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Differential expression of voltage-activated Na^+ currents in two prostatic tumour cell lines: contribution to invasiveness in vitro

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Abstract **The voltage-gated ionic currents of two rodent prostatic cancer cell lines were investigated using the whole-cell patch clamp technique. The highly metastatic Mat-Ly-Lu cells expressed a transient, inward Na^+ current (blocked by 600 nM tetrodotoxin), which was not found in any of the weakly metastatic AT-2 cells. Although both cell lines expressed a sustained, outward K^+ current, this occurred at a significantly higher density in the AT-2 than in the Mat-Ly-Lu cells. Incubation of the Mat-Ly-Lu cell line with 600 nM tetrodotoxin significantly reduced the invasive capacity of the cells in vitro. Under identical conditions, tetrodotoxin had no effect on the invasiveness of the AT-2 cells.**

Key words: Ion channels; Tetrodotoxin; Prostate cancer; Invasion; Metastasis; Electrophysiology

1. Introduction

Ion channels are involved in a wide variety of cellular functions such as membrane excitability, secretion, fluid transport, enzyme activity, adhesion, differentiation/growth and proliferation. Pathological states of cells have also been shown to relate to ion transport, as in cystic fibrosis [1], hyperkalemic periodic muscle paralysis [2,3], viral infection [4] and multi-drug resistance [5]. Although some cancer cells, mostly those derived from neuronal tumours, have been shown to possess voltage-activated ion channels (e.g. [6–8]), any relation between ion channel activity and metastasis (formation of secondary tumours by cancer cell migration, invasion and proliferation) has not previously been investigated.

In this study, we have used the whole-cell patch clamp technique [9] to make an initial comparison of the voltage-dependent ionic currents in two different Dunning epithelial cell lines derived originally from the same rat prostatic tumour: (1) the AT-2 cell line which is characterised by its very low rate (< 10%) of metastasis to non-specific sites; and (2) the Mat-Ly-Lu cell line which displays a high rate (> 90%) of metastasis specifically to the lungs and lymph nodes, when injected subcutaneously into rats [10]. The results showed that there are significant qualitative and quantitative differences in the electrophysiological properties of these cells. Furthermore, the observed electrophysiological differences of the two cell lines could be related to the cells' invasiveness in vitro. A brief report of the early part of this work has been published [11].

2. Materials and methods

Two Dunning prostatic carcinoma cell lines derived from a spontaneously occurring prostate tumour in an inbred Copenhagen rat [10,12] were used. Cells were cultured using the protocol described by Isaacs et al. [10] with a few modifications. Cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 1% foetal calf serum (stripped and dialyzed against isotonic saline), 2 mM L-glutamine, 250 nM dexamethasone, 10 mg/ml penicillin/streptomycin, and 0.25 μg/ml fungizone. Cells were seeded into 35 mm Petri dishes and grown in an incubator at 37°C, 100% humidity and 5% CO_2. Prior to each experiment, the growth medium was replaced with an external bathing Ringer solution containing (in mM): NaCl 144, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 2.5, D-glucose 5.6, and HEPES 5, adjusted to pH 7.2 with 1 M NaOH.

Patch pipettes of resistances between 5 and 15 MΩ were filled with a solution containing (in mM): NaCl 5, KCl 145, $MgCl_2$ 2, $CaCl_2$ 1, HEPES 10, and EGTA 11, adjusted to pH 7.4 with 1 M KOH. Whole-cell membrane currents were recorded using a Biologic RK 300 amplifier; cells that appeared 'isolated' in culture were used. Analogue signals were filtered at 10 kHz using a 5-pole Tchebicheff filter, digitised using an interface (CED, 1401) and sampled at 5 kHz. CED voltage clamp software version 5.5 was used for acquisition and analysis of whole-cell currents.

Drug solutions were made up in the external bathing Ringer and applied using a U-tube perfusion system. Sodium-free external bathing Ringer was made by replacement of NaCl with equimolar choline chloride. (Replacement of NaCl with choline chloride had no effect on the outward potassium current). The effect of a range of external tetrodotoxin (TTX) concentrations (0.1 nM to 1 μM) was studied, using single voltage pulses from −100 mV to −10 mV. In these experiments, an internal pipette solution in which KCl had been replaced with CsCl to abolish outward currents was used. The effect of TTX was determined by measuring the current 3 min after drug application. TEA solutions were made by equimolar replacement of NaCl to maintain osmolarity.

