Journal of Physiology (1993), **461**, *pp.* 601–618
With 8 figures
Printed in Great Britain

SINGLE CHANNEL STUDY OF A Ca^{2+}-ACTIVATED K^+ CURRENT ASSOCIATED WITH *ras*-INDUCED CELL TRANSFORMATION

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(*Received 4 February 1992*)

SUMMARY

1. *Ras*-transformed fibroblasts have a whole-cell Ca^{2+}-activated K^+ current which is either absent or unavailable for activation in their non-transformed counterparts. To better understand the physiological significance of this K^+ current the single channel basis for the current was characterized in *ras*-transformed cells.

2. More than 90% of inside-out patches from *ras*-transformed *balb* 3T3 cells had a channel type which was Ca^{2+}-activated (threshold $< 0{\cdot}2$ μM internal Ca^{2+}), K^+-selective (permeability ratio $P_{Na}:P_K < 0{\cdot}02$), and inwardly rectifying in symmetric 150 mM KCl solutions (conductances at -60 and 60 mV of 33 ± 1 and 17 ± 1 pS respectively). Channel opening probability increased 25–50% between -60 and 60 mV due to an increase in the frequency of opening. Single K^+ channels in outside-out patches were blocked by externally applied 10 mM TEA or 100 nM charybdotoxin, as were whole-cell Ca^{2+}-activated K^+ currents. The properties of this class of K^+ channel are sufficient to account for the whole-cell Ca^{2+}-activated current in *ras*-transformed cells.

3. Inside-out patches from C3H10T1/2 and NIH 3T3 fibroblasts transformed by the H-*ras* oncogene had Ca^{2+}-activated K^+ channels identical to those observed in K-*ras*-transformed *balb* 3T3 cells.

4. As predicted from whole-cell experiments Ca^{2+}-activated K^+ channels were not observed in inside-out patches from non-transformed *balb* 3T3 cells. The purpose of the excised patch recordings was, instead, to rule out potential technical complications with the whole-cell experiments. For instance A23187, which evoked whole-cell K^+ currents in transformed cells, may not have elevated Ca^{2+} sufficiently to allow K^+ channel activation in non-transformed cells. Another possibility was that trypsin pretreatment used to round-up cells for whole-cell recording may have preferentially disabled channels in non-transformed cells. The first problem was addressed by exposing patches from non-transformed cells to 100–1000 μM Ca^{2+}. Excised patches were also taken from non-transformed cells which had not been exposed to trypsin. K^+ channel activity was not observed under either condition.

5. Patches from both *ras*-transformed and non-transformed cells had a type of non-specific cation channel which was activated at internal Ca^{2+} concentrations

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$\geqslant 100\ \mu$M. This channel was sensitive to membrane voltage, mean open time increasing from 12 to 72 ms between -90 and 90 mV.

INTRODUCTION

Mitogenic stimulation by certain peptides and serum factors has been correlated with mobilization of intracellular calcium and activation of ion transport systems (Rozengurt, 1986). In particular stimulation or modulation of potassium channel activity has been observed in mitogenically activated cells (Deutsch, Krause & Lee, 1986; Decoursey, Chandy, Gupta & Cahalan, 1987), and in some instances cell division can be inhibited by potassium channel blockers (Decoursey, Chandy, Gupta & Cahalan, 1984; Deutsch *et al.* 1986; Price, Lee & Deutsch, 1989). These observations suggest a causal link between potassium channel activation and the initiation or maintenance of mitogenesis.

Constitutive stimulation of mitogenesis in cells transformed by *ras* oncogenes occurs via an as yet unidentified signalling pathway. Because *ras* protein products (p21-ras) are in many ways similar to the heterotrimeric GTP-binding proteins (Jurnak, 1988), targets for the latter, including second messenger paths and ion channels, have been investigated as potential sites of action for p21-ras (Chen, Corbley, Roberts & Hess, 1988; Stacey, Tsai, Yu & Smith, 1988; Collin, Papageorge, Lowy & Alkon, 1990; Estacion, 1990; Flamm, Birnberg & Kaczmarek, 1990; Yatani, Okabe, Polakis, Halenbeck, McCormick & Brown, 1990; Bollag & McCormick, 1991). To test the idea that *ras*-induced mitogenesis like serum- or peptide-stimulated mitogenesis may involve modulation of ion channel activity, we compared the electrophysiological properties of *ras*-transformed fibroblast lines with the properties of their non-transformed counterpart lines. *Ras*-transformed cells were found to have a Ca^{2+}-activated K^+ current which was either absent or unavailable for activation in confluent non-transformed cells (Rane, 1991). The chronic appearance of this current in transformed cells is consistent with evidence that p21-ras oncoprotein acts as an irreversibly-activated GTP-binding protein, which persistently interacts with its targets (Bollag & McCormick, 1991).

To begin to understand the physiological significance of the Ca^{2+}-activated K^+ current observed in *ras*-transformed cells we have herein characterized it at the single channel level. Consistent with the results from whole-cell macroscopic current recordings the channel is Ca^{2+} activated and K^+ selective, and is blocked by external application of charybdotoxin or high concentrations of TEA. In addition, single channel recordings have demonstrated a weak voltage sensitivity which was not immediately evident from the whole-cell work. The properties of this channel, therefore, are unlike those of the two general classes of Ca^{2+}-activated K^+channels already described (Rudy, 1988). However, this channel which we observe in *ras*-transformed fibroblasts is similar if not identical to channels described in other transformed or mitogenically activated cells. Some of the information reported here has been presented in preliminary abstract form (Huang, McDonald & Rane, 1991).

METHODS

Cell culture and preparation

Most experiments were performed using *balb* 3T3 cells stably transfected with the Kirsten-*ras* oncogene via infection by the Kirsten sarcoma virus. Cells were obtained from American Type Culture Collection (CCL 163, USA) and Dr L. C. Cantley, Department of Physiology, Tufts University Medical School, Boston, MA, USA. Non-transformed *balb* 3T3 cells were obtained from the same sources. NIH 3T3 and C3H10T1/2 cells transformed via chloroquine, calcium phosphate precipitate method with human H-*ras* oncogene were provided by Dr E. J. Taparowksy, Department of Biological Sciences, Purdue University. Cultures were maintained in a humidified, 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Gibco, USA) containing either 10% calf serum (*balb* 3T3 and NIH 3T3 lines) or 10% fetal bovine serum (C3H10T1/2 line) (Gibco). *Ras*-transformed C3H10T1/2 and NIH 3T3 cells were obtained by co-transfection with the *ras*-containing plasmid along with a plasmid containing the bacterial neomycin resistance gene, followed by selection of transfected foci using 400 μg/ml G418 (Gibco) (Taparowsky, Heaney & Parsons, 1987). Media for these cells was supplemented with 400 μg/ml G418 (Gibco). In preparation for electrophysiology cells were grown to confluence and received no fresh medium for at least the 2 days beforehand. To obtain individual rounded cells suitable for recording, cultures were treated with trypsin–EDTA (0·05%) for 3–5 min, mechanically triturated in growth medium and replated into 35 mm dishes with fresh medium. Recordings were made between 0·5 and 4 h after replating.

