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Whole-Cell Recording of Neuroblastoma x Glioma Cells during Downregulation of a Major Substrate, 80K/MARCKS, of Protein Kinase C

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Summary. Differentiated neuroblastoma cells exhibit both the delayed rectifier potassium current (I_K) and the M-current (I_M). The present study was designed to determine the roles of protein kinase C (PKC) and of the calmodulin-binding protein 80K/MARCKS, a prominent substrate for PKC and possible regulator of these currents. Neuroblastoma x glioma (NG108-15) hybrid cells transfected with m1 muscarinic receptors were grown with 1% fetal bovine serum (FBS) without the prostaglandin E_1 (PGE_1) and isobutylmethylxanthine (IBMX) usually added in preparation for electrophysiological studies. Under these conditions, the usual pleomorphism was largely abolished, leaving two populations of small cells with stellate and spherically symmetrical geometries. Whole-cell patch clamping indicated that the two cell types had identical electrophysiological properties, displaying: I_K, a small current through a "T-like" Ca^{2+} channel, and no M-current.

Stimulation with carbachol shifted the distribution of cells to a more stellate morphology within 24 hr and later (after 48 hr) reduced the PKC substrate 80K/MARCKS by 22 ± 7%. In contrast to the stimulation of I_K observed with cardiac cells, PKC activation produced only a small inhibition of I_K, which was independent of carbachol pretreatment. Thus, PKC and 80K/MARCKS can be dissociated from the regulation of I_K in neuroblastoma cells.

Key Words Patch clamping · neuroblastoma cells · PKC · 80K/MARCKS · muscarinic acetylcholine receptors · delayed rectifier

Introduction

The reversible phosphorylation of target proteins is a major, ubiquitous mechanism for signal transduction (Cohen, 1989). Protein kinase C (PKC) plays a substantial role in this transduction pathway (Bell, 1986). In particular, PKC has been implicated in the stimulus-response coupling of: channel modulation, endocrine and exocrine secretion, neurotransmitter release, muscle contraction, gene expression and cell proliferation (Nishizawa, 1986; Rozengurt, 1986; Rozengurt & Sinett-Smith, 1988). Some of these actions are mediated by direct phosphorylation of the effector target. For example, PKC downregulates the voltage-sensitive Na^+ channel of brain at least in part by directly phosphorylating the serine 1506 residue (West et al., 1991; Numann, Catterall & Scheuer, 1991). On the other hand, PKC has been found to both up and downregulate voltage-insensitive Na^+ channels from different epithelial cells (Civan et al., 1991). Regulation of these channels by kinases may not reflect a direct phosphorylation of the epithelial Na^+ channels (Lester, Asher & Garty, 1988).

In view of the very limited information available concerning the mechanisms of action, considerable attention has been focused on target substrates, which might serve intermediary, regulatory roles in expressing PKC-triggered effects. Of particular interest has been the 80K/MARCKS family of proteins, which migrate with molecular refractions of 80–87 kD in SDS-polyacrylamide gels, and which serve as PKC substrates in a wide variety of cells (Graff et al., 1989*a*,*b*; Stumpo et al., 1989; Brooks et al., 1991; Erusalimsky et al., 1991; Seykora, Ravetch & Aderem, 1991; Thelen et al., 1991; Herget et al., 1992). The potential importance of these substrates has been emphasized by the fact that 80K/MARCKS is phosphorylated in response to multiple extracellular factors, including growth factors, neuropeptides and direct activators of PKC, i.e., phorbol esters and externally applied diacylglycerols (Erusalimsky et al., 1991). Furthermore, Guadagno, Borner and Weinstein (1992) have reported that overexpression of PKC markedly altered the level and phosphorylation state of this protein, possibly leading to the observed change in cellular phenotype. The 80K/MARCKS proteins from murine, bovine and human cells have

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been cloned and sequenced (Graff et al., 1989*a*; Stumpo et al., 1989; Brooks et al., 1991; Erusalimsky et al., 1991; Seykora, Ravetch & Aderem, 1991; Herget et al., 1992). The inter-species conservation of consensus sequences for four PKC phosphorylation sites binding calmodulin (McIlroy et al., 1991) and actin (Hartwig et al., 1992) has stimulated two hypotheses concerning the physiologic action of the 80K/MARCKS proteins. One group has viewed these PKC substrates as calmodulin buffers, regulating calmodulin-stimulated activities (Graff et al., 1989*a*,*b*; McIlroy et al., 1991). Another group has considered the 80K/MARCKS proteins to be targets of calmodulin, whose interaction with actin is regulated by PKC and the free calmodulin concentration (Rosen et al., 1990; Thelen et al., 1991; Hartwig et al., 1992). In vitro evidence has been accumulated supporting both suggestions, but confirmatory evidence has not yet been obtained with living cells.

