrode contains ~45×10^8 and 21×10^8 c- and v-ras proteins, respectively, within 100 μm of the tip. An exact measure of the actual amount of protein injected is not possible with this technique. In a related study on a central *Hermissenda* neuron (Collin, C., A. G. Papageorge, D. Lowy, and D. L. Alkon, manuscript submitted for publication), pressure injection effects could be compared with the effect of iontophoretic injection to allow estimation of the number of molecules necessary for the observed effects. In some experiments, fast green (2 mg/ml) was also added to the ras solution to confirm injection of the electrode solution into the cell. Buffer alone or heat-inactivated ras (90° C for 2 h) were used as controls. After injections, the recording condition was switched back to the voltage clamp mode.

RESULTS

Effects of ras injections on I_A and I_C

In 100 mM TEA asw it was possible to observe both I_A and I_C in the absence of the delayed rectifier, which is minimally activated at membrane potentials ≤0 mV (15). Current amplitudes and current-to-voltage relationships were in agreement with data previously reported for this preparation, and are shown in Fig. 1 and Table 1. No significant changes of peak amplitudes of either current were observed immediately after ras injections or inactive controls. It was possible to keep viable recordings with no run-down for periods longer than 60 min. 10 min after v-ras (Fig. 2 *A*) and 20 min after c-ras injections, small and progressive reductions of I_A were observed, without significant changes of I_C. Reductions of I_A by ~30–40%, were clearly significant 30 min after either c- or v-ras injection ($p \leq 0.002$, student's *t*-test), (Table 1). 40–60% reductions of I_C developed progressively as well, and

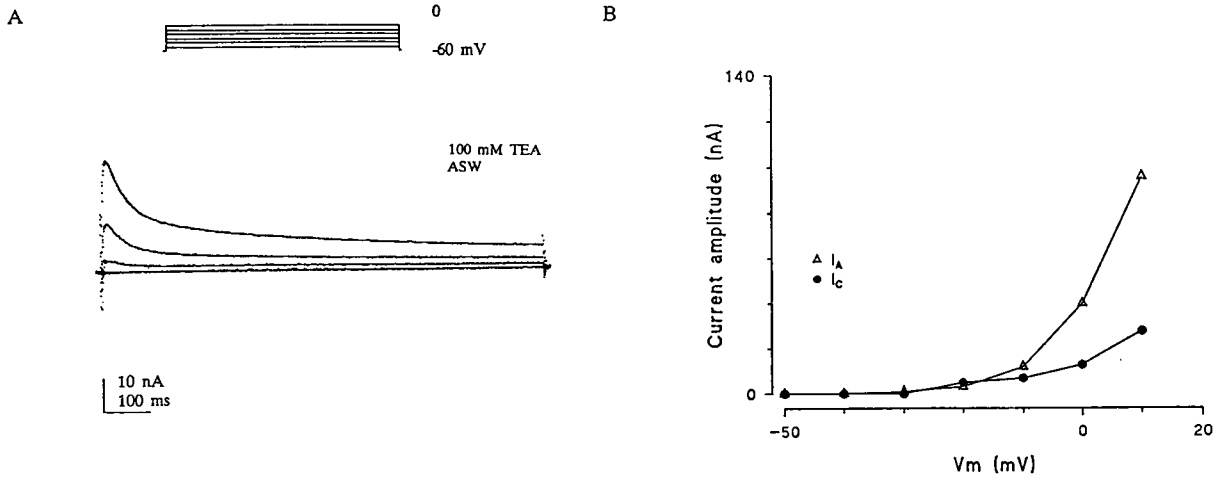

FIGURE 1 Two electrode voltage clamp recordings from an axotomized medial type B photoreceptor in darkness. (*A*) Under TEA containing ASW, cells were held at −60 mV and 10 mV-500 ms steps from −60 to 0 mV were delivered. A voltage dependent, rapidly activating and inactivating outward current, peaking at ~15 ms and blocked by 4-AP is followed by a later, slowly inactivating outward current that peaks at 200 ms. (*B*) Current to voltage relationship is shown for the same cell in *A*.

TABLE 1 I_A Amplitude at O mV (*nA*). Values are means ± SEM

	Baseline	10 *min*	20 *min*	30 *min*	60 *min*
c-ras ($N = 5$) 150 *μg/ml*	44 ± 5	42 ± 3	29 ± 4*	33 ± 4*	46 ± 3
v-ras ($N = 5$) 70 *μg/ml*	46 ± 3	31 ± 2‡	27 ± 4*	25 ± 3*	24 ± 3*
Boiled *c-* + *v*-ras 150 *μg/ml* ($N = 7$)	46 ± 3	43 ± 2	45 ± 4	46 ± 5	43 ± 4
Buffer alone ($N = 5$)	44 ± 5	46 ± 3	40 ± 4	45 ± 3	43 ± 5
I_C Amplitude at O *mV* (*nA*)					
c-ras ($N = 5$) 150 *μg/ml*	13 ± 2	12 ± 1	11 ± 2	6 ± 2*	12 ± 2
v-ras ($N = 5$) 70 *μg/ml*	13 ± 1	13 ± 2	8 ± 1*	8 ± 2*	6 ± 1*
Boiled c- + v-ras 150 *μg/ml* ($N = 7$)	14 ± 2	12 ± 3	14 ± 2	13 ± 2	12 ± 3
Buffer alone ($N = 5$)	12 ± 4	13 ± 2	12 ± 3	11 ± 3	13 ± 2

