T-Type Calcium Channels: Heterogeneous Expression in Rat Sensory Neurons and Selective Modulation by Phorbol Esters

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We report selective inhibition of low-threshold, T-type calcium channels by a phorbol ester in rat sensory neurons. Cells were exposed, either acutely or with 15–60 min preincubations, to low concentrations of phorbol 12-myristate 13-acetate, (PMA), an activator of protein kinase C; if the temperature was 29°C or higher, T-type Ca current was diminished without effect on high-threshold Ca current. In untreated cells, the amplitude of T-type Ca current varies widely among neighboring sensory neurons. T channels are absent in about 25% of cells, provide a small current near threshold for the majority of cells, and are a dominant pathway for calcium entry in a small subset of neurons. The results are of interest because, by selectively inhibiting a calcium channel expressed differently among subpopulations of sensory neurons, activation of protein kinase C might selectively suppress particular sensations.

T channels are a class of voltage-activated calcium (Ca) channel present on cardiac muscle, smooth muscle, secretory cells, fibroblasts, and neurons (for reviews, see Tsien et al., 1988; Bean, 1989), including the mammalian sensory neurons discussed in this paper (Bossu et al., 1985; Fedulova et al., 1985; Bossu and Feltz, 1986; Carbone and Lux, 1987; Kostyuk and Shirokov, 1989). They enhance the excitability and promote bursting of central neurons (Jahnsen and Llinás, 1984; McCormick and Prince, 1988; White et al., 1989) and help determine firing frequency of cardiac pacemaking cells (Hagiwara et al., 1988). Kinetic criteria distinguish T channels from the higher-threshold N- and L-type Ca channels (Carbone and Lux, 1984, 1987; Armstrong and Matteson, 1985; Nowycky et al., 1985; Matteson and Armstrong, 1986; Fox et al., 1987a, b). Activating at membrane voltages positive to -50 mV, T channels can be recorded in isolation from the other Ca channels, which activate above -30 mV. T channels inactivate during a maintained stimulus more rapidly than other Ca channels but turn off more slowly upon cessation of a brief stimulus. The 2 key functional properties of T channels are (1) activation, which occurs at membrane voltages near the threshold for action potentials, and (2)

inactivation, which occurs when cells are persistently held at voltages above -50 mV. Thus, variation of neuronal rest potential affects the availability of T channels, which in turn affects the excitability of the cell.

In embryonic chick sensory neurons, activation of protein kinase C with phorbol esters and diacylglycerol analogs inhibits nonselectively both high-threshold and T-type Ca channels (Rane and Dunlap, 1986; Marchetti and Brown, 1988; Rane et al., 1989). In this paper, we show a selective effect of phorbol esters on T channels in sensory neurons of newborn rat dorsal root ganglia and demonstrate heterogeneous expression of T channels among apparent subpopulations of these cells.

Materials and Methods

Tissue culture. Culture methods were modified from Yamamoto et al. (1981). Dorsal root ganglia from 5–10 d postnatal rats were dissociated in Ca/Mg-free Hanks solution (Sigma Chemical Co., St. Louis, MO) containing $25 \mu\text{M}$ CaCl_2 , 0.2% trypsin (GIBCO, Life Technologies, Inc., Grand Island, NY) and 0.01 or 0.1% (for 10 d rats) collagenase (Cis II Cooper Biomedical, Malvern, PA). Cells were grown on coverslips coated with collagen (Biomedical Technologies, Stoughton, MA) at 37°C and 5% CO_2 and studied after 1–14 d in culture. Culture medium was as follows: Ham's F12 (GIBCO) with 10% fetal calf serum (HyClone Laboratories, Logan, UT), 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 5 mg/ml glucose, and 100 ng/ml nerve growth factor (from Viper Libetina, Sigma).

Some cells studied after 1 d in culture did not have processes, but most did. Process formation was dramatically greater on laminin-coated coverslips compared with collagen; the extensive processes caused intolerable space-clamp problems after a day in culture on laminin coverslips. Diameters varied from 10–30 μm for those cells used for recordings.

