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An Electrophysiological Correlate of Protein Kinase C Isozyme Distribution in Cultured Cerebellar Neurons

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Protein kinase C (PKC) is a family of at least seven closely related molecules (isozymes) that vary in terms of their requirements for activation and their distribution among cells of the brain. A striking example of this differential distribution is seen in the cerebellum, where Purkinje cells express PKC-I, an isozyme that is strongly activated by both phorbol ester (PE), and low doses of *cis*-unsaturated fatty acid (c-UFA), while granule cells predominantly express PKC-II, an isozyme that is strongly activated by PE but not c-UFA. Both Purkinje and granule cells have large, easily recorded voltage-gated K currents. These currents are attenuated by PKC activators in several other varieties of neuron. We hypothesized that the effects of these two PKC activators would be predicted by the distribution of the relevant PKC isozyme, and that the delayed outward rectifier current, I_K, would be attenuated by both PE and c-UFA in Purkinje cells, but only by PE in granule cells. This hypothesis was confirmed in perforated-patch recordings. The attenuation produced by both activators could be blocked by application of a specific PKC inhibitor, RO-31-8220, and could not be mimicked by inert forms of PE or c-UFA. To our knowledge, this study represents the first report of an electrophysiological correlate of PKC isozyme distribution.

Protein kinase C (PKC) is found in high concentration in neural tissues and has been implicated in a broad spectrum of neuronal functions including process outgrowth, neurotransmitter release, synaptic plasticity, and the regulation of ion channels (Nishizuka, 1988; Linden and Routtenberg, 1989a; Malinow et al., 1989; Olds et al., 1989; Shearman et al., 1989b; Linden and Connor, 1991). It has been appreciated for several years that PKC is a family of isozymes (Coussens et al., 1986; Knopf et al., 1986; Ono et al., 1987) with different patterns of distribution in the brain as revealed by immunocytochemical (Ase et al., 1988; Hashimoto et al., 1988; Hidaka et al., 1988; Huang et al., 1988, 1990; Kose et al., 1988; Saito et al., 1988; Shimohama et al., 1990) or mRNA hybridization techniques (Brandt et al., 1987; Young, 1988). However, functional correlates of this differential distribution have been difficult to determine.

PKC was originally described as a Ca- and phospholipid-dependent enzyme activated by a diacylglycerol (DAG) second messenger produced by phosphoinositide hydrolysis (Kikkawa et al., 1982). The DAG activation pathway may be mimicked experimentally by addition of phorbol ester (PE), and has been shown to activate all PKC isozymes studied to date. More recently, a second activation pathway has been demonstrated in which *cis*-unsaturated fatty acids (c-UFAs) such as oleate and arachidonate activate PKC (McPhail et al., 1984) in the absence of Ca and phospholipid (Murakami and Routtenberg, 1985; Murakami et al., 1986).

This second pathway of activation is largely restricted to the PKC-I isozyme as demonstrated in purified PKC preparations made from cerebellar tissue. Shearman et al. (1989a) separated PKC activity from bovine cerebellum into three fractions by hydroxyapatite column chromatography. These fractions, called PKC-I, -II, and -III, correspond to the products of the γ, β-I/β-II, and α PKC genes, respectively. The greatest activation of PKC-I was achieved with 12 μM arachidonate (~55% of maximal stimulation produced with phosphatidyl serine/diolein/calcium). PKC-II and PKC-III were almost entirely inert with respect to arachidonate stimulation, being activated at ~7% and 10% of maximum, respectively, by 50 μM arachidonate (the highest concentration reported). As the present study was conducted in living cells with intact metabolic machinery, we chose to use oleic acid (OA) rather than arachidonate to allow for the separation of effects produced by direct PKC activation from those produced by arachidonate metabolites (Wolfe, 1982; Needleman et al., 1986).

Activation of PKC by PE or DAG has been shown to attenuate voltage-dependent K currents in several types of neuron (Baraban et al., 1985; Farley and Auerbach 1986; Higashida and Brown, 1986; Malenka et al., 1986; Grega et al., 1987; Apkon and Nerbonne, 1988; Colby and Blaustein, 1988; Doerner et al., 1988; Sawada et al., 1989). The Purkinje and granule cells of the cerebellum show well-developed voltage-gated K currents, including the delayed outward rectifier current, I_K. In addition, Purkinje cells strongly express PKC-I, while granule cells do not express this isozyme (Brandt et al., 1987; Ase et al., 1988; Hashimoto et al., 1988; Hidaka et al., 1988; Huang et al., 1988, 1990; Kose et al., 1988; Saito et al., 1988; Young, 1988; Shimohama et al., 1990). Hence, these two cell types serve as an ideal model system in which to test the hypothesis that PKC isozyme distribution predicts the electrophysiological response of neurons to different pathways of PKC activation.