*$P < 0.001$
‡$P < 0.002$
Peak amplitude of voltage-dependent outward currents measured at different times after injections with c-ras, v-ras, or control solutions. Values are means ± SD. Statistical significance was assessed comparing each current for a given time interval with its initial baseline level by two tailed *t*-tests.

became clearly significant 20 min ($p \leq 0.002$) after v-ras injection and 30 min after c-ras injections (Table 1). The effects were persistent and rather irreversible after v-ras injection, and only transient, lasting no more than 30 min after c-ras injection. No changes other than reductions in conductance were found in the current to voltage relationships of both currents. However, removal of the voltage-dependent inactivation with hyperpolarizing conditioning prepulses prevented any c- or v-ras (Fig. 3 *A*) effects upon I_A's peak amplitude. Removal of voltage-dependent inactivation did not alter ras injection effects on I_C. The findings prompted us to evaluate I_A's inactivation kinetics after ras injections with a twin pulse protocol, using variable pulses ranging from −100 to −10 mV and test pulses of 0 mV. As is shown in Fig. 3 *B*, 10 min after c- or v-ras injections, the midpoint of steady state inactivation was shifted from −55 ± 5 to −70 ± 3 mV, ($N = 5$). No effects on the inactivation kinetics were found after heat inactivated or buffer alone control injections (data not shown).

Ras effect on calcium currents

An inward sustained current, blocked by 1 mM Cd^{2+} similar to the currents previously described in 300 mM external K^+, 5 mM 4-AP, and 100 TEA was elicited with depolarizing steps to 0 mV (17, 18). A tail current, carried by reversed potassium flow, also provides a measure of I_C. 30 min after c-ras injection, I_{Ca}^{2+} did not change, but consistent reductions of 32 ± 7% ($n = 5$) were observed for the tail currents. The tail current reductions recovered spontaneously after 45 min. 20 min after v-ras injections, the tail currents were reduced by >46% ($N = 4$). Ras injections did not alter the light-induced inward Na^{2+} current. Heat inactivated c- and v-ras, or buffer alone injections failed to have any measurable effects on either current: I_{Ca}^{2+}(nA), 5.0 ± 1, and 5.2 ± 1, before and after heat inactivated ras, respectively.

Simultaneous measurements of I_{Ca}^{2+} and Ic were also performed 30 min after previous addition to the bathing solution of 10 μM of the Protein Kinase C (PKC) inhibitor Sphingosine. 40 min after ras injections there were no longer any measurable effects on the K^+ tail currents: tail current amplitude (nA), 33.5 ± 5 and 32.0 ± 6, before and after v-ras injection, respectively.

DISCUSSION

The two major voltage-dependent K^+ currents across the type B soma membrane, I_A and I_C, are shown here to be targets for v- and c-ras. The only measurable Ca^{2+} current, a voltage-dependent sustained inward current, was unaffected by ras injections. The light-induced inward Na^+ current was also unaffected. The sequence of

