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Native Kv1.3 Channels are Upregulated by Protein Kinase C

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Abstract. The voltage-gated potassium channel, Kv1.3, which is highly expressed in a number of immune cells, contains concensus sites for phosphorylation by protein kinase C (PKC). In lymphocytes, this channel is involved in proliferation—through effects on membrane potential, Ca^{2+} signalling, and interleukin-2 secretion—and in cytotoxic killing and volume regulation. Because PKC activation (as well as increased intracellular Ca^{2+}) is required for T-cell proliferation, we have studied the regulation of Kv1.3 current by PKC in normal (*nontransformed*) human T lymphocytes. Adding intracellular ATP to support phosphorylation, shifted the voltage dependence of activation by +8 mV and inactivation by +17 mV, resulting in a 230% increase in the window current. Inhibiting ATP production and action with ''death brew'' (2-deoxyglucose, adenylylimidodiphosphate, carbonyl cyanide-m-chlorophenyl hydrazone) reduced the K^+ conductance (G_K) by 41 ± 2%. PKC activation by 4β-phorbol 12,13-dibutyrate, increased G_K by 69 ± 6%, and caused a positive shift in activation (+9 mV) and inactivation (+9 mV), which resulted in a 270% increase in window current. Conversely, several PKC inhibitors reduced the current. Diffusion into the cell of inhibitory pseudosubstrate or substrate peptides reduced G_K by 43 ± 5% and 38 ± 8%, respectively. The specific PKC inhibitor, calphostin C, potently inhibited Kv1.3 current in a dose- and light-dependent manner (IC_{50} ~ 250 nM). We conclude that phosphorylation by PKC upregulates Kv1.3 channel activity in human lymphocytes and, as a result of shifts in voltage dependence, this enhancement is especially prevalent at physiologically relevant membrane potentials. This increased Kv1.3 current may help maintain a negative membrane potential and a high driving force for Ca^{2+} entry in the presence of activating stimuli.

Key words: Ion-channel phosphorylation — T-cell activation — Lymphocyte proliferation — Calphostin C — PKC peptides

Introduction

Protein phosphorylation can profoundly influence ion-channel activity and thus, ion fluxes and electrical properties of cell membranes. Evidence that voltage-gated K^+ channels in vertebrates can be regulated by protein kinase C (PKC) includes reports of inhibition of fast, transient (K_A) currents, and either an increase or decrease in delayed-rectifier K^+ currents by phorbol esters or diacylglycerol analogues (*reviewed in* Shearman, Sekiguchi & Nishizuka, 1989). cAMP-dependent protein kinase (PKA) also regulates delayed-rectifier K^+ currents; for example, PKA increased the current amplitude in vascular smooth muscle cells (Aiello, Walsh & Cole, 1995), and in squid axons dialyzed with ATP, or ATP plus the catalytic subunit of PKA (Perozo, Bezanilla & Dipolo, 1989). More recently, ion-channel regulation resulting from crosstalk between multiple protein kinases is beginning to be addressed, including dual regulation by PKC and PKA of delayed-rectifier K^+ channels in cardiac muscle (Walsh & Kass, 1988) and in the human Jurkat T-cell line (Payet & Dupuis, 1992). Little is known about phosphorylation of particular regions of K^+ channel proteins. PKC-dependent phosphorylation of one or two serine residues in the N-terminus of a cloned human K_A channel eliminated N-type inactivation (Covarrubias et al., 1994). Inactivation of *Shaker* K^+ channels was slowed by treatment with a protein phosphatase (Drain, Dubin & Aldrich, 1994). This effect was reversed by PKA and interpreted as phosphorylation of one or more serine residues at the C-terminus of the channel, but direct evidence for the site involved is lacking.