Materials and Methods

Cell culture

Cultured cerebellar neurons were prepared by a modification of the method of Hockberger et al. (1989b). Timed-pregnant rats were anesthetized with ether and the fetuses were removed. Cerebella were dissected, pooled, and chopped into ~1 mm chunks in ice-cold phosphate-buffered saline (PBS). The chunks were digested for 5 min at room

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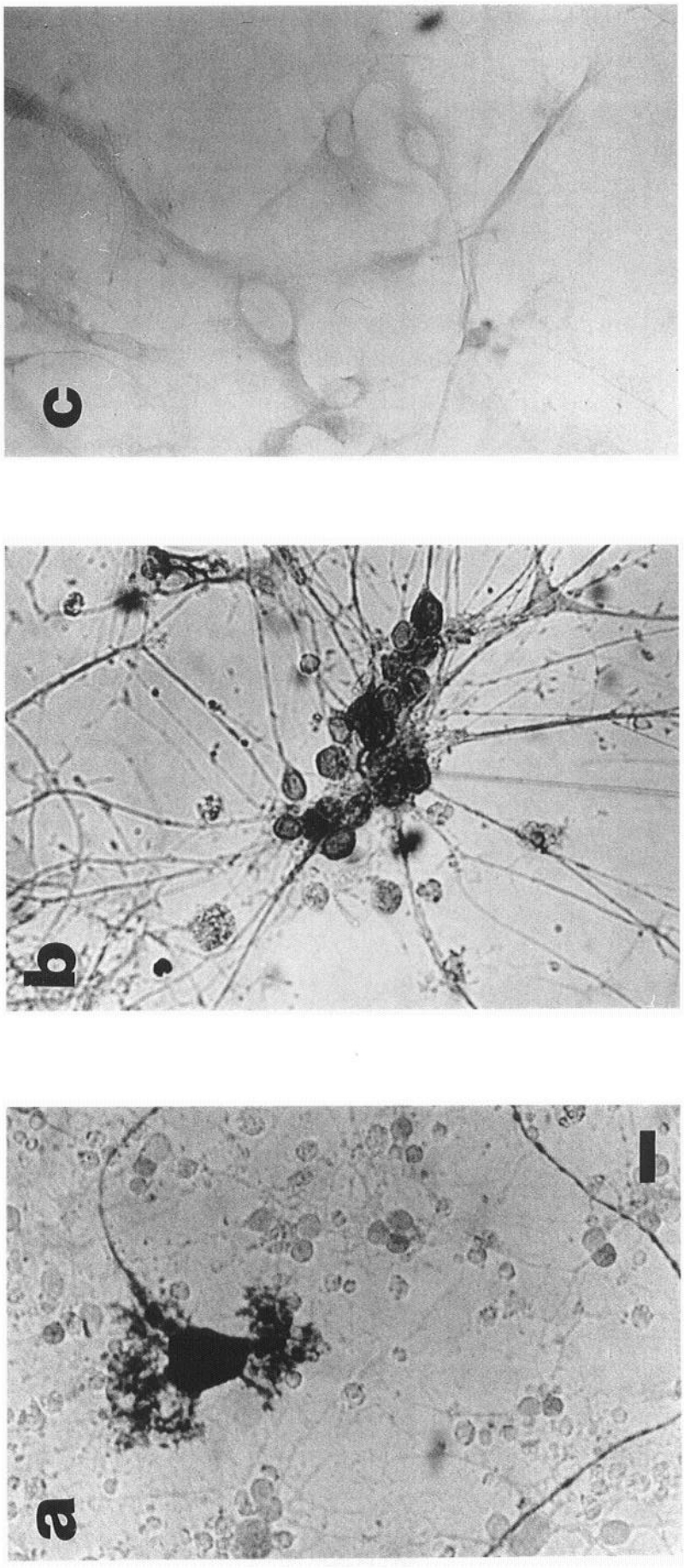
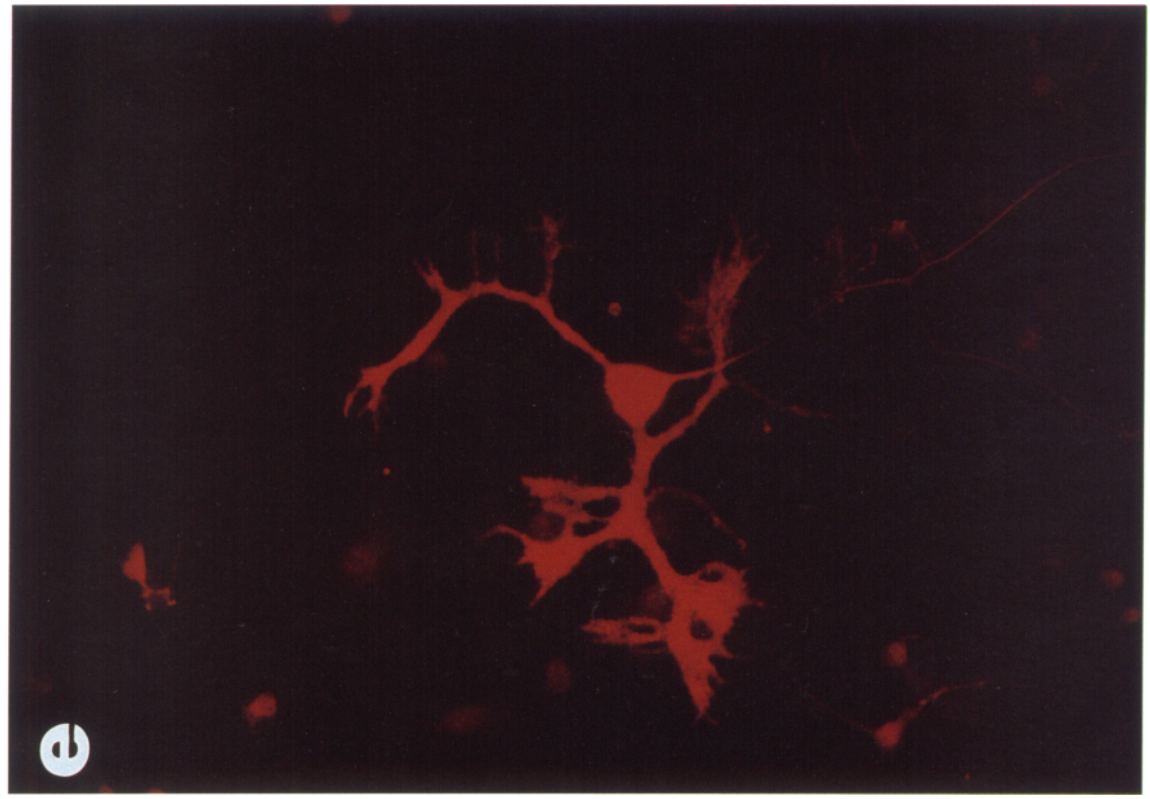
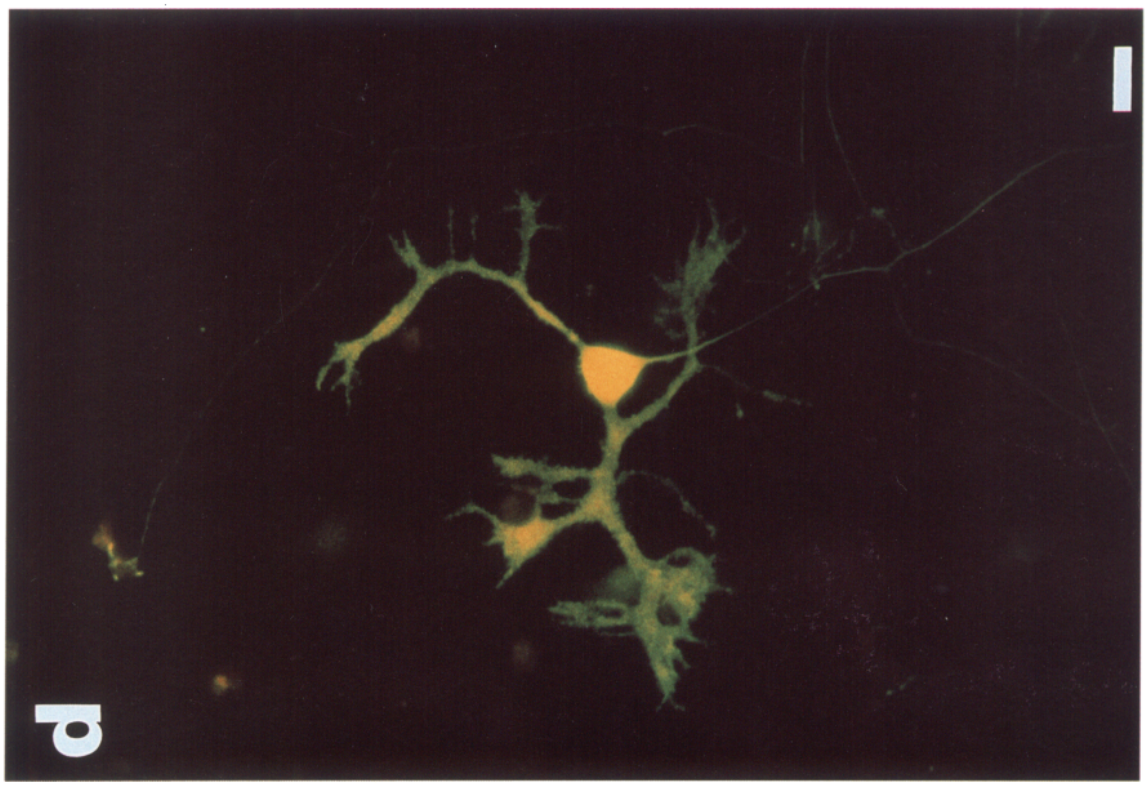