Recordings and experimental solutions. All recordings used the whole-cell patch clamp (Hamill et al., 1981). Leak and capacity currents were eliminated by digitally scaling and subtracting records obtained with subthreshold voltage pulses.

Experimental temperature was monitored with a bath thermistor and controlled by constant flow of solution (using a push-pull syringe) through a water-jacket heat exchanger.

The following extracellular solution (in mM) was used: 10 CaCl_2 , 135 tetraethylammonium-Cl (TEA-Cl); 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, adjusted with TEA-OH.

Intracellular (pipet) solution A was as follows (in mM): 100 CsCl, 1 Na_2ATP , 2.5 MgCl_2 , 0.3 Na_3GTP (Aldrich Chem. Co. Milwaukee, WI), 10 EGTA, 2.9 CaCl_2 ($\text{pCa} = 7.0$, $\text{pMg} = 2.7$), 40 HEPES, pH 7.0, adjusted with TEA-OH.

Intracellular solution B was as follows: 100 CsCl, 2 Na_2ATP (equine, Sigma), 5 MgCl_2 , 10 ethyleneglycol-bis-(β -aminoethyl ether)-tetraacetic acid (EGTA), 40 HEPES, pH 7.0 adjusted with TEA-OH.

PMA and 4 α -phorbol (both from Sigma) were dissolved in dimethylsulfoxide as 2 mM stock solutions and stored at room temperature no more than 2 weeks. Experimental solutions were obtained by dilution from a 10 μM stock (5% DMSO, 95% water) prepared on the day of the experiment.

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Figure 1. T current variation in 3 rat sensory neurons. Traces: Whole-cell currents evoked by steps from -70 to -30 mV (T current) or $+10$ mV (sustained Ca currents). Vertical scale: 250 pA (A), 1000 pA (B), 1500 pA (C). Graphs: peak current vs stimulus voltage. Cell capacitances: 31 pF (A), 97 pF (B), 53 pF (C). Temperature, 22°C ; intracellular solution A.

Results

Heterogeneous expression of T-type Ca channels

Figure 1 shows Ca currents recorded with the whole-cell patch-clamp technique (Hamill et al., 1981) from 3 sensory neurons. The traces show currents evoked by steps to the indicated voltage from a holding voltage of -70 mV; the graphs are plots of peak current against test voltage. Cell A has the classic signature of the T-type Ca channel: the step to -30 mV elicits a small, transient current, and there is a shoulder on the current-voltage curve at negative voltages. Steps to more positive voltages elicit a larger, more sustained current and result in a peak in the current-voltage curve at around $+10$ mV. In contrast, cell B has no T current, whereas cell C has a T current, which is larger than the high-threshold current. The large T current in Figure 1C has a similar inactivation rate but reaches its peak slightly faster than the current in Figure 1A; the faster activation may be due to imperfect voltage control of neuronal processes.

The records of Figure 1 are representative of 3 kinds of responses. In a survey of 63 cells, 23% had no T current (like Fig. 1B) and 5% had very large T currents (like Fig. 1C). The remainder had the more classical response in which T current amplitude is a fraction (ranging from 5 to 75%) of the peak high-threshold current. Scatter plots of T current density against cell size showed no correlation. Though we lump these different responses into 3 categories, the "classical response" contains a wide range of T channel amplitudes and may well have subsets within it.

There are 3 possible explanations of this variation in the level of T-type Ca current: (1) differences in hormonal modulation of T current in different cells; (2) variation in development *in vivo* or with time in tissue culture; and (3) different expression of T channels on subsets of sensory neurons. We favor the final explanation. Neighboring cells in a culture dish routinely exhibit radically different levels of T-type current, so differences in hormonal modulation seem unlikely. We have found T channel heterogeneity in adult sensory neurons, embryonic neurons and the newborns discussed here; thus, heterogeneity is not simply a manifestation of development. The differences exist among

cells that are freshly dissociated, as well as those maintained in culture, so the observation is not an artifact of tissue culture.