Electrophysiology

Inside-out and outside-out excised patch, cell-attached, and whole-cell tight-seal experiments (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) were performed with an EPC-7 patch clamp amplifier (List Electronics, FDR) and hard glass patch pipettes with resistances from 2 to 7 MΩ (1B150F-4 WP Instruments, USA). Pipette tips were dipped in Sigmacote (Sigma Chemical Co., USA) to reduce noise associated with pipette capacitance. Data acquisition and off-line analysis were performed with Atari MegaST and MegaST^e computers running Acquire, Review, and TAC software (Instrutech Corp., USA). Output from the current monitor of the patch clamp was filtered from 1 to 3 kHz (−3 dB) with an 8-pole Bessel filter (Frequency Devices Inc., USA), and was digitized (ITC-16 interface, Instrutech) at a rate at least five times the Bessel frequency. The single channel analysis software uses the 50% threshold method for detecting channel events and for determining open and closed times. The single channel amplitudes reported here represent means derived from Gaussian fits of amplitude histograms comprising 100–500 openings. Logarithmically binned histograms were used to display distributions of channel open times derived from at least 1000 openings. A theoretical exponential probability function was first manually fitted to the distribution. This fit provided starting parameters for computer-driven automatic maximum likelihood fitting which yielded τ_{open} (time constant for open channels) values (Sigworth & Sine, 1987). For some experiments, channel activity was expressed as the product of the number of channels times the open probability, NP_o, where

$$NP_o = \Sigma \frac{(\text{open time} \times \text{no. channels open})}{\text{Total time of record}}.$$

For multiple channel patches the probability of being open, P_o, for a single channel was determined by fitting the binomial distribution to the open times at each current level.

For certain ionic conditions non-linearities of the single channel current–voltage plots precluded the use of linear regression lines through the entire plot to determine reversal potentials. Instead a line (not shown) was fitted through just the points closest to the apparent reversal and the actual reversal potential was taken as the X-intercept of this line. Where applicable the Goldman equation was used to estimate relative ionic permeabilities. Transmembrane potentials are given as the potential at the intracellular membrane surface relative to the extracellular surface. For the purposes of describing solution changes or drug applications the intracellular face of the membrane patch is referred to as 'intracellular' or 'internal', and the extracellular surface as 'extracellular' or 'external'.

In the whole-cell configuration drugs were applied via pressure ejection from blunt-tipped (5–10 μm) puffer pipettes positioned about 20 μm from the cell. For excised patches the solution

at the exposed membrane surface was changed with a superfusion system. This system consisted of 10 ml reservoirs connected by small-bore tubing to a broken-off patch pipette, which had been fire-polished to a smooth, regular tip of about 100 μm i.d. The tip of this superfusion pipette was bent so that the recording patch pipette could be advanced about 150–200 μm down its length. In this way the patch was isolated from the bath solution both by the walls of the superfusion pipette and by bulk flow of perfusate. Gravity feed of solution from any one reservoir was controlled by a series of manually actuated valves.

Solutions and reagents

For single channel experiments with the Ca^{2+}-activated K^+ channel, pipette and bath solutions typically contained 150 mM KCl, 1·0 mM $MgCl_2$, 10 mM Hepes, and 0·1–1000 μM free Ca^{2+} as $CaCl_2$ with 100 μM EGTA (computer program for calculation of free Ca^{2+} values kindly supplied by R. J. Cork, Department of Biological Sciences, Purdue University). 'Ca^{2+}-free' solutions (free Ca^{2+} concentration less than 0·1 μM) consisted of 100 μM EGTA and no added Ca^{2+}. For determining the Ca^{2+}-activated channel's relative permeabilities to K^+ and Na^+ (Fig. 1) the intracellular membrane face was exposed to a solution containing 150 mM NaCl in place of KCl. The 150 mM NaCl solution was also used for certain experiments with the non-selective cation channel which will be described (Figs 7 and 8). To show how changes in extracellular K^+ concentration affected rectification of the Ca^{2+}-activated K^+ current an extracellular solution containing 37 mM KCl, 138 mM NaCl, 1·0 mM $MgCl_2$, 10 mM Hepes, and 1 mM $CaCl_2$ was used for whole-cell and outside-out patch experiments (Fig. 3). Potassium channel blockers, tetraethylammonium (TEA, Sigma) and charybdotoxin (Research Biochemicals Inc., USA), were applied to the extracellular face of outside-out patches via the superfusion system described above. TEA was diluted into the recording solution from a frozen 1 M stock. Charybdotoxin was stored frozen as a 25 μM stock in water. For each day of experiments an aliquot was removed from the stock and diluted into the recording solution to give a final toxin concentration of 100 nM. A23187 (Sigma, USA) was prepared by dilution from a frozen stock and was applied at a final concentration of 0·3 μM in all cases. All solutions were at pH 7·3.

RESULTS

Selectivity, conductance and Ca^{2+} dependence

Under whole-cell tight-seal recording conditions the prominent current observed in *ras*-transformed fibroblasts is K^+ selective and requires intracellular Ca^{2+} for activation (Rane, 1991). In this study more than 90% of over 225 membrane patches from transformed cells had a type of channel with properties consistent with those of the whole-cell Ca^{2+}-activated K^+ current. There were usually one to three channels per patch. Figure 1 shows a typical recording of this channel in an inside-out patch. Initial patch excision into a bath solution containing 150 mM NaCl and 0·15 μM Ca^{2+} revealed only inward current openings. In this patch and two others with 150 mM internal NaCl no outward openings were observed at potentials up to 100 mV (not shown). When the solution at the intracellular membrane surface was changed to 150 mM KCl and 0·15 μM Ca^{2+} outward currents were observed at all positive membrane potentials. With symmetric 150 mM KCl the reversal potential for the channel in Fig. 1 was $-0·4$ mV; therefore the maximal $P_{Na}:P_K$ (permeability ratio) value for this channel was 0·02. For seven patches exposed to symmetric 150 mM KCl and Ca^{2+} concentrations of 0·15–1 μM, the mean reversal potential was $0·7 \pm 0·5$ mV. Reversal potential was not affected by replacing KCl with potassium aspartate (two patches). The channel displayed inward rectification in symmetric KCl solutions. For the channel in Fig. 1 the conductance was 33 pS at -60 mV, and 18 pS at 60 mV. For thirteen patches exposed to symmetric 150 mM KCl mean channel conductances at -60 and 60 mV were 33 ± 1 and 17 ± 1 pS respectively.

Activity of the K^+-selective channel in patches from *ras*-transformed cells is strongly dependent on the concentration of intracellular Ca^{2+}. Figure 2*A* shows inward K^+ channel openings recorded at −60 mV with 0·2 μM Ca^{2+} present at the intracellular face of the patch. Channel activity ceased when the Ca^{2+} concentration was reduced below 0·1 μM, and it resumed when Ca^{2+} was restored to 0·2 μM. The same results were obtained in two other patches. The relationship between