Figure 1. PKC isozyme distribution in cultured rat cerebellar neurons. PKC-I immunoreactivity (*a*) is restricted to large cells with complex arborizations, presumptive Purkinje cells. In contrast, PKC-II immunoreactivity (*b*) is present in small, round, clustering cells, presumptive granule cells. PKC-III immunoreactivity (*c*) is not detectable in cerebellar neurons, but is present in glia. Double labeling for PKC-I (*d*) and the Purkinje cell marker calbindin-D_{28K} (*e*) shows these proteins to be colocalized in a large neuron with elaborated processes. Scale bars: *a*, 20 μm for *a–c*; *d*, 30 μm for *d* and *e*.





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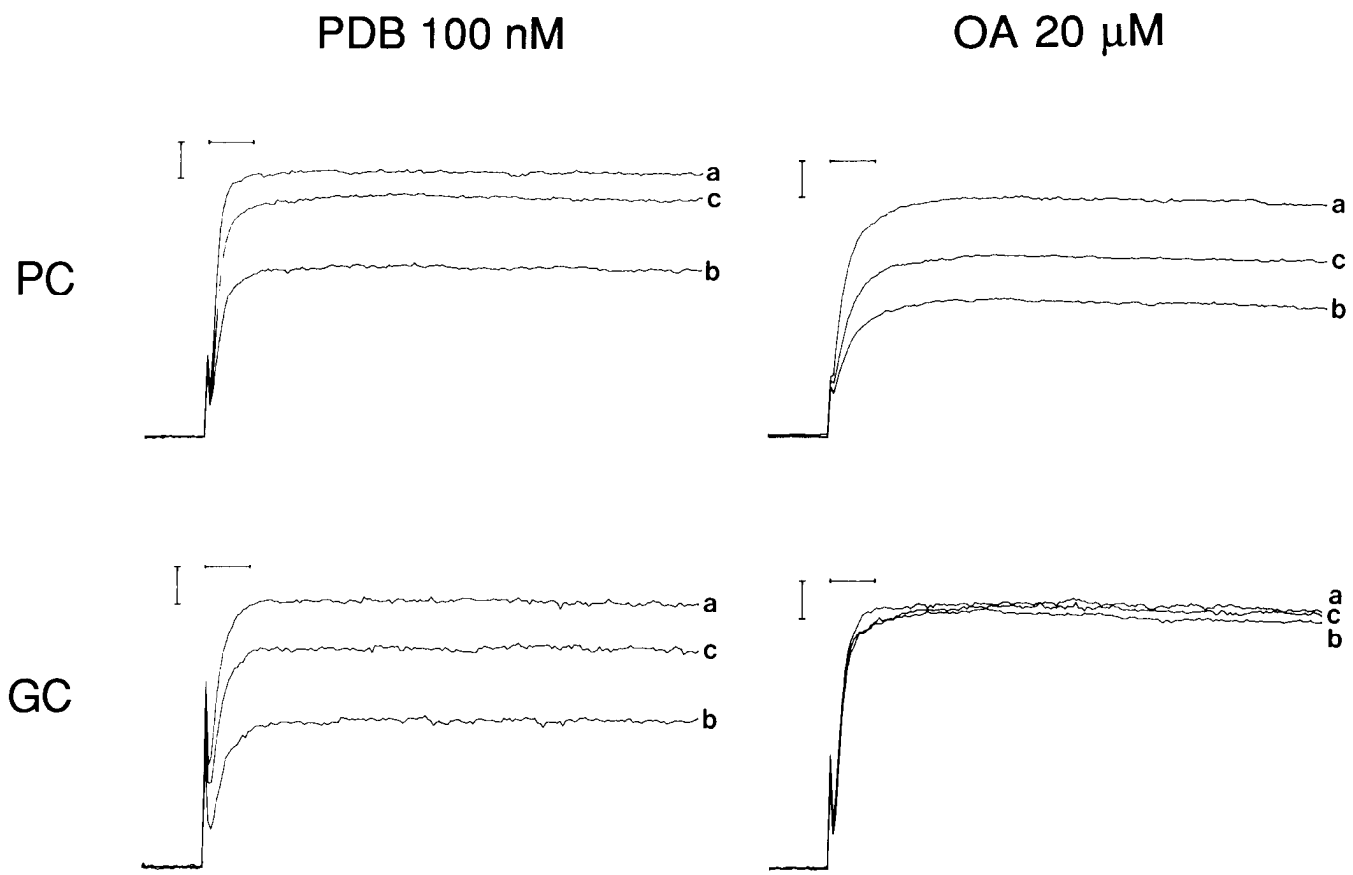

Figure 2. Effects of a PE and a c-UFA on I_K in cultured granule and Purkinje cells. I_K was elicited by a step from −30 mV to +30 mV in TTX-containing, nominally Ca^{2+}-free saline. I_K was attenuated by both classes of PKC activator in Purkinje cells (*PC*). However, in granule cells (*GC*), PE produced attenuation but c-UFA was without effect. Labels on current traces are as follows: *a*, control trace, immediately prior to application of test compound; *b*, test trace, 15 min after application of test compound; *c*, washout trace, 10–14 min after beginning of washout. Calibration: 100 pA, 8 msec.

temperature (20–23°C) in PBS containing 0.4% trypsin (type XI, Sigma, St. Louis, MO) and 0.5% DNase I (type IV, Sigma). Following digestion, the chunks were washed twice in PBS with 10% fetal calf serum and 12 mM $MgSO_4$ for 2 min, and twice again in the same solution without trypsin inhibitor. The chunks were then triturated with a fire-polished Pasteur pipette (~0.5 mm tip diameter) in PBS with 0.5% DNase I until the cells were completely dispersed (about 15 passages). The cell suspension was spun at 500 × *g* for 10 min, following which the pellet was resuspended at a concentration of 5 × 10^5 cells/ml in a growth medium containing Minimal Essential Medium (MEM) with Earle's salts (GIBCO, Grand Island, NY) and supplemented with 6.0 gm/liter glucose, 3.7 gm/liter $NaHCO_3$, 2 mM glutamine, 25 mM KCl (final concentration), N3 supplement (Romijn et al., 1982), 200 U/ml penicillin G (GIBCO), 200 μg/ml streptomycin (GIBCO), and 10% heat-inactivated horse serum (GIBCO). Cells were plated on glass coverslips coated with poly-D-lysine (Sigma) and were maintained in a humidified atmosphere containing 10% CO_2 at 37°C. Twenty-four hours after plating, the penicillin, streptomycin, and serum were withdrawn. Following this, cells were fed two or three times per week. In some cultures, 100 μM cytosine-β-D-arabinofuranoside HCl (Sigma) was added at the time of the second or third feeding.