Matrigel invasion chamber assay protocols were as previously described [13]. In summary, chambers (six-well inserts) were purchased from Collaborative Biomedical Products and were prepared as per instructions. Cells were plated at 1×10^5 cells/ml in chambers containing either the supplemented RPMI medium alone, or with added 600 nM TTX, and were left at 37°C for 48 h. All solutions were filtered prior to use. Non-invading cells were removed from the upper surface of the membrane with a swab and cell numbers on the underside were determined using the colourimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [14,15]. Briefly, 0.625 ml MTT (5 mg/ml) was added to the 2.5 ml of fresh medium in the bottom of the chambers. The plates were wrapped in foil and incubated at 37°C for 6 h. The medium was then removed from the chambers and replaced with 2.5 ml DMSO and 0.31 ml glycine buffer. Absorbance at 570 nm was determined 10 min after addition of the buffer. Results were calculated as the mean of six repeats of triplicate TTX vs. control spectrophotometer readings from individual invasion wells.

In order to determine possible toxic effects of TTX (or direct effects

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on the proliferation rate), cells were plated at 2×10^4 cells/well in supplemented RPMI only or with added 600 nM TTX in six-well plates (Falcon) and were left at 37°C for 48 h. Cell numbers were then determined using the MTT assay as described above.

The determination of cell numbers from the spectrophotometer readings was achieved by plotting standard curves for both cell lines for ten serial dilutions from a starting density of 1.25×10^5 cells/plate. Cells were plated at the required density in the supplemented RPMI medium in two six-well plates (Falcon) and were left at 37°C for two hours to settle before performing the MTT assay.

All quantitative data are presented in the text as mean ± S.E.M. Statistical significance was determined with Student's t-test.

3. Results

In the AT-2 cell line, application of depolarizing voltage pulses produced outward currents which activated slowly and inactivated partially over the depolarization period (Fig. 1a). The decay of the current, during a 10 s depolarization to +60 mV, could be fitted by a single exponential with a time constant of 660 ± 80 ms (n = 10). The threshold voltages for the activation of this outward current were in the range −60 to −30 mV, with a mean value of −48.3 ± 2.3 mV (n = 23). The current