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The present study concerns regulation by PKC of native Kv1.3 channels in normal (nontransformed) human T lymphocytes. Kv1.3, which is the most thoroughly studied in immune cells, is a voltage-gated K^+ channel that is highly expressed in resting and activated human T cells and some T-cell lines, such as Jurkat (*for review*, Lewis & Cahalan, 1995). As a result of progress in cloning a large number of K^+ channels, Kv1.3 was first cloned from rat brain, where it was called RCK3 (Stühmer et al., 1989), Kv3 (Swanson et al., 1990) or RGK5 (Christie et al., 1990), then from mouse brain, where it was called MK3 (Grissmer et al., 1990). cDNAs from mouse (Chandy et al., 1990), rat (Douglass et al., 1990) and human T lymphocytes (Attali et al., 1992*b;* Cai et al., 1992), when expressed in *Xenopus* oocytes, had the same biophysical and pharmacological characteristics, thus the same gene appears to produce the lymphocyte current. In the recently standardized nomenclature for K^+ channels (*for review*, Gutman & Chandy, 1995), this member of the *Shaker*-related subfamily is usually referred to as Kv1.3. The potential for direct Kv1.3 channel regulation by phosphorylation was reinforced with the cloning of this channel since its amino acid sequence contains several concensus sites for serine/threonine phosphorylation. The channel protein is partially phosphorylated *in vivo* in Jurkat T cells and can serve as an *in vitro* substrate for further phosphorylation by PKA and PKC, apparently at serine residues (Cai & Douglass, 1993).

Attempts to study PKC-dependent regulation of native Kv1.3 channels have yielded inconsistent results and the first reports showed only long-term effects. Phorbol esters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) had no effect on normal T cells when added during a whole-cell recording or after the cells were preincubated from 10 min to 4 hr before patch-clamp recording (Deutsch, Krause & Lee, 1986). However, after several hours treatment of T cells (Deutsch et al., 1986), or 2–5 days treatment of splenic B lymphocytes (Partiseti et al., 1992), increases in K^+ current occurred. In contrast with these long-term effects, acute TPA treatment during whole-cell recordings from Jurkat T cells decreased the K^+ current over a time course of 10–30 min (Payet & Dupuis, 1992).

Differences in these previous reports on PKC effects—including the different cell types used, and native vs. exogenously expressed channels—prompted us to examine acute PKC-dependent regulation of Kv1.3 channels in their native environment in normal human T lymphocytes. Kv1.3 channels are expressed mainly in nonexcitable cells which undergo only small excursions of membrane potential from the resting value. The functional importance of the Kv1.3 channel is best understood in lymphocytes, where it is required to maintain a negative membrane potential that promotes Ca^{2+} influx (*for review*, Lewis & Cahalan, 1995). Thus, this channel is essential for Ca^{2+}-dependent events of lymphocyte activation, including secretion of lymphokines and cytotoxic molecules. Our present results show that, owing to shifts in voltage dependence, enhancement of Kv1.3 current by PKC is especially prevalent around the resting potential. This upregulation should help maintain a large driving force for Ca^{2+} entry in the presence of lymphocyte-activating stimuli.

Materials and Methods

Cells

Heparinized human venous blood was collected from healthy donors. Mononuclear cells were first separated by centrifugation on Ficoll-Hypaque or Percoll (both from Pharmacia, Dorval, Quebec), then B lymphocytes and monocytes were removed by adhering them to nylon wool (DuPont, Toronto, Ontario). We find that this procedure yields >95% T lymphocytes as determined by fluorescence-activated cell sorter (FACS) analysis using OKT3 antibody (Ortho Pharmaceutical, Raritan, NJ). Cell viability was 95–100% as measured by trypan blue exclusion or a LIVE/DEAD assay (Molecular Probes, Eugene, OR).

Chemicals

Adenosine trisphosphate (dipotassium salt, K_2ATP), 2-deoxyglucose, adenylylimidodiphosphate (AMP-PNP), and carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) were purchased from Sigma (St. Louis, MO). L-ascorbic acid was from BDH (Toronto, Ontario), and adenosine-5′-O′-(3-thiotriphosphate) (ATPγS) and calphostin C were from Calbiochem (San Diego, CA). PKC pseudosubstrate and substrate peptides were from UBI (Lake Placid, NY), and 4α- and 4β-phorbol 12, 13-dibutyrate (PDBu) were from Biomol (Plymouth Meeting, PA). Margatoxin (MgTX) was a gift of Merck, Sharpe and Dohme Research Laboratories (Rahway, N.J.). Stock solutions of CCCP, calphostin C, 4α- and 4β-PDBu were prepared in DMSO (maximum final concentration of DMSO, 0.1%).