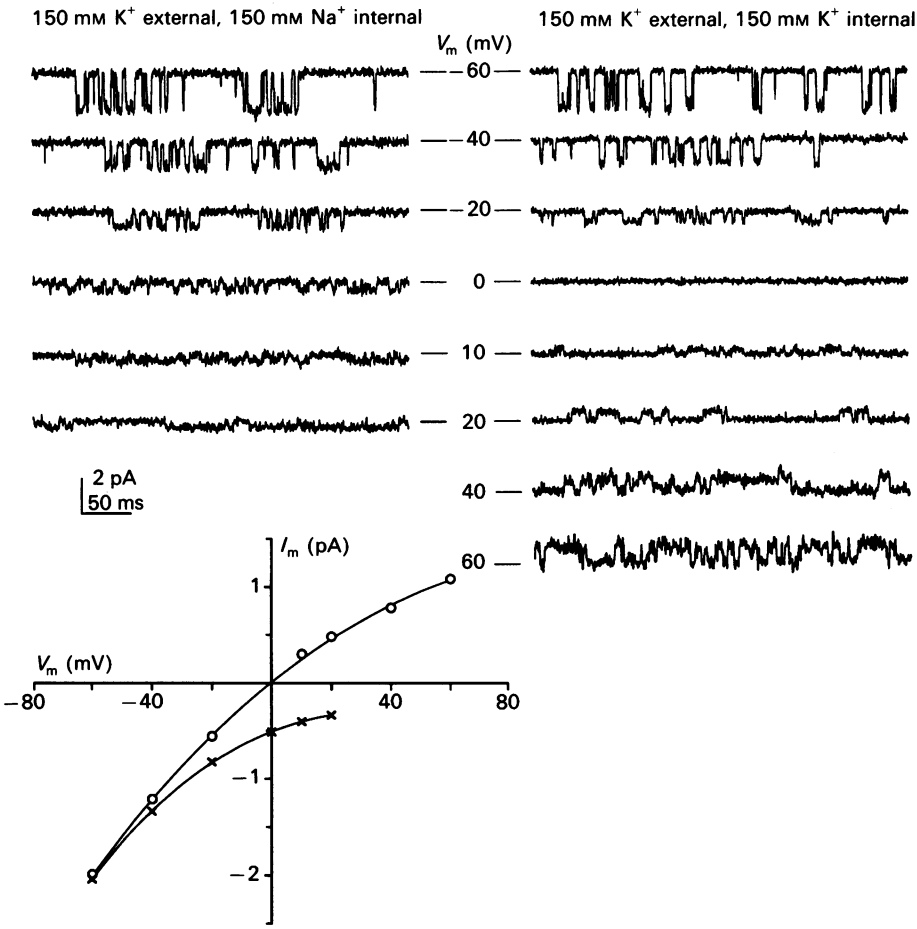

Fig. 1. Inside-out patch records of a Ca^{2+}-activated K^+ channel from a *ras*-transformed *balb* 3T3 fibroblast. Channels were first recorded with 150 mM intracellular NaCl, and then with 150 mM KCl. There was 0·15 μM free Ca^+ on both sides of the patch. Numbers between records show the holding potential and bars indicate the closed channel level. Lines were fitted by eye to the single channel current–voltage (I_m–V_m) relationships (○ for internal K^+, × for internal Na^+).

intracellular Ca^{2+} concentration and channel opening probability (P_o) is given in Fig. 2*B*. The threshold for channel activation was less than 0·2 μM Ca^{2+} and P_o approached a maximum at 10 μM Ca^{2+}. Four patches were exposed to Ca^{2+} concentrations from 10 to 1000 μM. Increases in internal Ca^{2+} above 10 μM did not appear to cause

significant changes in NP_o. Data from four single channel patches were analysed to determine the effects of increasing Ca^{2+} from 1 to 10 μM. The frequency of channel openings increased by 35–240% (105±49%) when Ca^{2+} concentration was raised. For the same four patches there was a non-significant increase in τ_{open} values from

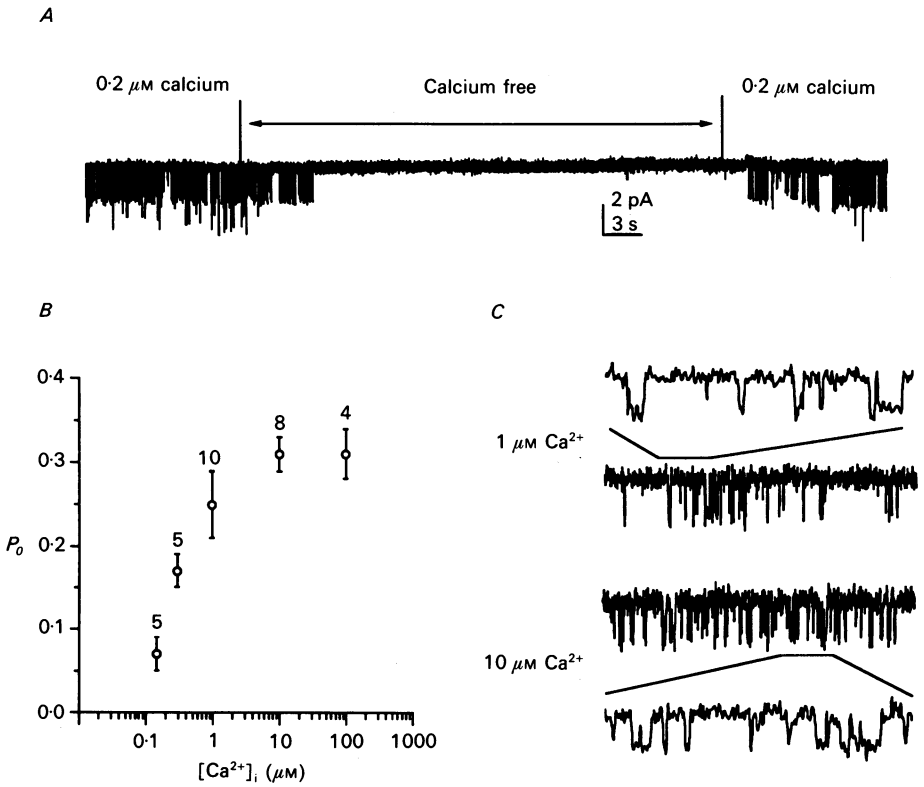

Fig. 2. Ca^{2+} dependence of the fibroblast K^+ channel. *A*, inside-out patch, from a *ras*-transformed *balb* 3T3 cell, with two active K^+ channels. Solutions were symmetric 150 mM KCl and the patch voltage was −60 mV. Upward deflections are switching artifacts that indicate when superfusion of the intracellular membrane surface was switched from 0·2 μM Ca^{2+} solution to a Ca^{2+}-free solution (see Methods), and then back to 0·2 μM Ca^{2+}. *B*, plot of opening probability (P_o) *versus* intracellular Ca^{2+} concentration for the fibroblast K^+ channel. For multiple channel patches P_o for a single channel was determined by fitting the binomial distribution to the open times at each current level. Each point is the mean±S.E.M. from the number of inside-out patches indicated (symmetric KCl, patch potential −60 mV). *C*, records from a patch under the same conditions as in *A* and *B*. The internal face of the patch was superfused first with 1 and then 10 μM Ca^{2+}. Records in centre show the increase in frequency of channel opening with application of 10 μM Ca^{2+} (each trace is 832 ms). Expanded records (130 ms each) show that channel open times were not affected by the increase in Ca^{2+}.

2·4±0·2 to 3·1±0·7 ms. The open time distributions were well-fitted by single exponential functions. Figure 2*C* illustrates the primary effect of an increase in Ca^{2+} from 1 to 10 μM to be an increase in the frequency of channel openings. For any given Ca^{2+} concentration there was variability in P_o values among different patches. In

addition channel activity in some patches declined abruptly or ceased altogether even though patch integrity was apparently unchanged. This was most often observed in long-term recordings (tens of minutes) or for patches which had been exposed to several superfusion changes at the intracellular surface.