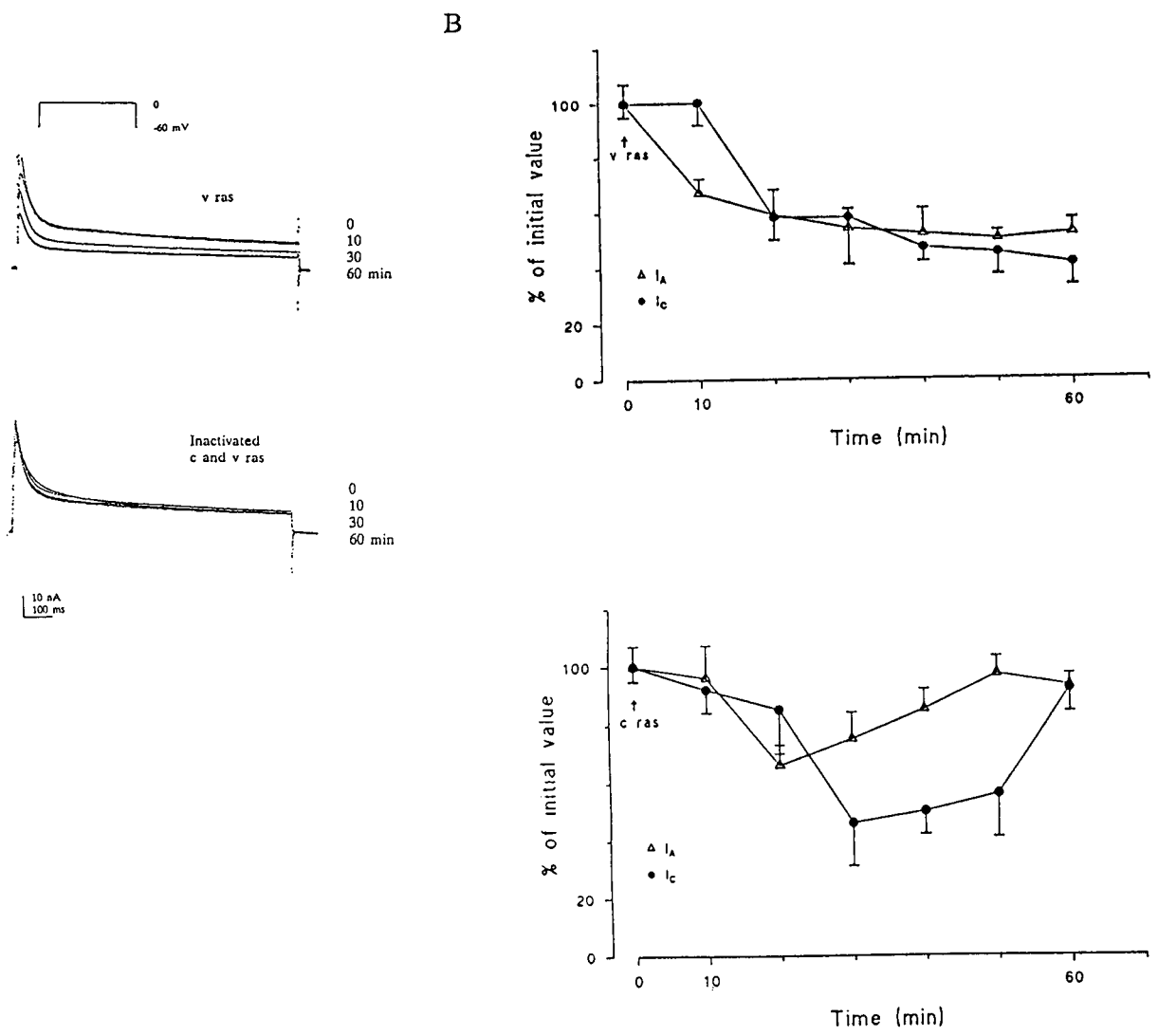

FIGURE 2 Effects of ras injections on I_A and I_C and its time course. (*A*) Top traces were obtained after injections with 70 μg/ml of v-ras. I_A reduced its peak amplitude, but not I_C, which was reduced 10 min later. Changes were sequential and progressive. No recovery was observed at 60 min after injections. Bottom traces are control injections with heat inactivated c- and v-ras and showed no measurable effects on either current. (*B*) Percentage change in I_A and I_C after injections of v-ras ($N = 5$; *top*) and c-ras ($N = 5$; *bottom*) c-ras transiently reduced both I_A and I_C (20 and 30 min after injections, respectively). v-ras effects were faster and irreversible.

potassium current changes after ras injection resembles the sequence observed with a progressively increased number of trials during classical conditioning (18). In both cases, I_A is the first current affected. I_C effects follow later. This parallel between ras and conditioning-induced effects raises the possibility that in both cases a common biochemical pathway is activated. The delay of ras effects, and the somewhat different nature of the conductance modifications for both forms of ras are consistent with involvement of a biochemical regulatory pathway. The role of phospholipid metabolites as primary targets of ras during normal or transforming activity is a matter of controversy, as previously discussed. However, the production of intermediaries that can induce mobilization of intracellular calcium has been ruled out in transformed cells (3, 9–11). Pathways involving Ca^{2+}-activated protein phosphorylation, however, could eventually lead to global modifications of cellular homeostasis during learning (18, 19), cell growth, and/or oncogenesis, such as the recently reported down regulation of T calcium channels in ras-mediated transformed NIH 3T3 cells (20), voltage sensitive sodium channels in cell-fusion hybrids (21) or voltage-sensitive calcium channels in BC_3H_1 monocytes (22). Sustained phospholipid-mediated PKC activation