Recently, Brooks et al. (1991, 1992) have succeeded in downregulating 80K/MARCKS by applying phorbol esters, bombesin or PDGF to Swiss 3T3 fibroblasts. Our current strategy has been to alter the 80K/MARCKS level in a cell line whose PKC- and/or calmodulin-dependent properties could be monitored electrophysiologically. For this purpose, neuroblastoma hybrid cells seemed promising. Hybrid NG108-15 cells are a colony clone of neuroblastoma x glioma cells, formed by Sendai virus-induced fusion of a subclone of mouse N18-TG2 neuroblastoma cells and a subclone of rat C6Bu-1 glioma cells. These cells can display both an M-current (I_M) and a delayed rectifier (I_K). I_M is physiologically important in controlling cell excitability and has been reported to be inhibited by activators of PKC (Brown & Higashida, 1986*b*; Schäfer, Béhé & Meves, 1991). I_K is important in the control of the firing rate of excitable cells (Hille, 1984) and has been reported to be increased by PKC activation of cardiac ventricular myocytes (Walsh & Kass, 1988).

The present manuscript describes conditions under which it is possible to downregulate 80K/MARCKS in neuroblastoma hybrid cells. Under those conditions, I_M is not expressed and I_K is dissociated from PKC regulation.

Materials and Methods

Electrophysiological Methods

Neuroblastoma x glioma (NG108-15) cells transfected with m1 muscarinic receptors (RM1) were continuously grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS), HAT (hypoxanthine, aminopterin and thymidine) and L-glutamine at 37°C in 10% CO_2. For electrophysiological studies, cells were plated onto 35-mm petri dishes precoated with polyornithine. One day after plating, the FBS concentration in the medium was reduced to 1% to reduce growth, without other additions. Cells were used 4–7 day later. In some cases, cells were preincubated with carbachol (0.1–1.0 mM) for 24–96 hr. As described in detail elsewhere (Robbins & Sim, 1990; Docherty, Robbins & Brown, 1991), the medium was replaced with a superfusate containing (in mM): 120 NaCl, 3 KCl, 5 HEPES (N-2-hydroxyethyl piperazine N'-2-ethane sulfonic acid), 22.6 $NaHCO_3$, 11.1 glucose, 1.2 $MgCl_2$, and 2.5 $CaCl_2$, and equilibrated with 5% CO_2/95% O_2 at 35°C and pH 7.4. Cells were patch clamped in the whole-cell mode, using the discontinuous voltage-clamp approach (Finkel & Redman, 1984). Electrodes (3–6 MΩ) were filled with (in mM): 90 K^+ acetate, 20 KCl, 40 HEPES, 3 $MgCl_2$, 3 EGTA [ethyleneglycol-bis-(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid], and 1 $CaCl_2$, and the pH adjusted to 7.4 with KOH. Under these conditions, the free calcium concentration was calculated to be 40 nM. In the experiments conducted with impalement electrodes, resistances of 25–40 MΩ were observed when the micropipettes were filled with 1 M $(K^+)_3$ citrate. Currents were evoked by voltage jumps generated by PC-based software (pCLAMP, Axon Instruments) via a voltage clamp amplifier (Axoclamp 2, Axon Instruments). Currents were digitally recorded on computer and simultaneously displayed on a pen recorder (Gould 2400S). The membrane potential was usually held at −50 mV, and intermittently clamped to a series of test voltages in 10-mV intervals, over the range from −100 to +80 mV, for 1 sec every 20 sec. Leak subtraction, when used, was an on-line P/5 protocol.

Drugs and Chemicals

Carbachol and 1-oleoyl-2-acetyl-sn-glycerol (OAG) were obtained from Sigma, staurosporin from Boeringer Mannheim, and purified protein kinase C (PKC) was a gift from Dr. Peter J. Parker. Carbachol was dissolved in water at 1,000-fold the applied concentration. OAG was dissolved in ethanol and staurosporin in dimethylsulfoxide (DMSO), both at 1,000-fold the final concentrations. When added to the micropipette filling solution, PKC was used at 100 nM, and accompanied with 50 μg/ml bovine serum albumin, 0.1 mM dithiothreitol, 2 mM ATP, 0.25 mM guanosine triphosphate (GTP) and an increased free Ca^{2+} concentration of 120 nM.