This laboratory has reported that for *ras*-transformed fibroblasts whole-cell Ca^{2+}-activated K^+ current evoked by external application of A23187 with 1 mM Ca^{2+} eventually subsided, and subsequent applications of A23187 failed to reactivate the current (Rane, 1991). One possible interpretation of this result was that internal Ca^{2+} increased to a level which blocked the channel in a manner analogous to the Ca^{2+} block observed for the maxi-K^+ channel in lacrimal cells (Marty, Tan & Trautman, 1984). This interpretation must be incorrect based on our present observations that K^+ channel activity in excised patches persisted with internal Ca^{2+} concentrations up to 1 mM. To confirm that turn-off of K^+ current following A23187 application could not be due to Ca^{2+} block, recordings were made of single K^+ channels in cell-attached and then inside-out recording configurations. The bath contained 150 mM KCl and 0·2 μM Ca^{2+}, the pipette had 150 mM NaCl, and the pipette potential was set to -50 mV to insure large outward K^+ channel openings. In the cell-attached configuration single channel K^+ currents were activated by extracellular application of A23187 and 1 mM Ca^{2+} from a puffer pipette. In a manner analogous to the whole-cell currents single channel activity eventually subsided and failed to reappear in response to a second challenge with ionophore and 1 mM Ca^{2+}. When the patch was excised into the bath K^+ channel activity immediately resumed. The puffer pipette was again lowered into the bath and the patch pipette tip was manipulated into the flow from the puffer to show that neither A23187 nor 1 mM Ca^{2+} inhibited or blocked the channel.

Voltage sensitivity of the Ca^{2+}-activated K^+ channel

In symmetric 150 mM KCl solutions the Ca^{2+}-activated K^+ channel displayed mild inward rectification, but macroscopic Ca^{2+}-activated K^+ currents recorded in whole-cell mode were reported to be nearly linear (Rane, 1991). However, the earlier whole-cell experiments were performed in asymmetric KCl solutions. To compare results from single channel and whole-cell experiments more directly, we first repeated the latter with 150 mM KCl bath and patch pipette solutions. Under this condition the whole-cell Ca^{2+}-activated current showed mild inward rectification (Fig. 3*A*). A comparison was also made of K^+ channel current–voltage relationships from outside-out patches exposed to 150 mM internal K^+ and either 150 or 37 mM external K^+ (Fig. 3*B*). Inward rectification was again more apparent for symmetric K^+ conditions (34 and 17 pS for ± 60 mV from the potassium reversal potential, E_K) than for asymmetric conditions (25 and 20 pS).

The whole-cell current rectification observed with either 37 or 150 mM external K^+ was, however, less than that predicted by the single channel current–voltage relationships alone. In symmetric 150 mM K^+ solutions, the whole-cell ramp current amplitude at 60 mV was $0{\cdot}75 \pm 0{\cdot}10$ times the amplitude at -60 mV ($n = 31$ ramp currents recorded from four cells). Single channel currents at 60 mV were $0{\cdot}50 \pm 0{\cdot}04$ times the size of the currents at -60 mV ($n = 9$ patches). This deviation could have been due to several factors, including the presence in the whole-cell recordings of an

additional outward current at positive membrane potentials, or a depolarization-dependent change in K^+ channel activity involving an increase in open probability or the number of active channels. We first looked to see if voltage-dependent changes in K^+ channel activity could by themselves explain the differences in rectification

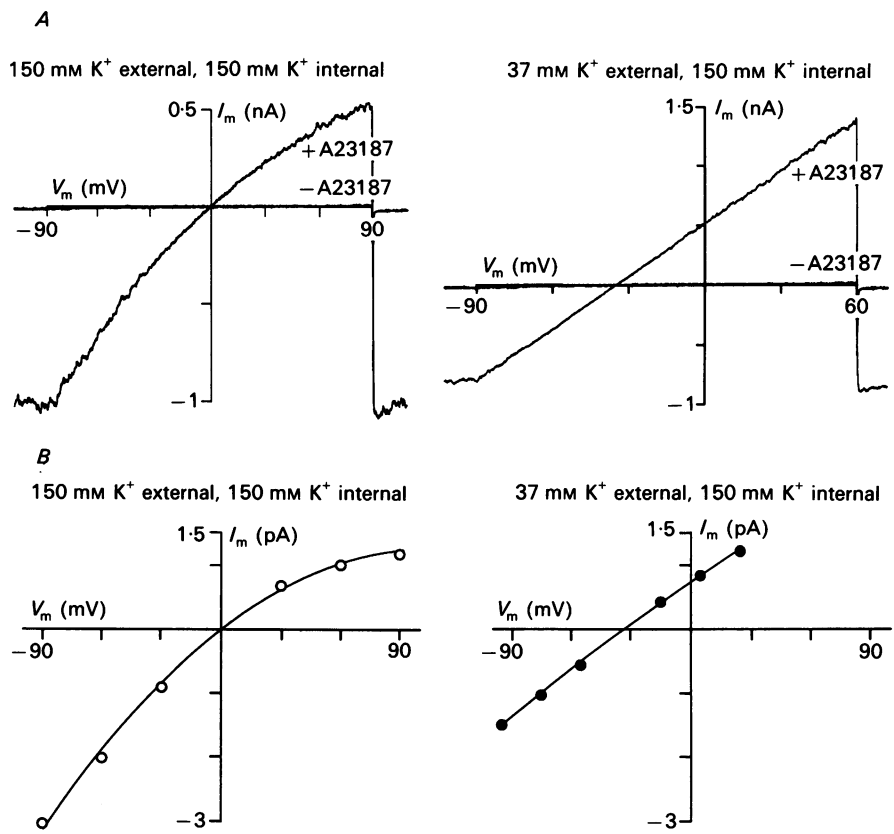

Fig. 3. Rectification for both whole-cell and single channel Ca^{2+}-activated K^+ currents was affected by the external K^+ concentration. *A*, whole-cell currents evoked by external application of A23187 (1 mM Ca^{2+} bath) during voltage ramps. Cells were held at −90 mV and voltage ramps (not shown) were applied at a rate of 1 mV/ms. Portions of the record beyond the extremes of the voltage axis represent currents recorded at the holding potential preceding and following the ramp. *B*, current–voltage relationships obtained from an outside-out patch recording of a single Ca^{2+}-activated K^+ channel from a *balb* 3T3 cell. The extracellular surface of the patch was superfused first with 150 mM KCl solution, and then with 37 mM KCl solution. The patch pipette solution was 150 mM KCl and 1 μM free Ca^{2+}.

observed for macroscopic and single channel currents. Ramp voltage commands like those used in whole-cell experiments were applied to inside-out patches exposed to symmetric KCl solutions. The Ca^{2+}-activated K^+ channel was identified by its characteristic twofold difference in amplitude at −60 and 60 mV (Fig. 4*A*). Forty to sixty ramp currents were recorded and then digitally averaged to simulate the summation of channel activity which constitutes whole-cell currents. Figure 4*B* is an

ensemble average in which the current amplitude at 60 mV is 0·82 times the amplitude at −60 mV. Mean values for this ratio in 1 μM and 10 μM internal Ca^{2+} were 0·69 ± 0·07 ($n = 8$) patches and 0·69 ± 0·09 ($n = 7$) respectively. Neither value is significantly different from the 0·75 ratio for whole-cell ramp currents (two-tailed t test at $t_{0.05}$).