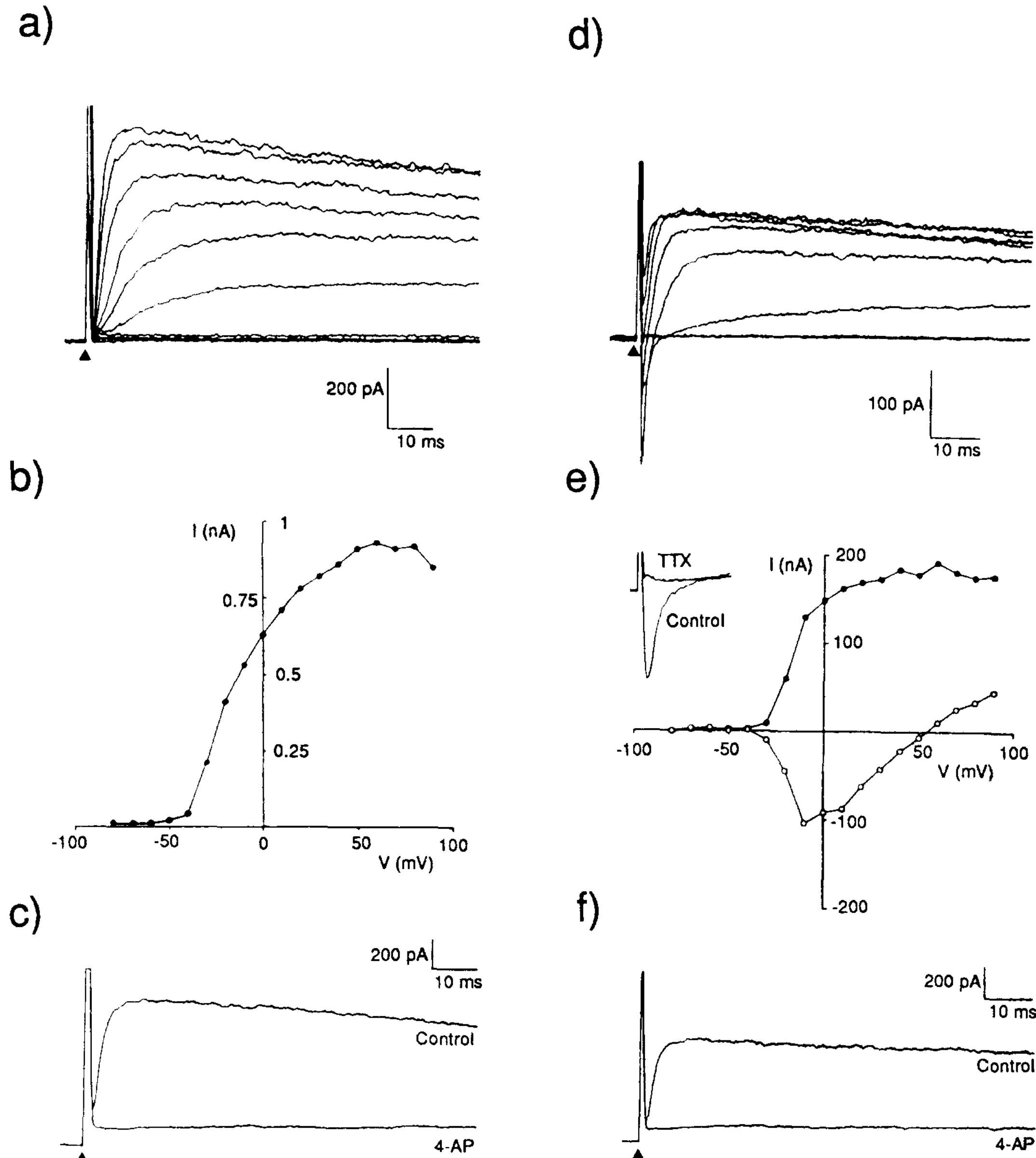

Fig. 1. Whole-cell currents recorded from AT-2 (a–c) and Mat-Ly-Lu (d–f) cells. (a) A typical family of voltage-activated membrane currents elicited by depolarizing voltage pulses of 80 ms duration, applied in 10 mV increments from a holding potential of −90 mV. The onset of the voltage pulses, which lasted for the whole duration of the traces, is indicated by the arrow-head (▲). In experiments aimed at curve-fitting of the inactivation kinetics, 30 s pulses (from −90 mV to +60 mV) were used. (b) The current–voltage (I–V) relationship of the outward currents measured as in (a). Current measurements were taken at the end of the 80 ms voltage pulses. (c) The effect of 25 mM 4-AP on the current elicited by a single depolarizing voltage pulse to +60 mV from a holding potential of −90 mV. (d) A typical family of voltage-activated currents recorded in a Mat-Ly-Lu cell (experimental details as in (a)). (e) The current–voltage relationship for the outward (●) and inward (○) currents recorded as in (d). For the outward current, measurements were taken at the end of the 80 ms voltage pulse, whereas for the inward current, the peak current for each voltage step was measured. Inset: the effect of TTX (1 mM) showing complete blockage of the inward current elicited by a single depolarizing voltage pulse to −10 mV from a holding potential of −100 mV. (f) The effect of 25 mM 4-AP on the current elicited by a single depolarizing voltage pulse to +60 mV from a holding potential of −90 mV.