Solutions

The bathing medium was NaCl saline consisting of (in mM); 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 N-hydroxyethyl-piperazine-N′-2-ethanesurfonic acid (HEPES), adjusted to pH 7.4 with NaOH. The standard pipette solution contained (in mM); 95 Kaspartate, 40 KCl, 10 ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), 1 $CaCl_2$, 10 HEPES, 2.5 $MgCl_2$, and 2 K_2ATP adjusted to pH 7.4 with KOH. Intracellular Ca^{2+} was buffered to a low concentration (~10 nM) to prevent possible activation of any Ca^{2+}-dependent currents. Free Ca^{2+} and Mg^{2+} concentrations in these pipette solutions were calculated using a computer program written by Dr. J. Kleinschmidt (New York University, NY). For certain experiments ATP was omitted and free Mg^{2+} was altered. Osmolalities of the bath and pipette solutions were 290–295 and 280–285 mosmol/kg H_2O, respectively, measured with a freezing point depression osmometer (Advanced Instruments, Needham Heights, MA).

Electrophysiological Recordings

All recordings were made in the whole-cell patch-clamp configuration using an Axopatch 1B patch-clamp amplifier (Axon Instruments, Foster City, CA). pClamp software (ver. 5.5.1, Axon Instruments) and Labmaster hardware were used to control voltage and to acquire and analyze data. We used borosilicate glass pipettes (A-M Systems, Everette, WA) with 5–7 MΩ resistance. The maximum uncompensated series resistance was <10 MΩ during whole-cell recordings, so the voltage error was <5 mV for a current amplitude of 500 pA. With the pipette in the bath, the zero-current potential was measured, then remeasured after seal formation and subtracted from the command voltage using the Axopatch internal circuit. Unless otherwise stated, whole-cell currents were allowed to stabilize for ~10 min before the K^+ current parameters were measured. This allowed for the shift in voltage dependence of the currents which occurs in the first few minutes (Cahalan et al., 1985; Pahapill & Schlichter, 1990) and for diffusion of the pipette contents into the cell. Currents were filtered at 5 kHz with the 8-pole Bessel filter of the Axopatch amplifier. Drugs were added manually by exchanging the entire bath using Pasteur pipettes or by continuous bath superfusion using a peristaltic pump. All recordings were made at room temperature (22–24°C). Where appropriate, results are presented as mean ± SEM (*n*, number of cells). The statistical significance of different mean values between control and treated cells was tested using the student's *t*-test and the accepted level was $P < 0.05$.

Results

The basic biophysical features of Kv1.3 current have been well characterized in human T lymphocytes. To compare them with results of the present study, the salient features of the whole-cell currents will be summarized (*see* Cahalan et al., 1985; Pahapill & Schlichter, 1990 for more detail). In lymphocytes, the native Kv1.3 current activates with time during voltage-clamp steps, with kinetics that are well described by an exponentially rising function, raised to the fourth power, multiplied by an exponentially declining inactivation function (so-called Hodgkin-Huxley type n^4j kinetics). Both voltage dependence and kinetics are affected by physical and biochemical regulation, including temperature (Pahapill & Schlichter, 1990) and phosphorylation (*this study*). For instance, depending on temperature the threshold for activation varies from ~−80 to −30 mV, the less negative values corresponding with temperatures below 37°C. Activation kinetics are fast and inactivation is much slower; for instance at room temperature (as used in the present study) the time constants are ~2 msec for activation and ~180 msec for inactivation (at +40 mV with 140 KF in the pipette). The current recovers slowly from inactivation, requiring as long as 30 sec to 2 min depending on voltage and on temperature. The voltage dependence of both activation and steady-state inactivation are sigmoidal functions that are well described by Boltzmann equations. From these equations (at room temperature with 140 mM KF in the pipette) the half-maximal voltage for activation is −33 to −38 mV and for steady-state inactivation is −58 to −64 mV.