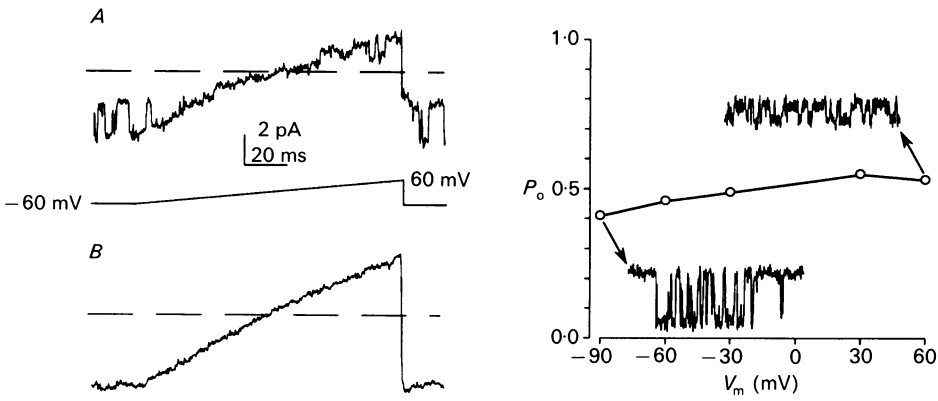

Fig. 4. The Ca^{2+}-activated K^+ channel has weak voltage sensitivity. *A*, leak-subtracted, inside-out patch record obtained in symmetric 150 mM KCl, 1 μM Ca^{2+} solutions. The channel was identified by its characteristic twofold smaller amplitude at 60 mV compared to −60 mV. There were two channels open through most of the ramp, and the dashed line indicates the zero current level. *B*, leak-subtracted, ensemble average of forty-five records as in *A*. Of the forty-five records, the trace in *A* showed the least amount of channel activity (particularly at 60 mV). It was chosen to show clearly resolved individual openings. For the ensemble current the amplitude at 60 mV was approximately 0·8 the amplitude at −60 mV. This difference from the 0·5 ratio predicted by single channel rectification was attributed to an increase in open probability as the patch was depolarized. *C*, opening probability (P_o) was calculated at the indicated holding potentials for records from another patch (examples shown inset, each record 155 ms long). Same solutions as in *A*, giving inward currents at −90 mV and outward currents at 60 mV. The increase in P_o at positive voltages was due to an increase in the number of openings per unit time.

NP_o or P_o measurements for K^+ channel openings recorded at different patch potentials confirmed that there was an increase in channel activity at positive voltages. Figure 4*C* shows data for a single channel patch in which P_o increased by about 25% between −90 and 60 mV. An analysis was made of five patches in which channel activity was recorded at −60 and 60 mV. NP_o or P_o values at 60 mV were 25–50% greater than those at −60 mV. This change could be entirely attributed to an increase in opening frequency of 32–106%, since τ_{open} values either decreased slightly or were unchanged in all five patches. At the internal Ca^{2+} concentrations used for these experiments (0·3 and 1 μM) there was no obvious recruitment of additional channels as patch potentials were made more positive.

The weak voltage sensitivity of the Ca^{2+}-activated K^+ channel appeared sufficient to explain differences in rectification between single channel current–voltage plots and whole-cell Ca^{2+}-activated currents. As noted above, however, an additional outward current at positive membrane potentials could have also made whole-cell

currents appear more linear. There was no evidence from either whole-cell or patch recordings for additional K^+ currents. A Ca^{2+}-activated non-specific cation conductance is present, however, and single channel recordings (see Figs 7 and 8) showed that open times for this channel were increased at positive membrane voltages. Initially, whole-cell recording conditions were designed to measure K^+ currents at 0 mV and cation currents at E_K (−70 mV) (Rane, 1991). To determine more accurately the contribution of the non-specific cation current to whole-cell Ca^{2+}-activated currents over a range of voltages, whole-cell ramp currents were recorded with 150 mM NaCl in both the pipette and bath solutions to eliminate K^+ current. The bath contained 1 mM Ca^{2+} and 0·3 μM A23187 was applied to activate currents. Consistent with earlier results the whole-cell non-specific cation current activated by A23187 and 1 mM extracellular Ca^{2+} was quite small, less than 50 pA outward current at 60 mV. With K^+ in the patch pipette solution whole-cell currents at 60 mV are typically greater than 1 nA. Thus, it is unlikely that the non-specific cation current made a significant contribution to outward current amplitudes when mixed K^+ and non-specific currents were recorded.

Pharmacology

Both TEA and the scorpion toxin component charybdotoxin block whole-cell Ca^{2+}-activated K^+ currents in *ras*-transformed fibroblasts (Rane, 1991). The effects of these blockers on single channel Ca^{2+}-activated K^+ currents were tested by applying them to the external face of outside-out patches from *balb* 3T3 cells (Fig. 5). TEA reduced *NP* and single channel amplitude in all seven patches tested and charybdotoxin dramatically reduced *NP* in four of four patches. Channel activity in the presence of charybdotoxin was characterized by short bursts of openings separated by long closures. Although the superfusion system was not designed for quantifying the onset of channel block, clear effects of both TEA and charybdotoxin were observed within 5–10 s of drug application. Maximal block was achieved within 60–90 s and for TEA recovery was complete within about 60 s of changing back to control solution. Recovery from charybdotoxin was incomplete. Figure 5 shows the most extensive recovery for the four patches tested. In two of the patches there was no recovery of channel activity for 5 min after drug application was ended.

Identification of the Ca^{2+}-activated K^+ channel in other ras-transformed cell lines

Ras transformation was correlated with the presence of Ca^{2+}-activated K^+ current in three distinct cell lines transformed by two different *ras* alleles. At the whole-cell level the currents in each of the lines had similar kinetics and sensitivities to channel blockers (Rane, 1991). To determine if the current in *ras*-transformed lines has the same single channel basis, *ras*-transformed C3H10T1/2 and NIH 3T3 cells were examined for the presence of the Ca^{2+}-activated K^+ channel identified in *balb* 3T3 cells. The figures in this paper thus far have shown K^+ channels recorded from *ras*-transformed *balb* 3T3 cells only. Figure 6 shows a current–voltage plot and selected records taken for a channel in an inside-out patch from a *ras*-transformed C3H10T1/2 cell. The figure is representative of single channel data obtained for fifteen of nineteen patches from C3H10T1/2 cells. In symmetric 150 mM KCl the average conductances for channels from these patches were 33 ± 1 and 17 ± 1 pS at −60 and

60 mV respectively ($n = 10$). As for the Ca^{2+}-activated K^+ channel observed in *balb* 3T3 cells, activity of the C3H10T1/2 channel was increased by depolarization (Fig. 6). Also, this channel could be turned on and off repeatedly by changing the internal Ca^{2+} concentration back and forth between > 1 and $< 0{\cdot}1$ μM (five patches, data not