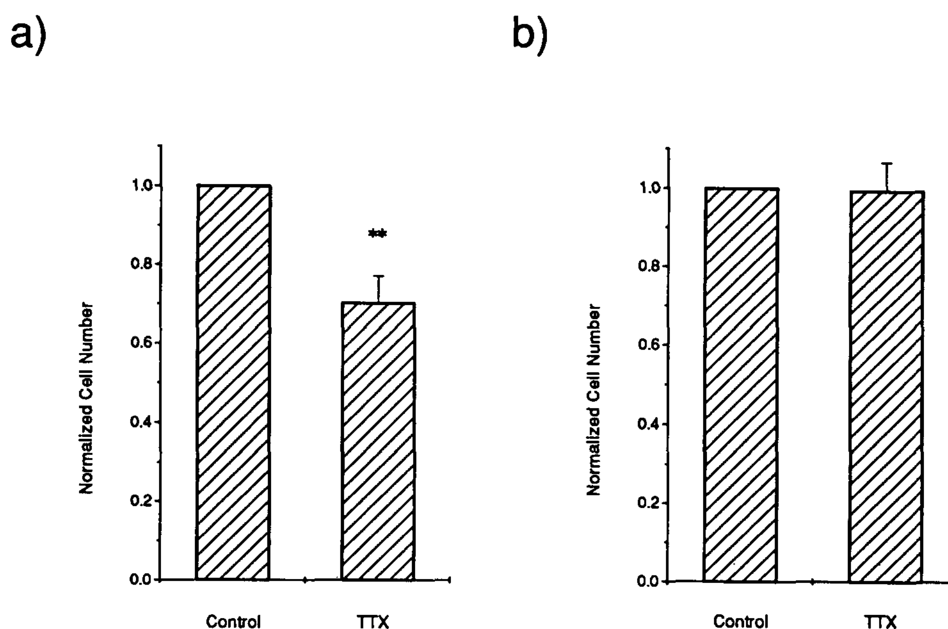

Fig. 2. Effect of 600 nM tetrodotoxin (TTX) on the invasiveness of Mat-Ly-Lu and AT-2 cells. Histograms showing the effect of incubation with 600 nM TTX for 48 h on the relative numbers of invasive cells for (a) Mat-Ly-Lu and (b) AT-2 cells, as determined from the Matrigel in vitro invasion assay. For individual assays, the numbers of invading Mat-Ly-Lu cells varied between 500 and 12,000 whilst for the AT-2 cell line the range was 4,000–14,000. **Indicates statistical significance at $P < 0.01$. Error bars = standard errors.

reached half-maximum at −20.9 ± 3.1 mV (n = 30) and typically saturated at higher depolarizing voltages (47.3 ± 3.7 mV; n = 30), whereby increasing the voltage steps led to no further increase in current (Fig. 1b). The maximum size of the current was 703.8 ± 93.9 pA (n = 30), corresponding to a peak current density of 19.1 ± 2.1 pA/pF (n = 28). This outward current was blocked in a dose-dependent manner by the K^+ channel blockers tetraethylammonium (TEA) and 4-aminopyridine (4-AP) with IC_{50}s of approximately 10.1 and 3.8 mM, respectively (Fig. 1c). Inward currents were not detected in any of the cells tested (n = 142).

The Mat-Ly-Lu cell line displayed markedly different channel characteristics. First, there was considerable heterogeneity in the channels expressed, with populations of cells displaying both inward and outward currents activated by membrane depolarization (Fig. 1d). Of the 151 cells studied, 71 (47%) possessed an initial, transient inward current, whilst all cells expressed a delayed outward current. The inward currents were activated by voltage steps in the range −50 to −30 mV, the mean threshold value of the activation voltage being −39 ± 2.0 mV (n = 20) (Fig. 1e). These currents had a time to peak of 1.77 ± 0.16 ms and reached maximal amplitude at −8.0 ± 2.1 mV (n = 20). The maximum size of the inward current was 355.5 ± 66.7 pA (n = 20), corresponding to a current density of 14.5 ± 3.2 pA/pF (n = 20). These inward currents were completely suppressed by the blocker of voltage-dependent Na^+ channels, tetrodotoxin (TTX) at concentrations of 100 nM – 1 μM (n = 18) (Fig. 1e inset); the currents were also abolished by removal of extracellular Na^+ (n = 8). Second, the outward currents recorded in the Mat-Ly-Lu cells had a maximum size of 340.7 ± 56.2 pA (n = 28) corresponding to a current density of 11.5 ± 1.9 pA/pF (n = 25), which was significantly lower than those found in the AT-2 cell line (P = 0.011). However, in three other respects, as follows, the outward currents in the two cell lines were similar. (i) *Voltage activation profile:* threshold voltages were in the range −60 to −30 mV, with a mean of −42.4 ± 3.3 mV (n = 17). Peak currents occurred at 44.9 ± 5.6 mV (n = 28) whilst the current was half-maximum at −18.0 ± 2.8 mV (n = 28). The respective values were essentially the same in the two cell lines (P = 0.14–0.72). (ii) *Time course:* current decay followed again a single exponential with a time constant of 630 ± 100 ms (n = 10) which was statistically similar to that found in the AT2 cells (P > 0.7). (iii) *Pharmacology:* the outward current was suppressed by TEA and 4-AP with similar relative potency, the IC_{50}s being approximately 29 and 1.1 mM, respectively (Fig. 1f).