Immunoblotting

Control and treated cells grown in 35-mm dishes were washed three times with ice-cold phosphate-buffered saline (PBS) and harvested in 100 μl extraction buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA (ethylene-diamine-tetraacetic acid), 2% Noniodet P-40 (NP-40), 1 mM phenylmethylsulphonylfluoride (PMSF) with or without 10 μg/ml leupeptin. In one of six preparations, the cells were incubated with DMEM for 15 min at 37°C under 10% CO_2, 90% air before harvesting. This procedure has been found to convert all of the 80K/MARCKS of 3T3 cells to the dephosphorylated form (Brooks et al., 1991). Similar immunoblots were obtained whether the 80K/MARCKS was in a primarily dephosphorylated or in a mixed dephosphorylated and phosphorylated state.

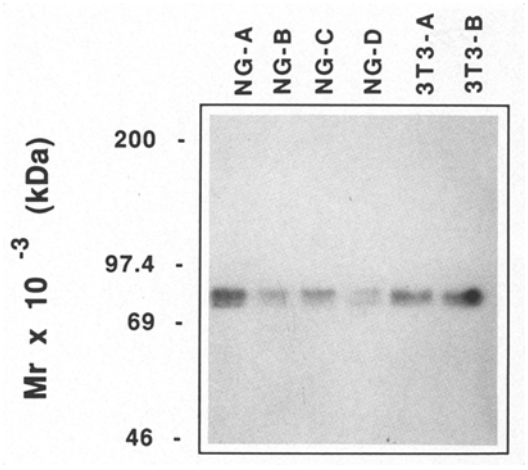

Fig. 1. Radioautograph of Western immunoblot for nontransfected NG108-15 neuroblastoma x glioma hybrid cells (NG) and for Swiss mouse 3T3 fibroblasts (3T3). (*NG-A*) and (*NG-B*): The NG cells had been grown for 1 day in 10% serum and then in 1% for 6 days (control NG conditions). In addition, the cells of *NG-B* had been exposed to 10 μM PGE_1 and 50 μM IBMX during days 1–7. (*NG-C*) and (*NG-D*): The NG cells had been grown in 10% serum for 6 and for 2 days, respectively. (*3T3-A*): The fibroblasts had been plated in 10% serum at low density (5×10^3/ml) and refed fresh medium. (*3T3-B*): The 3T3 cells had been plated at higher density (6×10^4/ml) without refeeding (control 3T3 conditions).

After incubation on ice, the preparation was centrifuged at 13,000 × *g* for 5 min at 4°C. The protein concentration was determined either with Coomassie blue or bicinchoninic acid protein assay reagent. Equal amounts of protein (6–10 μg) were loaded into each lane and separated by sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to nitrocellulose membranes and stained overnight at 4°C with a Swiss 3T3 fibroblast 80K/MARCKS antibody (at 1:400 or 1:500 dilution) (Erusalimsky et al., 1991). Under our experimental conditions, the integrated intensities of the bands are proportional to the intracellular contents of the protein (Stabel et al., 1987).