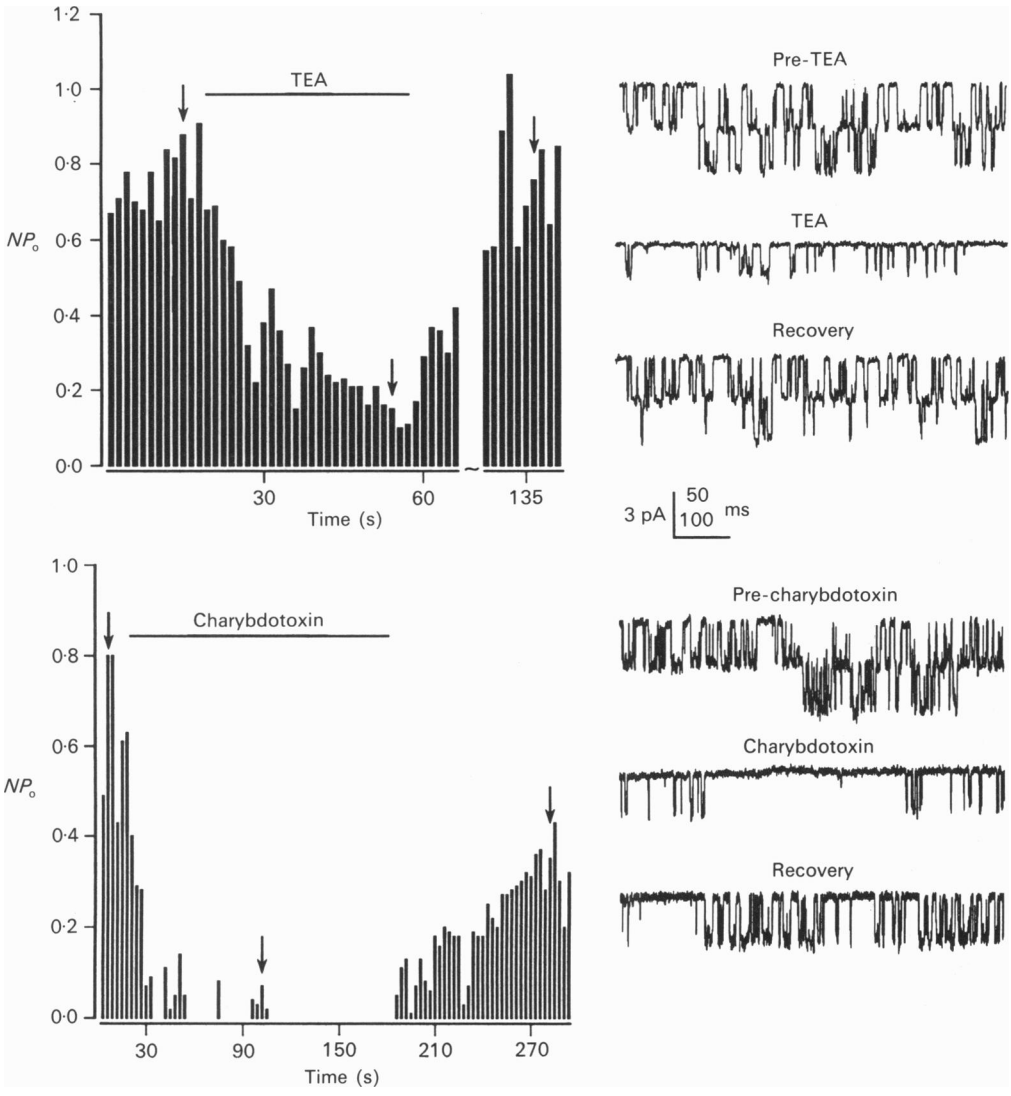

Fig. 5. The Ca^{2+}-activated K^+ channel is blocked by application of TEA or charybdotoxin to outside-out patches. Inward K^+ currents were recorded at a pipette potential of -90 mV in symmetric 150 mM KCl with 1 μM free Ca^{2+} in the patch pipette. Bin times for NP_o plots are 1·5 and 3 s for TEA and charybdotoxin respectively. Arrows indicate the bins from which sample single channel records were taken. The time calibration is 50 ms for the upper records and 100 ms for the lower.

shown). Ten of twelve inside-out patches from NIH 3T3 cells also had a similar channel with average single channel conductances of 32 ± 1 and 17 ± 1 pS at -60 and 60 mV respectively (symmetric 150 mM KCl and 1 μM internal Ca^{2+}). Channel activity in all ten patches was turned off reversibly when the internal Ca^{2+}

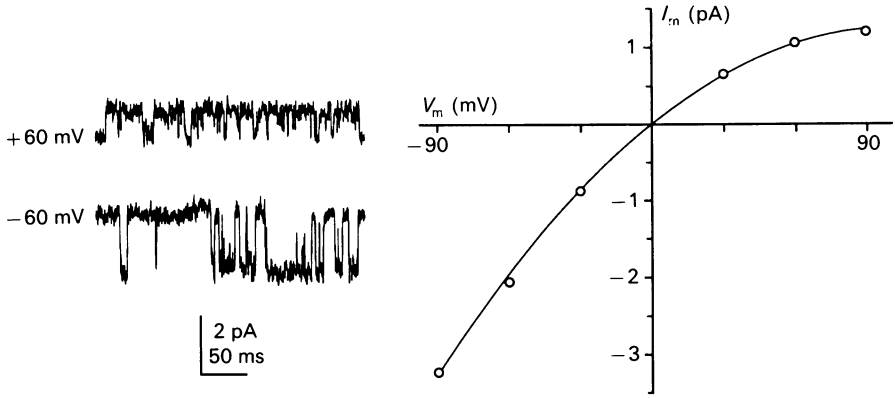

Fig. 6. Current–voltage relationship and selected records from an inside-out patch recording of a Ca^{2+}-activated K^+ channel from a *ras*-transformed C3H10T1/2 fibroblast. External and internal solutions were both 150 mM KCl, 1 μM Ca^{2+}, etc. Conductance and rectification were the same as for the Ca^{2+}-activated K^+ channel observed in *ras*-transformed *balb* 3T3 fibroblasts.

concentration was reduced from 1 to $< 0{\cdot}1$ μM. The similarities in conductance, mild rectification in symmetric 150 mM KCl, Ca^{2+} dependence and weak voltage sensitivity among the channels in each of the *ras*-transformed lines suggests that these channels are similar if not identical.

Absence of Ca^{2+}-activated K^+ channels in non-transformed cells

To confirm our earlier whole-cell experiments that showed a lack of Ca^{2+}-activated K^+ current in non-transformed cells relative to their *ras*-transformed counterparts, excised patch experiments were performed on non-transformed *balb* 3T3 cells. For the previous whole-cell experiments either trypsin–EDTA or EDTA alone followed by vigorous trituration was used to cause cells to detach and round-up. This pretreatment was particularly necessary for the non-transformed cells because in their normally flattened state application of suction to obtain whole-cell access invariably resulted in breakdown of the gigaseal. A variety of controls had been used to show that trypsin or EDTA pretreatment was probably not responsible for the lack of K^+ current in non-transformed cells (Rane, 1991). Having identified the K^+ channel in *ras*-transformed cells it was possible to look for it in excised patches from confluent non-transformed cells which had not been pretreated with enzyme or EDTA. Thus the pretreatment would not be a factor in whether the channel was present. Thirty-two inside-out patches from non-transformed *balb* 3T3 cells were exposed to internal Ca^{2+} concentrations ranging from 10 to 1000 μM, and to 150 mM KCl in either the internal or external solutions or both. No K^+ channel activity was

observed in any of these patches. However, patches from non-transformed cells had the same Ca^{2+}-dependent non-specific cation channel which was observed in *ras*-transformed cells (see below). It appears, therefore, that neither trypsin–EDTA treatment nor trituration was responsible for the absence of whole-cell Ca^{2+}-activated K^+ currents in non-transformed fibroblasts (Rane, 1991).