Since Na^+ currents were recorded only in the Mat-Ly-Lu cells, the possible involvement of these channels in the metastatic process was investigated using an in vitro Matrigel invasion chamber assay. Incubation of the Mat-Ly-Lu cells with 600 nM TTX for 48 h reduced the invasive capacity of the these cells by 14–51% (mean reduction = 32.9 ± 6.5%; P < 0.01) (Fig. 2a). In contrast, TTX had no significant effect on the invasiveness of the AT-2 cell line (mean reduction = 2.5 ± 7.4%; P = 0.55) (Fig. 2b). The size of the observed TTX-induced reduction in invasion appeared to correlate with the fraction of Mat-Ly-Lu cells expressing Na^+ channels in different cultures, although this relationship was not studied systematically. In one experiment, for example, where every cell tested (n = 13) was found to express Na^+ channels, 600 nM TTX suppressed invasion by the maximal level seen (51%). On another occasion, for a batch of cells showing only 50% expression of Na^+ channels (n = 18), equimolar TTX caused only some 16% reduction in invasion.

Incubation of cells with 600 nM TTX for 48 h had no effect on cell density when compared to those incubated in supple-

mented medium alone for either the Mat-Ly-Lu (mean reduction = 1.3 ± 0.04%; $P = 0.30$) or the AT-2 cell line (mean reduction = 0.3 ± 0.02%; $P = 0.75$), indicating that the observed reduction in the invasion rate of the Mat-Ly-Lu cell line could not be due to any TTX-induced toxicity or an alteration in the proliferation rate.

4. Discussion

Our recordings showed clearly that TTX-sensitive Na^+ channels were found only in the Mat-Ly-Lu cells which have previously been shown to be much more metastatic, in a tissue-specific manner, than the AT-2 cell line [10]. In order to test whether the Na^+ channels could indeed be involved in the cells' overall metastatic behaviour, an in vitro Matrigel invasion chamber assay was carried out. This test showed that 600 nM TTX, which was found earlier by patch clamp recording to block the Na^+ currents present, significantly reduced the cells' invasive capacity, whilst having no effect on the AT-2 cells. The mechanism by which Na^+ channel activity could contribute to invasion is not yet clear. However, such channels and the action potentials they can generate, commonly have an intermediary role in cellular responses, including chemical secretion (i.e. stimulus-secretion coupling) [16,17] and motility (e.g. muscle contraction, neurite extension) [18,19] which are considered to be components of metastasis [20].

Voltage-gated, delayed rectifier type K^+ channels were present in both of the prostatic epithelial carcinoma cell lines studied. The pharmacological, as well as the electrophysiological characteristics of the outward currents in the AT-2 and the Mat-Ly-Lu cells are consistent with the underlying K^+ channels being of the same type. The cellular function(s) subserved by K^+ channels in the two cell lines is not yet clear. In analogy with excitable cells, however, the higher density of the K^+ channels present would imply that the AT-2 cells could have a relatively stable membrane potential. In contrast, the lower density of the K^+ channels present in the Mat-Ly-Lu cells coupled with the occurrence of the Na^+ currents, would suggest that the membranes of the latter would be capable of generating much more dynamic responses. Whilst the role of K^+ channels, if any, in the metastatic process remains to be elucidated, we should note that the product of the metastasis suppressor gene nm23, nucleoside diphosphate kinase, has been shown to be a regulator of K^+ channels [21,22].