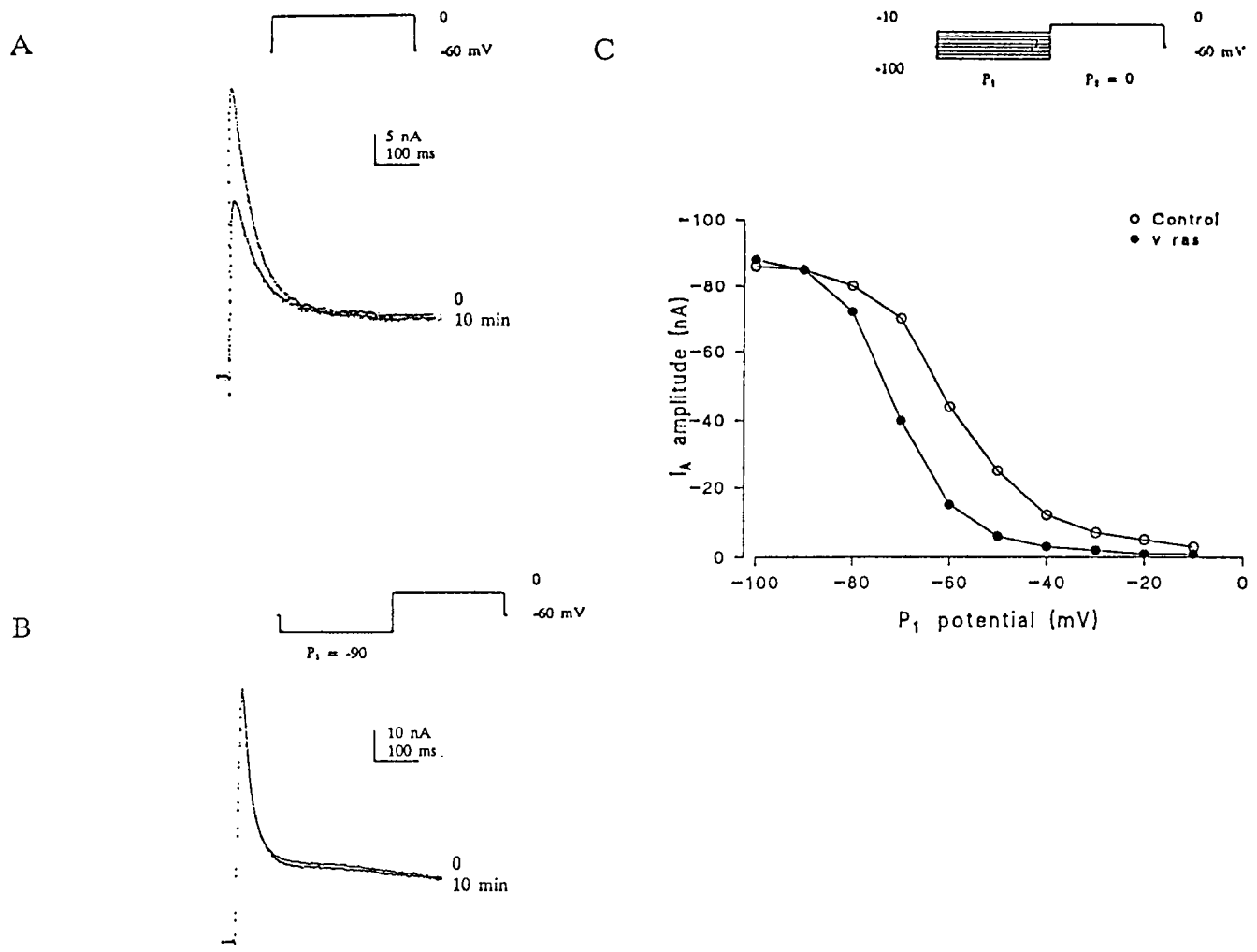

FIGURE 3 c-ras changes inactivation kinetics on I_A but not I_C. (*A*) 20 min after c-ras injections, only I_A showed a 25% reduction of its peak amplitude when elicited with depolarizing steps to 0 mV from a holding of −60 mV (*top traces*). With a hyperpolarizing pre-pulse (*bottom traces*), c-ras-induced reduction of I_A was eliminated. I_C was unchanged in both conditions. A lower gain display was used because of the overall larger current amplitudes elicited with this protocol. (*B*) Voltage dependence of the steady state inactivation of I_A was obtained with a double pulse protocol. A variable 100-ms prepulse (P_1) from −100 to −10 mV was followed by a 200-ms test pulse to 0 mV (P_2). I_A peak amplitude to the P_2 was plotted against P_1. 10 min after c-ras injections but not controls, a shift from −55 to −70 mV in the midpoint of inactivation was observed.

may be induced by oncogenic ras (but not proto-oncogenic ras). Calcium-activated protein phosphorylation has also been implicated in conditioning-induced reduction of I_A and I_C (23, 24). A substrate of such phosphorylation, a 20–21 Kd G-protein, reduces I_A and I_C (12) as well as regulates axonal transport (13). Therefore, the function of cp20 during learning may share common features with ras function in oncogenic contexts.

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