Intracellular ATP Levels Affect the K^+ Conductance and Voltage Dependence

Before studying the modulation of Kv1.3 current by PKC we first examined the effects of augmenting or depleting intracellular ATP. Figure 1*A* illustrates the K^+ current recorded without (−ATP, left) and with ATP (+ATP, right) in the pipette solution. Because nucleotides and Mg^{2+} are inextricably linked by complex formation, and Mg^{2+} has direct effects on a number of ion channels (O'Rourke, 1993), we maintained a constant free Mg^{2+} concentration in the pipette, unless otherwise stated. For example, when 5 mM K_2ATP was added to the pipette solution containing 2.5 mM $MgCl_2$, the free Mg^{2+} concentration was 0.08 mM. To maintain the same free Mg^{2+} concentration in the control pipette solution lacking ATP, only 0.16 mM $MgCl_2$ was added.

For each set of treatments, activation (*g-V*) and inactivation (h_∞-*V*) were plotted as a function of voltage and the data fitted with Boltzmann equations. The sigmoidal *g-V* and h_∞-*V* relations overlap and produce a voltage window which extends from the threshold of the *g-V* curve to the potential at which the current is completely inactivated. This overlap or ''window current'' predicts tonically active channels throughout this voltage range, i.e., a component of the current remains activated even during sustained depolarizations. The magnitude of the window current (pA) was calculated from the area under each actual *g-V* and h_∞-*V* curve (pA/mV times mV), using the theoretical fits to these curves and the intersection set over the full voltage range. This value cannot simply be read from the area under the ''crossover'' of these curves in the figures, since the summarized h_∞-*V* and *g-V* curves were normalized to the maximal conductance of each cell. The same result can be obtained by multiplying the area under these curves (a unitless number times mV), by the average maximal K^+ conductance (pA/mV) given in Fig. 5 and in the text. In addition to this measure of total, tonically activated current, the voltage at which the *g-V* and h_∞-*V* curves cross is of interest, since it corresponds with the voltage at which tonic K^+ channel activity is expected to be maximal (i.e., best able to oppose depolarizing currents).

In the absence of added ATP (Fig. 1*C*, left), activation was half maximal at −47 mV and maximal at about −10 mV. Steady-state inactivation was half-maximal at −74 mV and maximal at −50 mV. The mean maximal K^+ conductance (G_{max}) at +30 mV was 4.0 ± 0.3 nS ($n = 11$). From these results, the calculated magnitude of the window current was 844 pA. Peak channel activity is expected to occur at about −65 mV. With added intracellular ATP, G_{max} was 3.9 ± 0.4 nS ($n = 8$), which is not significantly different from the conductance without added intracellular ATP ($P > 0.05$). The average G_{max} for all recordings containing 2 mM ATP was 4.0 ± 0.2 nS ($n = 60$, *see* Fig. 5). However (*see* Fig. 1C, right), the