Immunocytochemistry

Single-labeled cells. Cells were briefly rinsed with PBS and then fixed in 4% formaldehyde in PBS buffer for 15 min. Cells were then washed in 10% methanol, 0.3% H_2O_2 to saturate endogenous peroxidase activity, permeabilized with 0.1% Triton X-100, and blocked with 5% normal goat serum prior to application of the primary antibodies. Mouse monoclonal antibodies directed against PKC isozymes I, II, and III (produced by the method of Hidaka et al., 1988) were purchased from Seikagaku America (Rockville, MD) and were applied at a dilution of 1:75, overnight at 4°C. Immunoreactivity for a single isozyme was visualized using the anti-mouse IgG Vectastain ABC kit (Vector Labs, Burlingame, CA) with 3,3′-diaminobenzidine as the substrate for HRP bound to the secondary antibody (e.g., see Fig. 1*a–c*). To reduce cross-reactivity between the anti-mouse secondary antibody and the cells from rat, 2% normal rat serum was used as a blocking agent.

Double-labeled cells. Cells were fixed, permeabilized, and blocked as above. The cultures were incubated overnight at 4°C in a mixture of two primary antisera: a mouse monoclonal antiserum directed against calbindin-D_{28K} (Sigma) at 1:500 and a rabbit polyclonal antiserum directed against a synthetic oligopeptide from the variable region of PKC-I (GIBCO/Bethesda Research Laboratories) at 1:800. Following thorough washing, the cells were incubated in fluorescein-conjugated anti-mouse IgG at 1:75 for 60 min, biotinylated anti-rabbit IgG (Vector) at 1:500 for 30 min, and avidin-Texas Red (pH 8.2, Vector) at 1:250 for 60 min. All the secondary reactions were performed at room temperature, in the dark, with 5 × washing in PBS between each step. Cells were mounted in glycerol with 5% propyl gallate and observed on a Zeiss Axioplan microscope (e.g., see Fig. 1*d,e*).

Electrophysiology

Perforated-patch recording was performed according to the method of Horn and Marty (1987). Patch electrodes were pulled from N51A glass (1.65 mm o.d., 1.20 mm i.d.; Garner Glass Co., Claremont, CA) and polished on a microforge to yield a resistance of 1–2 MΩ when recorded with normal internal and external solutions (see below). We used a List EPC-7 patch-clamp amplifier and membrane capacitance neutralization was optimized. Membrane currents were filtered at 3 kHz, digitized with a 12-bit A/D converter operating at a sampling frequency of 20 kHz, and stored on the hard disk of an LSI-11/23 computer for later analysis. Linear leakage current was determined by hyperpolarizing

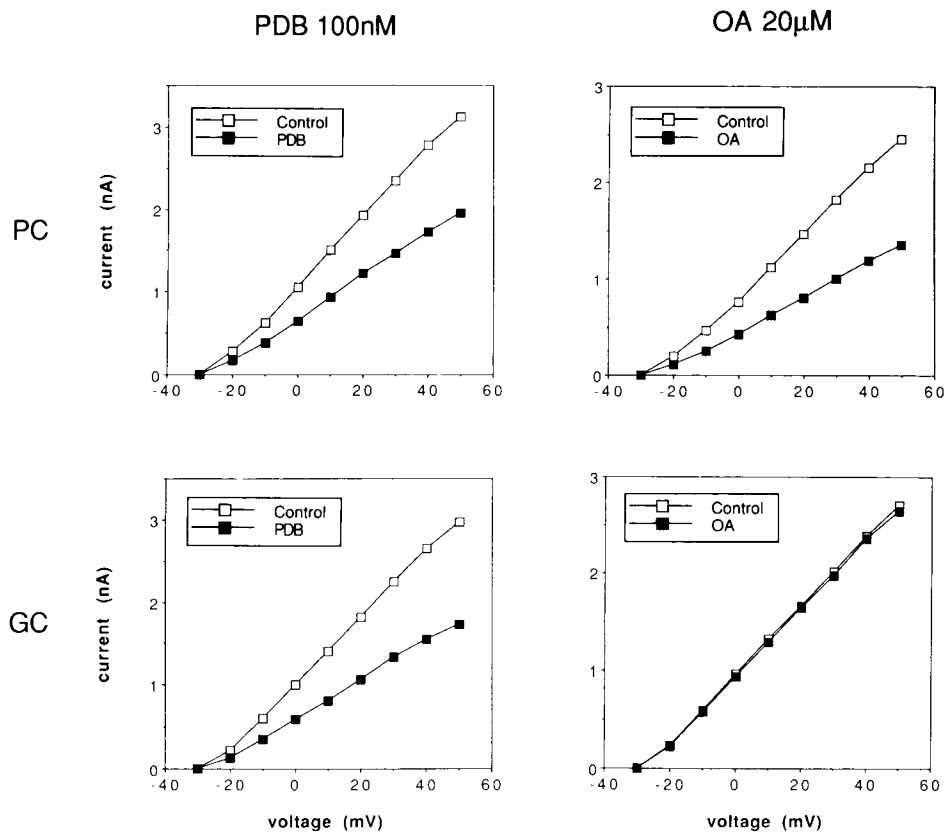

Figure 3. Current–voltage relations of I_K during application of a PE and a c-UFA to cultured cerebellar neurons. Families of currents were elicited by depolarizing steps from a holding potential of −30 mV. The current was measured at the point of greatest positivity, 15 min after drug application. In the three cases in which an attenuation of I_K was observed, it took the form of a decrease in slope conductance without alteration in the voltage dependence of activation. Current–voltage relations shown are from single neurons, representative of a sample of n = 6/group. *PC*, Purkinje cells; *GC*, granule cells.