The available data taken together would suggest broadly that ion channels may well be involved in some aspects of cell behaviour potentially related to cancer. Accordingly, several groups have shown that expression of oncogenes can affect Na^+ channels [23,24], K^+ channels [25–28] and Ca^{2+} channels [23,29,30] and that these changes may in turn bring about profound effects on cell behaviour. In particular, the *ras* family of oncogenes, which can be involved in metastasis [31,32], also plays an important role in nerve growth factor-induced neuronal differentiation and induction of voltage-gated Na^+ channels [33–35]. Developmental differentiation of human retinoblastoma cells in vitro has also been found to be accompanied by expression of Na^+ channels [36]. Metastasis is a complex, multistep process involving several types of cell behaviour such as adhesion, secretion, interaction with the extracellular matrix (including enzymatic degradation), migration, invasion and proliferation. Ion channels could be involved in any of these steps, either indirectly (by causing changes in gene expression) [37] or via a more direct route affecting the intra-cellular and/or extra-cellular environment [18,19,38,39]. Cell adhesion [40], motility [18,19], interaction with components of the extracellular matrix [41] and proliferation [23,28,39,42–45] have indeed been shown to involve ion channel activity.

In summary, our results showed qualitative and quantitative variability in the expression of Na^+ and K^+ channels in prostatic epithelial carcinoma cell lines. In particular, pharmacological blockage of Na^+ channels, expressed only in the highly metastatic Mat-Ly-Lu cells, suppressed the cells' invasiveness in vitro. These results raise the possibility that voltage-gated Na^+ channels could contribute to the metastatic behaviour of particular carcinomas of the prostate, perhaps those metastasizing to a specific tissue site, as in the case of the Mat-Ly-Lu cells. However, Na^+ channels are unlikely to be a unique feature of invasive cells in prostate cancer, since the AT-2 cells, which are similarly invasive, completely lacked such channels. Thus, several questions remain. First, are other ion channels (e.g. K^+ and Ca^{2+}) also involved in cellular mechanisms that may contribute to the overall metastatic behaviour of prostatic tumours? Second, do different patterns of metastasis involve activities of different ion channels? Third, what is the role of ion channels, if any, in other cancers? Clearly, further work is required to answer these and other related questions.

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References

[1] Quinton, P.M. (1990) FASEB J. 4, 2709–2712.
[2] Cannon, S.C., Brown, R.H. and Corey, D.P. (1991) Neuron 6, 619–626.
[3] Lehmann-Horn, F., Iaizzo, P.A., Hatt, H. and Franke, C. (1991) Pflügers Arch. 418, 297–299.
[4] Pinto, L.H., Holsinger, L.J. and Lamb, R.A. (1992) Cell 69, 517–528.
[5] Gill, D.R., Hyde, S.C., Higgins, C.F., Valverde, M.A., Mintenig, G.M. and Sepulveda, F.V. (1992) Cell 71, 23–32.
[6] Champigny, G. Verrier, B. and Lazdunski, M. (1991) Biochem. Biophys. Res. Commun. 176, 1196–1203.
[7] Marx, A., Siara, J. and Ruedel, R., (1991) Pflügers Arch. 471, 537–539.
[8] Pancrazio, J.J., Viglione, M.P., Tabbara, I.A. and Kim, Y.I. (1989) Cancer Res. 9, 5901–5906.
[9] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F. (1981) Pflügers Arch. 391 85–100.
[10] Isaacs, J.T., Isaacs, W.B., Feitz, W.F.G and Scheres, J. (1986) Prostate 9, 261–281.
[11] Grimes, J.A., Djamgoz, M.B.A., Downing, J.E.G., Laniado, M.E., Foster, C.S. and Abel, P.D. (1993) Urol. Res. 21, 459.
[12] Dunning, W.F. (1963) Monogr. Natl. Cancer Inst. 12, 351–369.
[13] Albini, A., Iwamoto, Y., Kleinman, H.K., Martin, G.R., Aaronson, S.A., Kozlowski, J.M. and McEwan, R.N. (1987) Cancer Res. 47, 3239–3245.
[14] Freshney, R.I. (1994) Culture of Animal Cells: a Manual of Basic Technique, Wiley-Liss, New York, pp. 296–299.
[15] Hansen, M.B., Neilsen, S.E. and Berg, K. (1989) J. Immunol. Methods 119, 203–210.
[16] Tiligad, E. and Wilson, J.F. (1988) Neurosci. Lett. 95, 318–322.
[17] Wang, X., Inukai, T., Greer, M.A. and Greer, S.E. (1994) Brain Res. 662, 83–87.
[18] Komuro, H. and Rakic, P. (1992) Science 257, 806–809.
[19] Silver, R.A. and Bolsover, S.R. (1991) J. Physiol. (Paris) 85, 79–83.
[20] Aznavoorian, S., Murphy, A.N., Stetler-Stevenson, W.G. and Liotta, L.A. (1993) Cancer 71, 1368–1383.