Heterogeneous expression suggests that the T channel might be a key determinant of different electrophysiological behavior of sensory neuron subpopulations. In particular, the giant T current (like Fig. 1C) is similar in amplitude to Na currents seen in these neurons, indicating that the T channel can have a controlling influence on the threshold and shape of the action potential in a subset of sensory cells (White et al., 1989).

T channels are inhibited by phorbol esters

Figure 2 shows T currents, recorded at 32°C , just before and within 100 sec after application of the indicated phorbol esters. Phorbol 12-myristate 13-acetate (PMA), a phorbol ester which activates the calcium and phospholipid-dependent enzyme, protein kinase C (Nishizuka, 1986), diminishes the T current (Fig. 2a, b). 4 α -Phorbol, an analog which does not affect the kinase (Nishizuka, 1986), has no effect on the T current (Fig. 2c). The average decrease in current with 10 nM PMA was 27% ($\pm 6\%$ SD, $n = 4$). Elevated temperatures ($\geq 29^{\circ}\text{C}$) are required for inhibition by PMA; no effect was seen at room temperature, but inhibition could be induced by raising the temperature in the presence of PMA. The experimental conditions required for inhibition are reasonable for protein kinase C effects: The temperatures are similar to those used for *in vitro* assays of protein kinase C activity, 10 nM is a submaximal PMA concentration for phosphorylation *in vitro* (Castagna et al., 1982), and the pipet solution provides a physiological intracellular Ca concentration ($0.1 \mu\text{M}$). These results contrast with those from *Aplysia* bag cell neurons, in which acute effects of PMA were lost with internal perfusion (DeRiemer et al., 1985).

The inhibition is selective

Figure 3 compares the effects of PMA on T-type and high-threshold Ca currents. The insets of Figure 3, a, b, show T currents a or high-threshold currents b before and after application of PMA to 2 different cells. The graphs express peak current against the time of recording. Inhibition of T channels occurs within 20 sec of application of 10 nM PMA, and there

Figure 2. Acute effects of phorbol esters on T current at elevated temperature. Recordings are just before and within 100 sec after application of the indicated phorbol ester. Holding potential: -70 mV. Test potentials: -30 mV (a, b) -20 mV (c). Vertical scale: 400 pA (a), 1000 pA (b), 200 pA (c). Temperature, 32°C ; intracellular solution A.

is no indication of recovery in 3 min following washout, suggesting that dephosphorylation may be relatively slow. In contrast, 50 nM PMA has no effect on high-threshold current.

Figure 3c shows results from a third cell from which current-voltage curves were obtained before and after 5 min in 10 nM PMA. The traces show superimposed currents at the indicated test voltage in the absence and presence of PMA (traces at $+40$ mV exactly superimpose). The T currents at -40 and -20 mV show the inhibition typical of 10 nM PMA, but there is no significant decrease in the high-threshold currents elicited at $+20$ and $+40$ mV. In each of 4 experiments, we saw no inhibition of high-threshold Ca current by either 10 or 50 nM PMA.

Selective inhibition in a population study

Two concerns led us to verify the results of Figures 2 and 3 with an alternate method not involving recordings at elevated temperature. Cells tended to be unstable and short-lived at the elevated recording temperatures, limiting the number of successful experiments. Moreover, the instability led to selection of larger-amplitude T currents since variation in holding current was less of a problem at the lower gain. Table 1 and Figure 4 describe data from a protocol in which cultures were preincubated at 37°C , followed by recordings from many cells at room temperature. There was no evidence of damage to cells or selective cell death due to PMA incubations. Since all incubations were done at 37°C , we did not show that this protocol was temperature-dependent, as was the acute application of phorbol ester in the previous section.