command pulses and was digitally subtracted from current records. The recording electrodes were filled with a solution containing (in mM) K_2SO_4, 95; KCl, 15; $MgCl_2$, 8; and HEPES, 10; pH 7.35 with KOH. The cells were bathed in a solution containing NaCl, 150; KCl, 5; $MgCl_2$, 0.8; HEPES, 10; glucose, 10; TTX, 0.005; and picrotoxin, 0.01, pH 7.35 with NaOH, that flowed at a rate of 3 ml/min (bath vol, 1.5 ml). For perforated-patch recording, nystatin (Sigma) was added to the internal saline to achieve a final concentration of 250 μg/ml in 0.5% dimethyl sulfoxide. However, a small amount of nystatin-free solution was used to fill the tip of the recording pipette to facilitate a seal formation. Typically, a 20 min waiting period was required after gigaseal formation to achieve stable I_K recordings. Conventional whole-cell recordings were made using the same electrodes, external saline, and recording equipment detailed above. The internal saline contained (in mM) KCl, 140; EGTA, 11; $CaCl_2$, 1; HEPES, 10; and Mg-ATP, 2. In choosing cells for recording, care was taken to select those that most completely fit the morphological profile of a given cell type. Only small (soma diameter, <10 μm), round, clustering cells were selected for granule cell recording, and only large (soma diameter, >25 μm) cells with complex dendritic arborizations were selected for Purkinje cell recording. When recordings were made from the latter cells in current-clamp mode, with 2 mM Ca^{2+} present in the external saline, these neurons showed spontaneous Ca^{2+} spiking typical of Purkinje cells. Only cells with resting potentials less than −50 mV and I_K that remained stable for at least 10 min prior to drug application were used. Experiments were performed at 20–24°C. I_K amplitudes were measured at a point 60 msec after the onset of the voltage step. Percentage attenuation measures were a comparison of I_K amplitudes determined immediately prior to, and 15 min following, application of the test compounds.

Drugs

Protonated forms of the fatty acids oleate and methyloleate (Sigma) were dispersed in external saline by vigorous vortex mixing and ultrasonication. The active (4-β) and inactive (4-α) forms of phorbol-12,13-dibutyrate (PDB; LC Services, Woburn, MA) and the protein kinase C inhibitors RO-31-8220 (a gift of Dr. G. Lawton, Roche Products Ltd., Welwyn Garden City, UK), calphostin C (Kamiya Biomedical Co., Thousand Oaks, CA), and staurosporin (Boehringer Mannheim) were prepared as stock solutions in 10% dimethyl sulfoxide before their addition to external saline.

All drugs were applied by switching the bath solution to one containing the drug at a point upstream from the bath chamber. In the cases where a PKC inhibitor was used together with a PKC activator, the inhibitor was introduced into the bath 5 min prior to the activator and remained in the bath for the duration of the experiment.

Results

Cerebella from embryonic Sprague–Dawley rats (E19–E21) were dissected, dissociated with trypsin, and cultured in high-K MEM for 12–22 d. Initially, we sought to determine if the distribution of PKC isozymes reported in sections of the adult rat cerebellum would be preserved in tissue culture. To this end, we used polyclonal antisera directed against PKC-I, -II, and -III with standard immunocytochemical techniques. As it is not always possible to identify cell type in a mixed culture based on morphological criteria alone, we also employed antisera directed against calbindin-D_{28K} (Thomasset et al., 1984), a marker shown to recognize Purkinje cells selectively in sections of adult rat cerebellum.

Figure 1*a* illustrates the localization of PKC-I immunoreactivity in a large cell with a complex arborization (a presumptive Purkinje cell), and the absence of PKC-I immunoreactivity in small, round, clustering cells (presumptive granule cells). Double labeling for PKC-I and calbindin-D_{28K} shows immunoreactivity for this isozyme and this Purkinje cell marker to be colocalized in a presumptive Purkinje cell. (Fig. 1*d,e*). In marked contrast, PKC-II immunoreactivity may be clearly seen in presumptive granule cells (Fig. 1*b*). This distribution corresponds to that observed in sections of rat (Ase et al., 1988; Hashimoto et al., 1988; Huang et al., 1988, 1990; Kose et al., 1988; Saito