[21] Leone, A., Flatow, U., King, C.R., Sandeen, M.A., Margulies, I.M.K., Liotta, L.A. and Steeg, P. (1991) Cell 65, 25–35.
[22] Otero, A.S., Breitwiesser, G.E. and Szabo Gabor (1988) Science 242, 443–445.
[23] Caffrey, J.M., Brown, A.M. and Schneider, M.D. (1987) Science 236, 570–573.
[24] Flamm, R.E., Birnberg, N.C. and Kaczmarek, L.K. (1990) Pflügers Arch. 416, 120–125.
[25] Hemmick, L.M., Perney, T.M., Flamm, R.E., Kaczmarek, L.K. and Birnberg, N.C. (1992) J. Neurosci. 12, 2007–2014.
[26] Huang, Y. and Rane, S.G. (1993) J. Physiol. 461, 601–618.
[27] Repp, H., Draheim, H., Ruland, J., Seidel, G., Beise, J., Presek, P. and Dreyer, F. (1993) Proc. Natl. Acad. Sci. USA 90, 3403–3407.
[28] Teulon, J., Ronco, P.M., Geniteau-Legendre, M., Baudouin, B., Estrade, S., Cassingena, R. and Vandewalle, A. (1992) J. Cell Physiol. 151, 113–125.
[29] Chen, C.F., Corbley, M.J., Roberts, T.M. and Hess, P. (1988) Science 239, 1024–1026.
[30] Collin, C., Papageorge, A.G., Lowy, D.R. and Alkon, D.L. (1990) Science 250, 1743–1745.
[31] Thorgiersson, V.P., Turpeeniemi-Hujanen, T., Williams, J.E., Westin, E.H., Heilman, C.A., Talmadge, J.E. and Liotta, L.A. (1985) Mol. Cell. Biol. 5, 259–262.
[32] Treiger, B. and Isaacs, J. (1988) J. Urol. 140, 1580–1586.
[33] Dichter, M.A., Tischler, A.S. and Greene, L.A. (1977) Nature 268, 501–504.
[34] Haleguoa, S., Armstrong, R.C. and Kremer, N.E. (1991) in: Current Topics in Microbiology and Immunology (Bothwell, M. ed.) Springer-Verlag, Berlin, pp. 119–170.
[35] Mandel, G., Cooperman, S.S., Maue, R.A., Goodman, R.H. and Brehm, P. (1988) Proc. Natl. Acad. Sci. USA 85, 924–928.
[36] Gomez, M.P., Waloga, G. and Nasi, E. (1993) J. Neurophysiol. 70, 1487–1496.
[37] Bading, H., Ginty, D.D. and Greenberg, M.E. (1993) Science 260, 181–186.
[38] Honn, K.V., Onoda, J.M., Pampalona, K., Battaglia, M., Neagos, G., Taylor, J.D., Diglio, C.A. and Sloane, B.L. (1985) Biochem. Pharmacol. 34, 235–241.
[39] Wegman, E.A., Young, J.A. and Cook, D.I. (1991) Pflügers Arch. 417, 562–570.
[40] Asou, H. (1992) J. Cell Physiol. 153, 313–320.
[41] Becchetti, A., Arcangeli, A., Del Bene, M.R., Olivotto, M. and Wanke, E. (1992) Proc. R. Soc. Lond. B 248, 235–240.
[42] Barres, B.A. and Raff, M.C. (1993) Nature 361, 258–260.
[43] Leonard, R.J., Garcia, M.L., Slaughter, R.S. and Reuben, J.P. (1992) Proc. Natl. Acad. Sci. USA 89, 10094–10098.
[44] Nilius, B. and Wohlrab, W. (1992) J. Physiol. 445, 537–548.
[45] Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) Nature 295, 68–71.