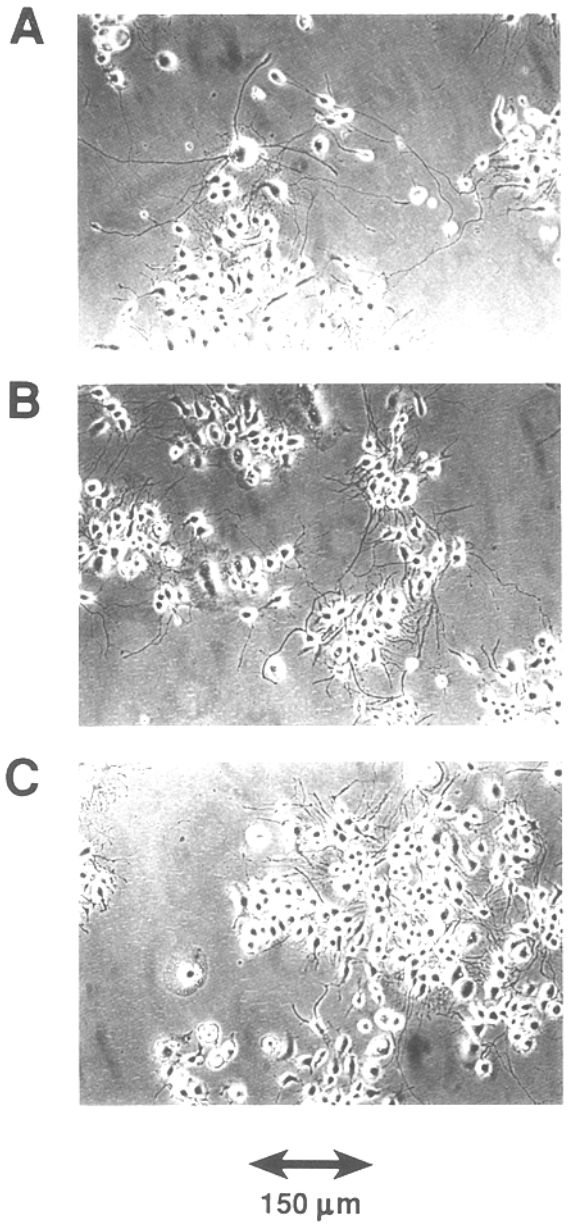

Fig. 2. Transfected neuroblastoma x glioma hybrid cells displaying pleomorphism after 48 hr of exposure to 10 μM PGE_1 and 50 μM IBMX.

Results

Morphology

Electrophysiological measurements are commonly conducted with neuroblastoma hybrid cells preincubated for 48 hr in 1% fetal bovine serum (FBS) with PGE_1 and IBMX (Robbins & Sim, 1990; Docherty et al., 1991). Under these conditions, the level of cellular 80K/MARCKS is reduced, as measured by immunoblotting using a specific antibody directed to the C-terminus of the protein (Fig. 1). In the present study we wished to correlate the electrophysiological parameters of a single cell with the total content of 80K/MARCKS of the entire cell population. For these purposes, the usual pretreatment was unsatisfactory because the IBMX and PGE_1 induce great cellular pleomorphism. The spectrum of cellular morphology is illustrated by Fig. 2. Both highly differentiated cells with well-developed dendrites and small cells with relatively homogeneous cytoplasm can be distinguished.

In contrast, when the cells were grown to quiescence in 1% FBS (Fig. 3*F–J*), the neuroblastoma cells were far more homogeneous than those dis-