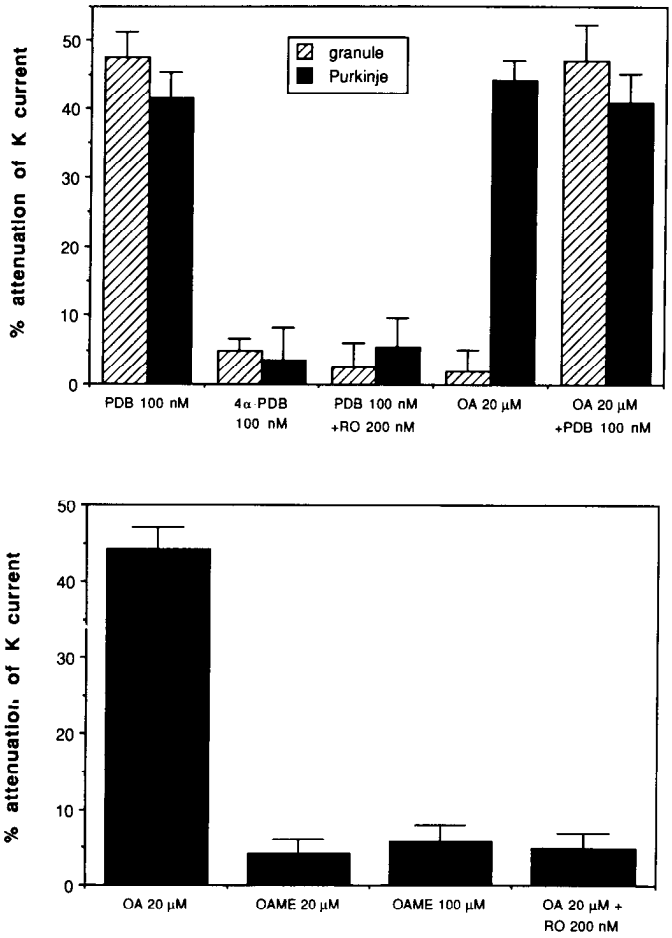

Figure 4. Experiments to determine if attenuation of I_K by c-UFA and PE are produced by PKC activation. *Top*, The attenuation of I_K produced by PE in both granule and Purkinje cells is not produced by application of an inert PE (*4α-PDB*) and is blocked by coapplication of RO-31-8220 (*RO*), a PKC inhibitor. Application of PE and c-UFA together did not produce an additive effect on I_K attenuation in either cell type. *Bottom*, Attenuation of I_K produced in Purkinje cells by a c-UFA is not produced by an inert c-UFA, OAME, and is blocked by coapplication of RO-31-8220 (*RO*). Each *bar* represents the mean ± SD of six separate observations.

et al., 1988) or rabbit (Hidaka et al., 1988) cerebellum. PKC-III immunoreactivity was very weak in all neuronal cell types, but was present in glia (Fig. 1*c*).

The main finding of the study is shown in Figure 2: outward current, evoked by a step from −30 mV to +30 mV in TTX-containing, nominally Ca^{2+}-free saline, was attenuated in Purkinje cells by both a PE (PDB) and a c-UFA (OA), but only by PE in granule cells. Thus, the distribution of the PKC-I isozyme predicts the response of the neurons to these two different modes of PKC activation. The attenuation produced by both PE and c-UFA took the form of a simple decrease in total conductance, as indicated by a reduction in the slope of the current–voltage relation (Fig. 3). In contrast, we saw no evidence for an alteration in the voltage dependence of activation. When the experiments illustrated in Figures 2 and 3 were repeated using external saline containing 200 mM EGTA to buffer trace Ca^{2+}, no differences were seen in any of the following measures: the percentage attenuation by the activators, the current–voltage relation, or the shape or amplitude of the evoked current (data not shown). Under these conditions, Na and Ca currents, as well as $I_{K(Ca)}$ and I_A, should be absent, leaving predominantly delayed rectifier current, I_K.

Recordings were routinely made using the perforated-patch technique (Horn and Marty, 1987), in which electrical continuity between the recording pipette solution and the cytoplasm is achieved by the incorporation of nystatin molecules into the membrane patch. The nystatin molecules form voltage-insensitive pores that are selective for small, monovalent ions. Consequently, the molecular machinery of second messenger signaling and calcium buffering remains relatively undisturbed. This is particularly relevant in the present case, as the PKC-I isozyme is reported to be ~90% cytosolic (Shearmen et al., 1989a) and hence susceptible to washout in conventional whole-cell recording. An examination of the effects of PKC activators on I_K measured in Purkinje cells using conventional whole-cell recording techniques showed that the attenuation produced by both PE and c-UFA was smaller in this case. c-UFA (20 μM oleate) produced a 44.2 ± 3.0% (mean ± SD; $n = 6$) attenuation of I_K measured with perforated patch and a 19.8 ± 3.7% ($n = 5$) attenuation measured with conventional whole-cell recording. Similarly, PE (100 nM PDB) produced a 41.3 ± 4.2% ($n = 6$) attenuation with perforated patch and a 23.6 ± 4.8% ($n = 5$) attenuation with conventional whole-cell recording. The elapsed time between the formation of the gigaseal and the application of the PKC activator was the same for both recording methods, ~20 min.