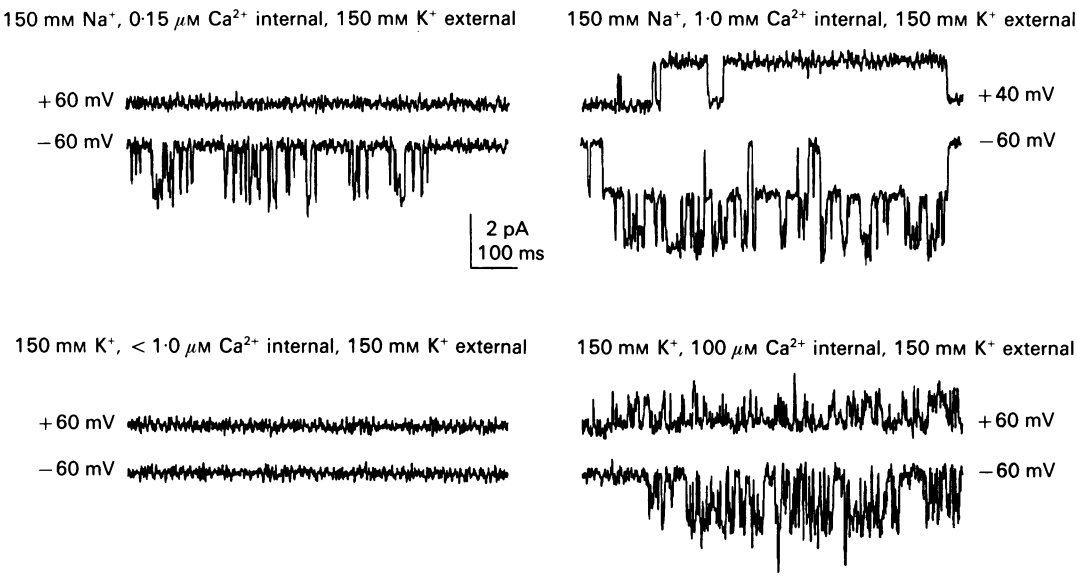

Fig. 7. Inside-out patch recording of a non-specific Ca^{2+}-activated cation channel and the Ca^{2+}-activated K^+ channel from a *ras*-transformed *balb* 3T3 fibroblast. Sequential changes of the internal solution are shown from top to bottom, left and then right columns. Only the K^+ channel opened at 0·15 μM Ca^{2+}, and outward K^+ currents were absent due to internal Na^+. No activity was observed at < 0·1 μM free Ca^{2+}. Long duration openings of the non-specific cation channel appeared when internal Ca^{2+} was increased to 1 mM. Finally, the non-specific channel failed to open at 100 μM Ca^{2+}; however, both inward and outward K^+ channel openings were present with K^+ in the internal solution.

Non-specific cation channels in balb 3T3 cells

In *ras*-transformed *balb* 3T3 fibroblasts another channel was observed which was easily distinguished from the K^+ channel by its lack of selectivity for K^+ *versus* Na^+, its much lower sensitivity to Ca^{2+}, and a significant effect of voltage on channel open time. Both the K^+ channel and this second channel type were present in the inside-out patch shown in Fig. 7. Only the K^+ channel was observed at 0·15 μM internal Ca^{2+} and its activity was eliminated when Ca^{2+} was < 0·1 μM. When Ca^{2+} was increased to 1 mM short duration inward K^+ channel openings reappeared at −60 mV, and long duration inward current openings also became apparent. Coincident with these long duration inward currents, long duration outward openings were recorded at 40 mV. These outward openings could not have been the K^+ channel since the internal solution contained 150 mM NaCl. When the internal Ca^{2+} concentration was lowered to 100 μM the long duration openings at positive and negative potentials were eliminated, but the inwardly rectifying Ca^{2+}-activated K^+ channel remained active.

With 150 mM NaCl external and 150 mM KCl, 1 mM Ca^{2+} internal, the mean reversal potential for the cation channel was $-1{\cdot}4 \pm 0{\cdot}9$ mV (four patches) and mean conductance at this potential was 29 ± 2 pS. When internal NaCl was substituted by sodium citrate in one inside-out patch the reversal potential changed from $-2{\cdot}7$ to

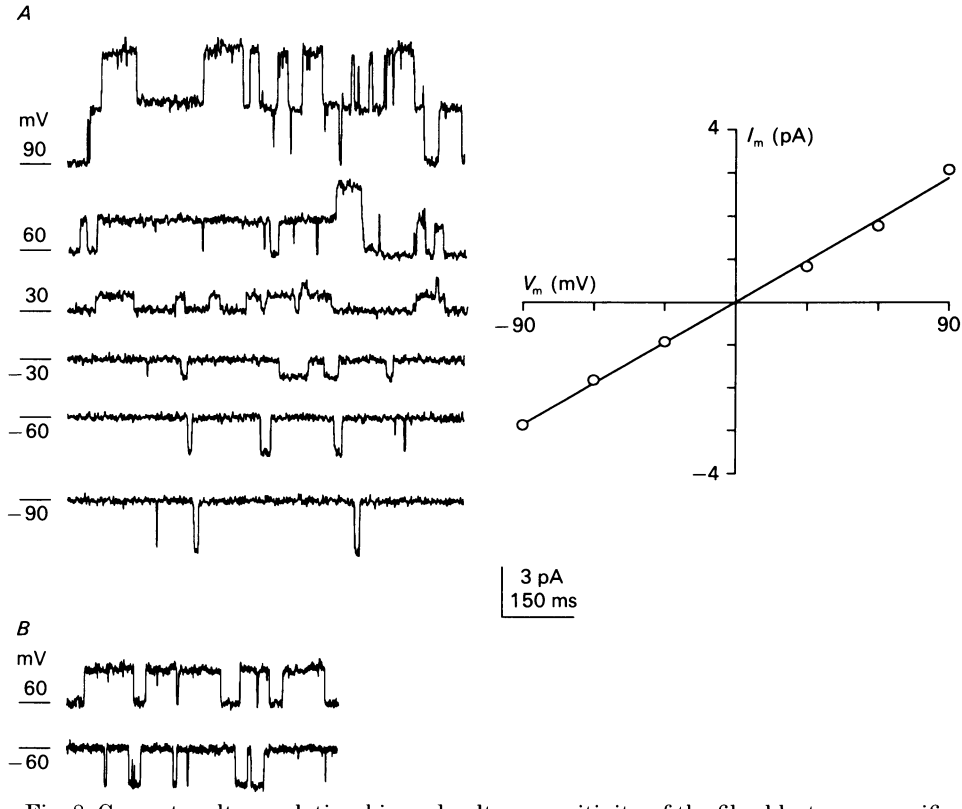

Fig. 8. Current–voltage relationship and voltage sensitivity of the fibroblast, non-specific cation channel. *A*, inside-out patch records of the channel from a *ras*-transformed 3T3 cell, with 150 mM K^+, 1 mM Ca^{2+} internally, and 150 mM Na^+, 1 mM Ca^{2+} externally. Current reversed at 0 mV and the current–voltage plot was characteristically linear. Also typical was the increase in channel open time as membrane voltage was made more positive. *B*, inside-out patch records from a non-transformed *balb* 3T3 cell under the same ionic conditions as in *A*. The channel had the same conductance, voltage sensitivity and Ca^{2+} dependence (not shown) as the channel observed in *ras*-transformed cells.

$-3{\cdot}0$ mV. Although the K^+ channel was usually present, a rare patch in which only non-specific cation channels were active is shown in Fig. 8*A*. With 150 mM NaCl externally and 150 mM KCl, 1 mM Ca^{2+} internally, the lack of sensitivity for Na^+ *versus* K^+ was clear. Changing membrane voltage from -90 to 90 mV increased *NP* from 0·04 to 1·10, and increased τ_{open} from 12 to 72 ms. In four of four patches cation channel activity was eliminated by lowering the internal Ca^{2+} concentration from 1 mM to $\leqslant 100$ μM. There is one caveat to this last point. In less than 5% of patches from *ras*-transformed *balb* 3T3 cells, a non-specific cation channel was observed which was not sensitive to the internal Ca^{2+} concentration. Like the Ca^{2+}-dependent

channel, this channel was non-selective for Na^+ *versus* K^+, its conductance was 32 ± 2 pS (four patches), and τ_{open} was increased at positive membrane voltages. However, channel activity was invariant over internal Ca^{2+} concentrations from 100 μM to $< 0{\cdot}1$ μM (four of four patches). It may be that there is only one non-specific cation channel in these cells but that it is subject to modulatory influences which affect its Ca^{2+} sensitivity. In the absence of data to either support or refute this idea, however, the dominant non-specific cation conductance in these cells should be considered Ca^{2+} dependent.