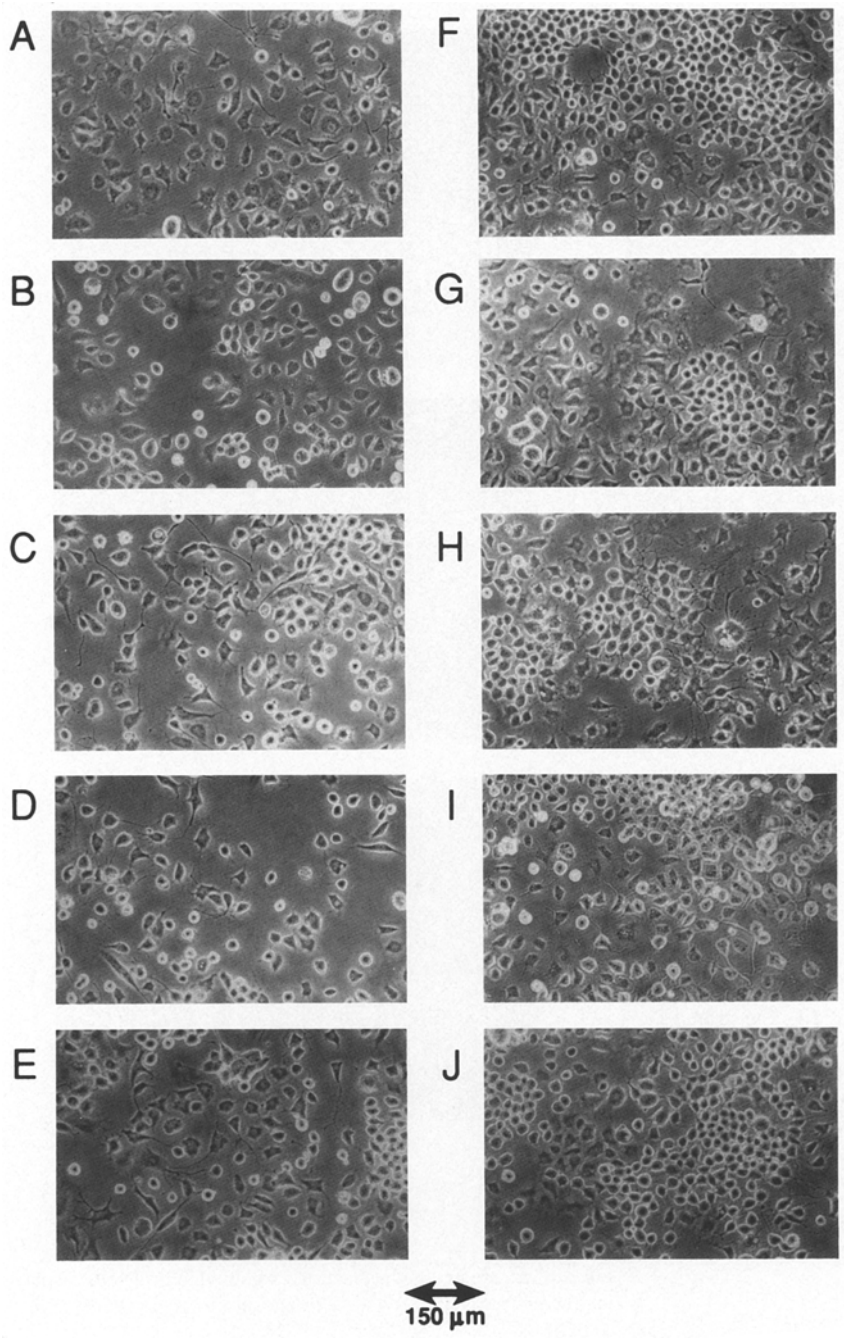

Fig. 3. NG108-15 transfected cells grown under baseline conditions (1% FBS and no PGE_1 or IBMX), with (*A–E*) or without (*F–J*) exposure to 0.1 mM carbachol for 48 hr. The exposure to carbachol shifted the distribution of cell morphologies from a predominately spherical to a predominantly stellate form.

played in Fig. 2. Two types of small cells can be distinguished. One is spherically symmetrical and the other is more stellate in appearance. Under these baseline conditions, the spherical form predominates. These cells can respond to stimulation with carbachol, because the muscarinic receptors of the transfects do not undergo desensitization (Fukuda et al., 1988). Upon pretreatment with 0.1–1.0 mM carbachol, the distribution is shifted to a predominately stellate form (Fig. 3*A–E*). However, in neither case are the large differentiated cells of Fig. 2 noted. Increasing the dose of carbachol added from 0.1 to 1.0 mM, carbachol produced no further detectable change in the cellular morphology.

Immunoblot Analysis

After preincubation with 1% FBS alone ("baseline conditions"), the 80K/MARCKS concentration of

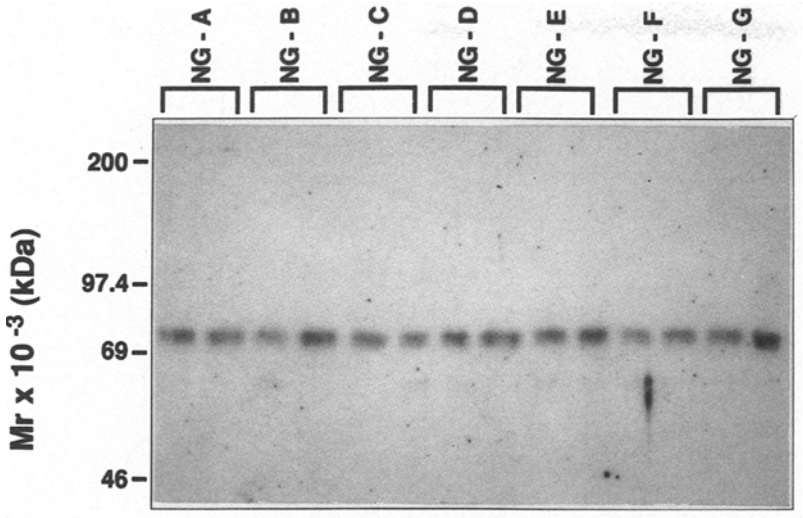

Fig. 4. The NG cells were PM1 (m1) transfects grown: under control conditions (*NG-A*), after exposure for 19 hr to 1.67 mM carbachol (*NG-B*), exposure for 48 (*NG-C*) and 72 hr (*NG-D*) to 1 mM carbachol, exposure for 48 hr to 1 mM carbachol and 10 μM atropine (*NG-E*), exposure to 10 μM PGE_1 and 50 μM IBMX (as in Fig. 1 legend) without (*NG-F*) or with the addition of 1 mM carbachol (*NG-G*) for 48 hr. The duplicate wells were loaded with protein obtained from separate dishes of identically treated cells.