Table 1 compares the average Ca currents in cells incubated in 100 nM PMA for 15–60 min with those in cells either untreated or incubated in the inactive phorbol ester, 4 α -phorbol. In all, 128 cells in paired sets from 7 rat preparations were studied. The data are presented for each rat preparation to show the variability of average currents. Despite this variability, incubation in PMA consistently increased the proportion of cells without detectable T current (41 vs 24%, column 4) and decreased the average amplitude of T current among those cells in which it was measurable (column 5). The average high-threshold (“peak”) current of PMA-treated cells was greater than control when all data were combined. This effect seemed to be due to one preparation (R32) which had an unusually large difference. Comparison of high-threshold current preparation by preparation shows that in 3 cases the average current was higher with PMA, in 2 cases it was lower, and in 2 it was within the standard error; this is different from the T current, which was diminished in each of the 7 preparations. The histogram of Figure 4 is a plot of the percentage of cells with the indicated T : peak current ratios (for each preparation except R32). χ^2 analysis indicates with $>90\%$ certainty that the increase in cells without T current is due to PMA. For cells which had T current, PMA reduced the average T : peak current ratio from $0.43 (\pm 0.09 \text{ SEM})$ to $0.11 (\pm 0.02 \text{ SEM})$.

Discussion

The physiological effects of activation of protein kinase C vary greatly among different kinds of neurons (Kaczmarek, 1987).

Figure 3. Selective inhibition of T current. a and b, Peak T current (a) or high-threshold current (b) as a function of time during applications of 10 nM (a) or 50 nM (b) PMA to 2 different cells. Insets: Current records at the indicated times, before and after PMA application. Holding potentials: -70 mV. Test potentials: -30 mV (a); $+20$ mV (b). Temperatures: 33°C (a), 32°C (b). c, Peak current vs stimulus voltage before (solid symbols) and with (open symbols) 10 nM PMA. Traces: superimposed currents at the indicated test voltages before and with (arrows), except last traces which superimpose) 10 nM PMA. Temperature, 29°C ; intracellular solution A.

Figure 4. Histogram of the percentage of cells exhibiting the indicated ratio of peak T current to peak total current. Pretreated cells (shaded bars) were incubated in culture medium containing 100 nM PMA at 37°C for 15–60 min. Control cells (solid bars) are from the 6 preparations which received no pretreatment. Recording temperature, 22°C. Extracellular recording solution for pretreated cells also contained 100 nM PMA; intracellular solution B.

This is exemplified by the effects of protein kinase C on Ca channels: whereas Ca channel activity is enhanced in *Aplysia* bag cell neurons by unmasking an otherwise silent Ca channel (Strong et al., 1987), protein kinase C inhibits Ca channels in chick dorsal root ganglion neurons (Rane and Dunlap, 1986; Marchetti and Brown, 1988; Rane et al., 1989), snail neurons (Hammond et al., 1987), and secretory cell lines (Harris et al., 1986). Sensory neurons within the dorsal root ganglion are themselves a heterogeneous group of cells. They transduce and encode a variety of sensations and have been divided into subpopulations based on sensory receptor, conduction velocity,

projection sites, peptide content, and surface antigens (Light and Perl, 1984; Dodd and Jessel, 1985). These subpopulations might be expected to display different physiological and pharmacological properties. The results of this paper suggest that protein kinase C selectively inhibits a type of voltage-dependent Ca channel that is absent on a subpopulation of rat dorsal root ganglion neurons and expressed at widely different degrees among others. Thus, activation of the kinase could diminish excitability of some sensory cells while leaving others unaffected, suggesting the potential for selective modulation of particular sensations.

Phorbol esters and diacylglycerol analogs have been shown to reduce Ca currents independently of protein kinase C activation (Hockberger et al., 1989). There are several differences between our results and the non-kinase effect. Both PMA and 4 α -phorbol caused the non-kinase inhibition, whereas 4 α -phorbol was ineffective in the present work. The non-kinase effect involved phorbol ester concentrations 1000 times greater than we have used, affected nonspecifically both T-type and high-threshold currents, did not require elevated temperature, and was rapidly reversible. In all these respects, our results differ from the non-kinase effect of phorbol esters. With regard to temperature sensitivity, our results are similar to the phorbol ester-induced potentiation of a cardiac K channel (Walsh and Kass, 1988).