The Ca^{2+}-dependent non-specific cation channel was also observed in non-transformed *balb* 3T3 cells. For inside-out patches taken from confluent 3 day post-passage cells which had not been pretreated with trypsin–EDTA (see previous section of Results), the cation channel was observed in three of three patches with 1 mM internal Ca^{2+}, eleven of nineteen patches with 200 μM Ca^{2+}, and none of seven patches with 10 μM Ca^{2+}. Cells pretreated with trypsin gave similar results. In general, patches from *ras*-transformed *balb* 3T3 cells also had a high frequency of cation channel occurrence. However, over the course of this study there were brief periods when the channel was observed in as little as 10% of patches from these cells. The reason for this variability is not known. Similar variability was not observed in non-transformed cells. Since the K^+ channel was the main focus of the work far fewer experiments were performed on non-transformed cells and therefore periods of low channel density may have been missed. The channel observed in non-transformed cells reversed at $-2{\cdot}2 \pm 1{\cdot}2$ mV in symmetric 150 mM NaCl (1 mM Ca^{2+} internally, 1 μM Ca^{2+} externally), and the conductance was 29 ± 2 pS (4 patches). Channel open times increased as membrane potential was made more positive. Based on physiological criteria the non-specific cation channels in non-transformed and *ras*-transformed *balb* 3T3 cells were indistinguishable.

DISCUSSION

In this study we have attempted to identify the single channel basis for a current associated with *ras* transformation of several fibroblast cell lines. The whole-cell current is activated by the calcium ionophore A23187, it is highly selective for K^+ over Na^+, and it is blocked by externally applied charybdotoxin (100 nM) and TEA (10 mM) (Rane, 1991). The predominant channel observed in *ras*-transformed cells is Ca^{2+} dependent, K^+ selective, and is blocked by charybdotoxin and TEA. In symmetric KCl whole-cell currents show mild inward rectification while rectification for single channel currents is more pronounced. The appearance of the whole-cell currents can be accounted for by the fact that depolarization causes an increase in the frequency of channel openings. Consequently, digital averaging of single channel ramp currents produces macroscopic currents that are identical to Ca^{2+}-activated currents recorded in whole-cell mode. It is clear, therefore, that the Ca^{2+}-dependent, K^+-selective channel is entirely responsible for the large Ca^{2+}-activated K^+ currents which correlate with expression of the p21-ras oncoprotein.

Application of A23187 to confluent, non-transformed fibroblasts fails to evoke whole-cell K^+ currents (Rane, 1991). Thus it was not surprising that during this study we failed to observe K^+ channels in excised patches from these cells. The

excised patch experiments were important, however, for several reasons. First, whole-cell recordings were technically feasible only on cells that had been rounded with trypsin–EDTA, and it was possible that this pretreatment selectivity disabled channels in the non-transformed cells. Flattened cells which had not been enzymatically treated were suitable for making excised patches, and the absence of K^+ channels in these patches could not be attributed to enzyme action. Second, it was possible that the failure to observe whole-cell K^+ currents in non-transformed cells was mainly due to inadequate elevation of intracellular Ca^{2+} levels by A23187. That is, non-transformed cells may buffer changes in internal Ca^{2+} more effectively than do transformed cells. K^+ channels in excised patches from transformed cells were fully activated at 10 μM Ca^{2+}, while application of 100–1000 μM Ca^{2+} to inside-out patches from non-transformed cells produced no evidence of K^+ channel activity. Therefore, the differences in whole-cell K^+ currents between transformed and non-transformed cells are probably not due to a failure to adequately increase intracellular Ca^{2+} concentration in the latter. Ca^{2+} concentrations greater than 100 μM did activate a type of non-specific cation channel in a large percentage of patches from non-transformed cells. Whole-cell non-specific cation currents elicited by A23187 were small, however, suggesting that internal Ca^{2+} levels did not rise much above 100 μM in response to external application of A23187 and 1 mM Ca^{2+}. A physiologically identical cation channel was observed in *ras*-transformed cells at a similar frequency of occurrence as in non-transformed cells. Thus it appears that *ras* transformation has little effect on the functional expression of this channel. The presence or absence of the Ca^{2+}-activated K^+ channel may be due to several factors. Its expression or insertion may be suppressed in non-transformed cells relative to their mitogenically active *ras*-transformed counterparts, or the channel may be present in non-transformed cells but rendered quiescent by some modulatory influence. Additional work is required to test these hypotheses.

What role, if any, the Ca^{2+}-activated K^+ channel has in *ras*-induced transformation remains to be determined. It is interesting that the same or a very similar channel has been identified in other mitogenically active cells. These include HeLa cells in exponential growth (Sauve, Simoneau, Monette & Roy, 1986), lymphocytes and thymocytes (Mahaut-Smith & Schlichter, 1989) and a mammary tumour cell line (Furuya, Enomoto, Furuya, Yamagishi, Edwards & Oka, 1989; Enomoto, Furuya, Maeno, Edwards & Oka, 1991). Each of these reports describes an inwardly rectifying, K^+-selective channel which activates at submicromolar Ca^{2+} concentrations. Enomoto *et al.* (1991) have also reported block of this channel by external TEA and charybdotoxin at concentrations similar to those reported here. The selective appearance of the K^+ channel in constitutively proliferating *ras*-transformed cells, together with its absence from confluent normal cells, suggests that it may be a marker or determinant of a mitogenically active state. Further support for this idea comes from preliminary results showing mitogenic stimulation of non-transformed fibroblasts induces the appearance of low-density, whole-cell Ca^{2+}-activated K^+ currents coincident with the onset of cell proliferation (Huang, McDonald & Rane, 1991). In T-lymphocytes mitogenic stimulation has been associated with rapid changes in the kinetics of one type of voltage-dependent potassium channel and over long periods of time with alterations in the number and

types of potassium channels expressed (Deutsch *et al.* 1986; Decoursey *et al.* 1987). In addition, mitogenic stimulation of DNA synthesis in lymphocytes was found to be inhibited by potassium channel blockers (Decoursey *et al.* 1984; Deutsch *et al.* 1986; Price *et al.* 1989), and in other systems changes in membrane potential and intracellular ion concentrations have been associated with altered rates of DNA synthesis and amino acid transport (Villereal & Cook, 1978; Moolenar, de Laat & van der Saag, 1979; Moolenar, Mummery, van der Saag & de Laat, 1981; Rozengurt, 1986; Kojima, Matsunaga, Kurokawa, Ogata & Nishimoto, 1988). Taken together these studies suggest that stimulation of cell proliferation, either by applied mitogens or by incorporation of the *ras* oncogene, may involve as a critical component the modulation of ion channel activity.

We thank Dr J. Strong for helpful suggestions on this manuscript. This investigation was supported by an Epilepsy Foundation of America research grant (S.R.), and by a graduate stipend (Y.H.) from the Purdue University Cancer Center supported by the American Cancer Society (IN-17 31).

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