the cells is higher than with growth under nonquiescent conditions (Fig. 1). Under baseline conditions, the concentration even exceeds that of quiescent 3T3 cells (Fig. 1, Table), in which 80K/MARCKS is the principal PKC substrate (Brooks et al., 1991). The transfects used contained m1 receptors, which mediate phosphoinositide hydrolysis and release of diacylglycerol, activating PKC (Fukuda et al., 1988). The application of 1.0 mM carbachol for 48 hr consistently lowered the baseline value (lanes *NG-C*, Fig. 4). The effect was dose dependent, with 0.1 mM carbachol exerting no effect on the 80K/MARCKS concentration, despite producing a clear morphological change (Fig. 3). The effect was also clearly mediated by muscarinic receptors since inclusion of 10 μM atropine in the incubation medium abolished the morphological and immunoblot (lanes *NG-E*, Fig. 4) effects of the carbachol.

The effect of the carbachol was also time dependent (lanes *NG-B* to *-D*, Fig. 4). A significant change in the 80K/MARCKS level was noted 48 hr after initiating exposure to carbachol. The densitometric measurements of the immunoblots are summarized in the Table. The mean carbachol-induced reduction in 80K/MARCKS was 22 ± 7%. The results illustrated by Figs. 1–4 and the Table indicated that the PKC substrate level could be significantly reduced by carbachol without substantially altering the morphology of the baseline neuroblastoma cells. These conditions appeared favorable for our electrophysiological studies to determine: whether baseline cells displayed I_M and I_K, whether either current was affected by PKC activation or inhibition, and whether a downregulation of the 80K/MARCKS substrate affected the absolute or PKC-induced change in either current.

Table. Densitometric analyses of Western immunoblots

Conditions	Mean ± SE (*N*)
Control	1.00 ± 0.05 (10)
Carbachol	
1 mM, 24 hr	0.92 ± 0.14 (5)
1 mM, 48 hr	0.78 ± 0.07 (10) ($P < 0.02$)
1 mM , 72 hr	1.07 ± 0.14 (6)
0.1 mM, 48 hr	1.29 (2)
Carbachol + atropine	1.38 ± 0.20 (6)
PGE_1 + IBMX	
Alone	0.64 ± 0.14 (3)
+ Carbachol	1.38 (2)
10% FCS	0.45 (2)
3T3 cells	0.73 ± 0.08 (5) ($P < 0.05$)

Electrophysiological Results

Two morphological cell types were seen under these conditions, spherical and stellate, which were subsequently found to be electrophysiologically identical. The cells predominantly used for the study were of the small (30–60 μm) spherical type to reduce space-clamp errors. Figure 5 shows a typical example of the currents seen in these cells which are dominated by a large, slowly inactivating current previously documented to be a potassium current (Robbins & Sim, 1990). This current has been described in chemically differentiated (Robbins & Sim, 1990; Dubois & Rouzaire-Dubois, 1991) and undifferentiated (Reeve & Peers, 1992) NG108-15 cells. There was evidence of a small inward "T-

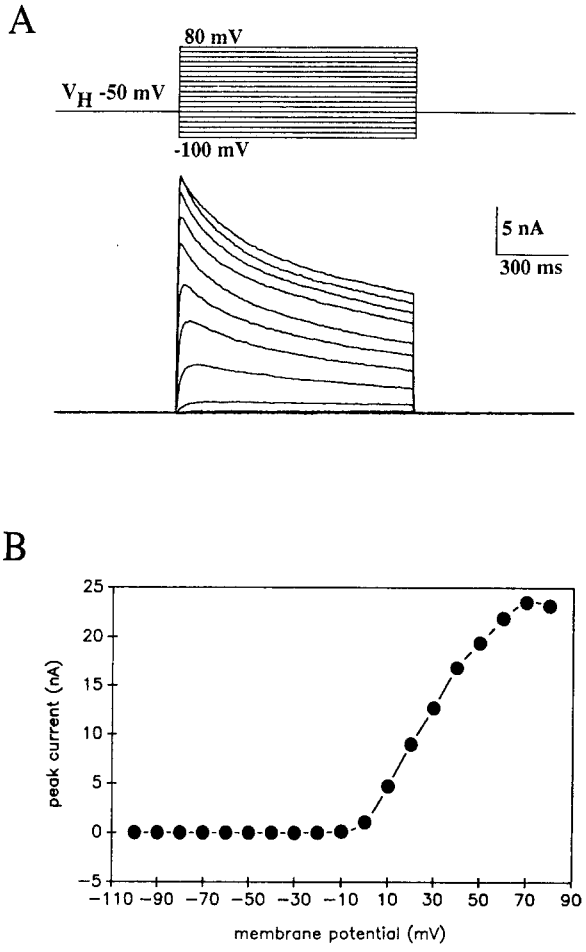

Fig. 5. Inactivating potassium current evoked in undifferentiated NG108-15 cells. Example of a typical cell which was clamped at −50 mV and stepped for 1 sec from −100 to 80 mV in 10-mV increments (upper trace). In this example, on-line P/5 leak subtraction was used (pCLAMP) to give the evoked current. The *I/V* curve (*B*) showed no time- or voltage-dependent currents until approximately −10 mV when, at potentials positive to this value, the *I/V* curve was dominated by the slowly inactivating K^+ current.