These results in newborn rat sensory neurons differ from those in chick embryonic neurons. Phorbol esters and diacylglycerol analogs inhibit both high-threshold and T-type Ca currents in chick sensory neurons (Rane and Dunlap, 1986; Marchetti and Brown, 1988; Rane et al., 1989), rather than the specific T channel effect we report in rat. The results on chick neurons certainly are not due to the non-kinase effect of Hockberger et al. (1989): a phorbol ester which does not affect protein kinase C was used as a control (Rane and Dunlap, 1986) and inhibitors of protein kinase C block the effect (Rane et al., 1989). Are the differences in the chick and rat results due to species or exper-

Table 1. Comparison of Ca currents in cells preincubated in PMA (100 nM for 15–60 min) to either untreated cells or cells incubated in 4 α -phorbol

Prep.	Incubation	Cells	Cells w/o T	Average T (pA)	Average peak (pA)	Average T:Peak
R08	PMA	4	3	−23	−1510 ± 218	0.020
	None	2	0	−257 ± 165	−1950 ± 115	0.198 ± 0.148
R14	PMA	2	1	−114	−880 ± 114	0.149
	None	5	0	−114 ± 27	−983 ± 177	0.132 ± 0.04
R17	PMA	14	8	−72 ± 10	−1860 ± 297	0.053 ± 0.007
	None	10	3	−130 ± 40	−1060 ± 201	0.153 ± 0.031
R18	PMA	10	7	−55 ± 16	−1470 ± 288	0.054 ± 0.016
	None	10	5	−173 ± 33	−1030 ± 144	0.233 ± 0.085
R20	PMA	18	4	−100 ± 18	−1290 ± 21	0.155 ± 0.45
	None	7	2	−457 ± 314	−1510 ± 490	0.329 ± 0.155
R21	PMA	8	3	−112 ± 15	−1420 ± 404	0.173 ± 0.042
	None	13	4	−341 ± 164	−731 ± 122	0.607 ± 0.282
R32	PMA	10	1	−98 ± 32	−2364 ± 431	0.058 ± 0.020
	4 α -phorbol	15	1	−881 ± 297	−1248 ± 213	0.695 ± 0.203
All cells	PMA	66	27	−92 ± 10	−1619 ± 135	0.108 ± 0.02
	Control	62	15	−437 ± 107	−1106 ± 93	0.429 ± 0.09

The total number of cells from each preparation and incubation is in column 3 and the number without detectable T current is in column 4. The average amplitude of T current (column 5) and average T : peak current ratio (column 7) consider only those cells which had measurable T current. The amplitude of T current was measured with pulses to −30 mV; peak current was measured with pulses to +10 mV. SEM is used throughout.

imental differences? The results on chick neurons were obtained at room temperature and involved higher phorbol ester concentrations than were used in our studies. In contrast, we see no effect of PMA on any rat Ca current at room temperature, even with 100 nM PMA. Nonspecific effects of phorbol esters at elevated temperature and higher PMA concentrations were difficult to rule out since these conditions generally killed our cells within tens of seconds during recordings. However, cells tolerated incubation in 100 nM PMA if they were not being patch-clamped, allowing preincubation experiments at this higher concentration. Since preincubation with high concentrations and acute application of low concentrations of PMA both cause selective loss of T current, we are convinced of a real difference in the specificity of phorbol ester effects between rat and chick sensory neurons.

Selective effects on T channels have been reported in 3 other cases, but the biochemical mechanisms are not known: (1) transformation of fibroblasts with several unrelated oncogenes leads to a loss of T channel activity without effect on L channels (Chen et al., 1988); (2) T current increases upon binding of monoclonal antibodies to the Thy-1 receptor on mouse sensory neurons (Saleh et al., 1988); (3) low concentrations of glutamate selectively inhibit T current in rat sensory neurons (Lovinger and Weight, 1988). All these may be candidates for involvement of protein kinase C.

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