Experiments to determine if the effects of PE and c-UFA on I_K were mediated by PKC activation are shown in Figure 4. Only minimal attenuation of I_K was observed with application of 4α-PDB, a PE inert with respect to PKC activation. Likewise, preincubation of the cells in RO-31-8220, a PKC inhibitor (Davis et al., 1989; Linden and Connor, 1991), almost entirely blocked the PE effect on I_K. A similar blockade was produced by the PKC inhibitor calphostin C applied at 2.0 μM (Kobayashi et al., 1989) or the nonspecific protein kinase inhibitor staurosporin applied at 20 nM (Tamaoki et al., 1986; data not shown). OA methyl ester (OAME) is a compound that does not activate PKC (Murakami and Routtenberg, 1985), but that shares many of the physical properties of OA, including the ability to fluidize membranes (see Linden and Routtenberg, 1989b, for a complete discussion of control procedures for PKC-activating fatty acids). Application of OAME produced only minimal attenuation of I_K in Purkinje cells. Preincubation of Purkinje cells in RO-31-8220 also blocked the c-UFA effect. Finally, if c-UFA and PE are both producing their effects on Purkinje cells through activation of PKC-I, then one would expect that application of these two compounds together would produce a nonadditive attenuation of I_K. This notion was confirmed in the present experiments (Fig. 4, top).

Discussion

These observations indicate that the effects of PE and c-UFA in the present study are mediated by PKC activation. However, it has been reported that the attenuation of voltage-gated Ca currents in chick dorsal root ganglion cells by PE or DAG results from a direct interaction with a site on the outside of the cell membrane, in a manner independent of PKC activation (Hockberger et al., 1989a). A similar mechanism is unlikely to be operative in the present case, as inactive PE or c-UFA produced <6% attenuation of I_K (Fig. 4). In addition, any PKC-independent mechanism would have to explain the differential effects produced by PE and c-UFA in granule and Purkinje cells.

The present results suggest that the presence of PKC-I in Purkinje cells confers upon them the ability to respond electrophysiologically to c-UFA and, by extension, to activating factors that will not affect granule cells. Specific functions of the PKC-I isozyme remain largely unknown. However, certain roles are suggested by its localization. PKC-I appears to be localized exclusively in the CNS (Shearman et al., 1988). Within the CNS, it is most enriched in the cerebellar cortex (Purkinje cells), hippocampal formation (pyramidal and dentate gyrus granule cells), amygdala, and neocortex (Ase et al., 1988; Hashimoto et al., 1988; Huang et al., 1988; Kose et al., 1988; Saito et al., 1988), areas known to exhibit various forms of use-dependent synaptic plasticity. A striking gradient of increasing PKC-I (but not PKC-II or -III) expression may be seen along the occipital-temporal-limbic visual processing pathway of the monkey (Huang et al., 1989), which correlates positively with the importance of these regions in storage of visual information, as determined in behavioral studies (Mishkin, 1982; Murray and Mishkin, 1986). Thus, it is tempting to speculate that PKC-I is somehow involved in information storage.

A model of information storage has been demonstrated in Purkinje cells in which coactivation of climbing fiber and parallel fiber inputs to the Purkinje cell leads to a long-term depression (LTD) of the parallel fiber input (Ito et al., 1982; Ito, 1989). Cerebellar LTD is thought to be mediated postsynaptically, as it may also be demonstrated as a depression of the Purkinje cell response to iontophoretically applied glutamate or AMPA (Ito et al., 1982; Kano and Kato, 1987; Crepel and Krupa, 1988; Linden and Connor, 1991; Linden et al., 1991) but not aspartate or NMDA (Crepel and Krupa, 1988; Linden and Connor, 1991). It has been suggested that PKC activation is necessary for LTD induction because PKC inhibitors have been shown to block this phenomenon (Linden and Connor, 1991) while PE application induces a similar depression (Crepel and Krupa, 1988; Crepel and Jaillard, 1991; Linden and Connor, 1991). The presence of strong PKC-I immunoreactivity in Purkinje cell dendritic cytoplasm as revealed by electron microscopy (Kose et al., 1988) is consistent with this notion.

The observation that the distribution of PKC isozymes in sections of adult rat cerebellum was preserved in a culture of embryonic tissue is interesting, but not easily interpretable. It might indicate that the cell type–specific expression of PKC isozymes is already determined by E19–E21. Alternatively, this pattern of isozyme expression might require certain signals during postnatal development (synaptic input, growth, or differentiation factors) that are also present in our cell-culture conditions. A developmental study of PKC isozyme expression in rat cerebellum (Huang et al., 1990) showed PKC-I immunoreactivity restricted to Purkinje cells, with an abrupt increase between the second and third postnatal weeks, corresponding to the development of the Purkinje cell dendritic arbor and the arrival of parallel fiber input. PKC-II immunoreactivity was seen in granule cell precursors in the external granular layer of 1-week-old rats, and appeared to track the migration of granule cells to the internal granular layer during the following 2 weeks of development. PKC-I immunoreactivity did not occur in granule cells or their identifiable precursors at any postnatal stage. Likewise, PKC-II immunoreactivity did not occur in Purkinje cells during postnatal development, suggesting that the distribution of these PKC isozymes is determined before birth in rat cerebellum.

The main finding of this study, that the distribution of PKC-I predicts the response of Purkinje and granule cells to c-UFA and PE, is important in that it provides a bridge between a purely anatomical observation, the differential distribution of PKC isozymes among cell types, and a well-known architect of neuron electrical signaling, the delayed outward rectifier current, I_K. A more inclusive issue, determining the net effect of c-UFA application on Purkinje cell electrical function, awaits further study. As previous work has shown c-UFA–induced PKC activation to attenuate voltage-gated Na and Ca currents (Linden and Routtenberg, 1989b; Keyser and Alger, 1990) as well as K currents, the total effect of c-UFA is likely to be complex.

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