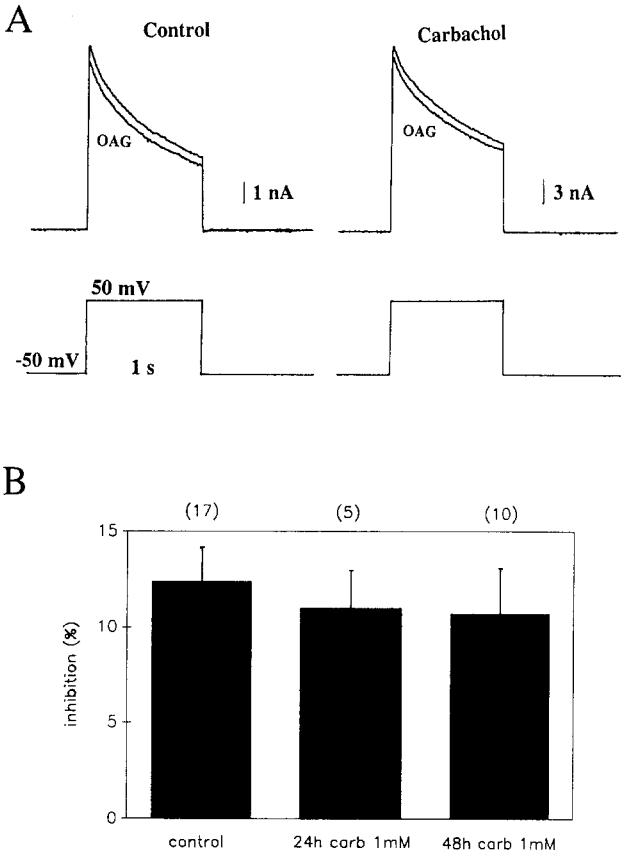

Fig. 6. Evoked currents (*A*, from control cell (left) and a cell exposed to 1 mM carbachol for 48 hr (right). In each panel are currents evoked before and after application of 30 μM OAG for control cells and cells preincubated for 24 and 48 hr in 1 mM carbachol. Numbers in brackets refer to the number of cells tested.

like'' calcium current in some cells (Buisson et al., 1992), but this was only about 0.1–0.2 nA in amplitude. This ''T-like'' current was observed prior to addition of OAG, and persisted in its presence. No other voltage- and/or time-dependent currents were evident.

The large outward current was inhibited to a very small extent by exposure to 10–100 μM OAG, usually for 6 min but as long as 15 min. In most cases, this inhibition reversed on washing. As shown in Fig. 6*A*, this inhibition was similar in control cells and cells pretreated with 1 mM carbachol for 48 hr. From the pooled data, the inhibition of the current was not different from control cells (12.4 ± 1.8%, $n = 17$) at 24 or 48-hr exposure to carbachol (Fig. 6*B*). Furthermore, the effect was not modified by preincubation of the cells with 100 nM staurosporin ($n = 8$) for as long as 90 min.

Under the conditions used, PKC may be dialyzed from the cell. We addressed this possibility in two ways. First, we used impalement electrodes and found that the inhibitory responses to 30 μM OAG were no larger (8.8 ± 4.0%, $n = 6$). Second, we optimized the conditions for PKC activity by including in the micropipette filling solution 100 nM PKC, together with ATP, GTP and 120 nM Ca^{2+}. Once again, the responses were not larger than under control conditions (7.8 ± 1.0%, $n = 6$).

Discussion

Whole-cell patch-clamp studies of neuroblastoma cells are commonly conducted with the large, differentiated cells which appear after preincubation with

PGE_1 and IBMX. These large cells constitute a minority fraction of a morphologically heterogeneous cell population (Fig. 2). Therefore, chemical measurements of all the cells cannot be readily correlated with the electrophysiological properties of the minority-cell population. For this reason, we chose to grow cells under conditions (1% FBS without PGE_1 or IBMX) minimizing morphological and electrophysiologic heterogeneity, so that a single cell could be considered representative of the entire population of cells. With this approach, we could then address the question whether changes in the PKC substrate (80K/MARCKS) were associated with changes in electrophysiological properties.

Two chemical techniques have been successfully applied to downregulate 80K/MARCKS: PKC activation and peptide-hormone stimulation. Phorbol esters have been reported to reduce this PKC substrate in fibroblasts (Brooks et al., 1991). This treatment induces the concurrent down-regulation of PKC, so that any observed functional effects are not readily ascribed to the reduction in PKC substrate, *per se*. The alternative approach of applying a peptide hormone to down-regulate 80K/MARCKS seemed more promising since it has not been associated with a reduction in PKC level (Brooks et al., 1992). The transfects used in the present study have muscarinic acetylcholine receptors which do not display desensitization (Fukuda et al., 1988). Since acetylcholine, like bombesin, probably exerts its muscarinic effects partly through PKC stimulation (Brown, 1988), we applied this agent in a hydrolysis-resistant form (carbachol).

The decrease in 80K/MARCKS was also mediated by occupancy of the muscarinic acetylcholine receptors, since it was prevented by addition of atropine at a 100-fold lower concentration than the carbachol. The shift in substrate level was not a nonspecific change accompanying the multiple effects comprising the change in morphology. At a concentration of 0.1 mM, carbachol induced the change in morphology, but no accompanying change in 80K/MARCKS level could be detected. This is the first time that the expression of 80K/MARCKS has been found to be regulated by carbachol and in cells other than fibroblasts. The change in the PKC substrate may in part explain the observation that carbachol stimulates calmodulin translocation in SK-N-SH human neuroblastoma cells (Mangels & Gnegy, 1992). Phosphorylation of the 80K/MARCKS reduces its affinity of binding calmodulin (Graff et al., 1989*b*; McIlroy et al., 1991), releasing the peptide for binding to other sites within the cell.

The electrophysiological properties of the small cells observed under our baseline conditions have not been previously described. Both cell types observed, the stellate and spherical forms, displayed the same characteristics. The predominant channel observed was a delayed rectifier (Fig. 5), accompanied by only a small inward "T-like" calcium channel without any M-current.

PKC can stimulate current through the delayed rectifier of cardiac myocytes (Walsh & Kass, 1988), and some delayed rectifiers do have a PKC-substrate sequence (Tempel, Jan & Jan, 1988). In the present study, PKC activation with OAG had only a small, reversible inhibitory effect on the I_K of the neuroblastoma hybrid cells (Fig. 6), unaffected by preincubation with staurosporin. Furthermore, no additional effect was observed even when the micropipette was filled with solution containing PKC, ATP, and GTP as well as Ca^{2+}. Thus, the current results indicate that PKC and 80K/MARCKS can be dissociated from regulation of the delayed rectifier of neuroblastoma cells. This finding is consistent with the report that two species of voltage-gated K^+ channels cloned from NG108-13 cells (termed NGK1 and NGK2) do not display a PKC-phosphorylation sequence (Yokoyoma et al., 1989).

M-current is not expressed in the homogeneous cell preparation, but is consistently detected in the differentiated cells appearing after incubation with PGE_1 and IBMX (Brown & Higashida, 1988*a*; Robbins et al., 1992). It is noteworthy that the pleomorphic preparation induced by PGE_1 and IBMX (Fig. 2) displayed a lower level of 80K/MARCKS than that of the homogeneous preparation, even after carbachol stimulation (Fig. 1, Table). The 80K/MARCKS concentration of the differentiated cells may not be the same as that of the averaged pleomorphic cell population. Nevertheless, it is tempting to speculate that the low 80K/MARCKS level may be linked to the expression of M-current. One possible link could be through the actin cytoskeleton, whose state of polymerization partially regulates the activity of an epithelial Na^+ channel (Cantiello et al., 1991) and possibly other channels. Activation of PKC depolymerizes actin (Kiley et al., 1992), in part by phosphorylating 80K/MARCKS and thereby reducing the substrate's affinity of binding for actin (Rosen et al., 1990; Hartwig et al., 1992). To the extent that muscarinic stimulation of neuroblastoma cells is PKC mediated (Brown & Higashida, 1988*b*; Schäfer et al., 1991), the hypothesis predicts that acetylcholine could inhibit I_M both acutely (by phosphorylating 80K/MARCKS) and in more sustained fashion, by downregulating the 80K/MARCKS level altogether.

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