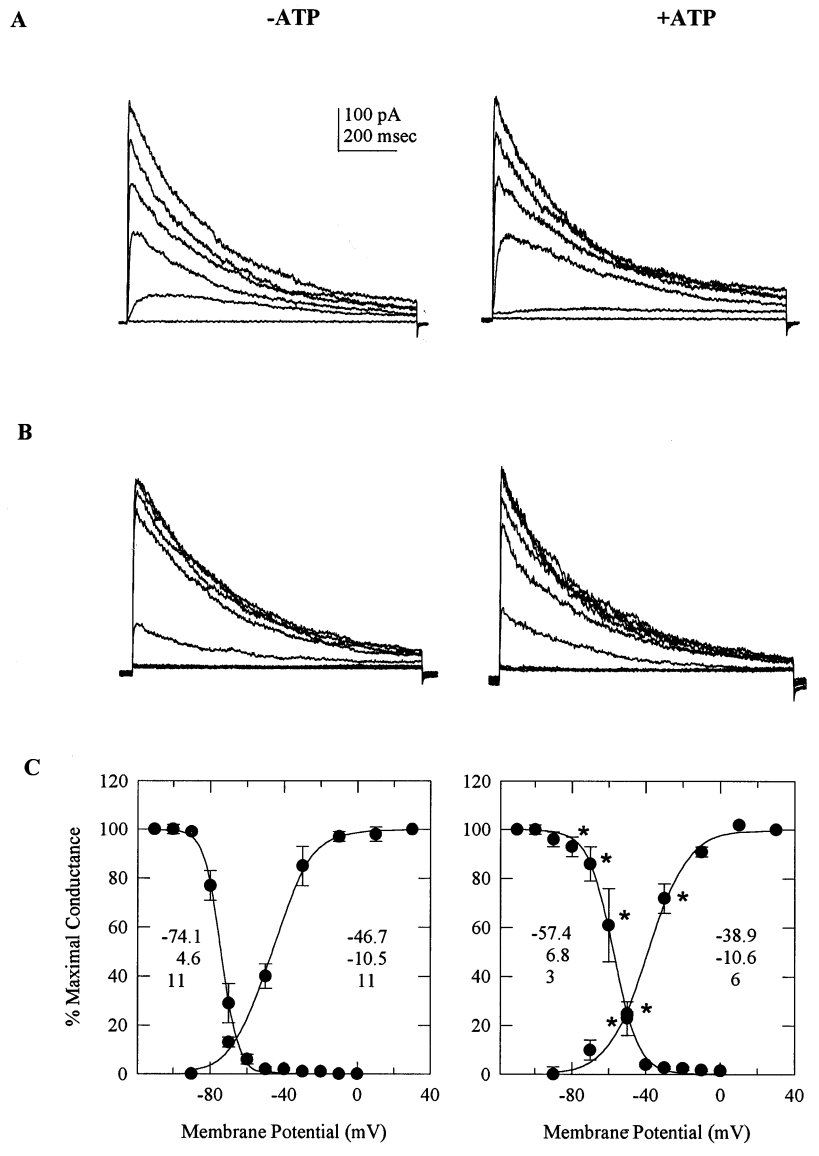

Fig. 1. Adding intracellular ATP shifts the voltage dependence of whole-cell Kv1.3 currents. Currents were recorded without added ATP (−ATP, left panel) or with 5 mM K_2ATP added to the intracellular solution (+ATP, right panel). The same intracellular free Mg^2 concentration (0.08 mM) was obtained in the two pipette solutions by adding 0.16 mM $MgCl_2$(−ATP) or 2.5 mM $MgCl_2$ (+ATP). Cells were bathed in standard NaCl saline (*see* Materials and Methods). (*A*) K^+ currents were recorded during voltage steps from V_m = −70 mV to +30 mV, in 20-mV increments (applied every 1–2 min) from a holding potential of −90 mV. (*B*) Steady-state inactivation was determined by measuring the peak current at V_m = +30 mV following 3-min long test pulses between −100 mV and 0 mV, applied in 10-mV increments. (*C*) Voltage dependence of activation and steady-state inactivation. At each voltage, the conductance (G_K) was calculated as $I_{peak}/(V_m-E_K)$, where E_K (calculated and measured) was −80 mV. For inactivation, the peak G_K at +30 mV following each holding potential was normalized to the maximal G_K following a holding potential of −100 mV. Activation and inactivation curves were then fitted to Boltzmann equations, using nonlinear least squares fits of $g(V) = g_{max}/\{1 + \exp[(V - V_{1/2})/k_m]\}$. Beside each curve (from top to bottom) are values of the parameters obtained from the curve fits: $V_{1/2}$, the voltage producing half-maximal G_K; k_n, the slope factor indicating the steepness of the voltage sensitivity; *n*, the number of cells tested. Significant differences in normalized G_K were observed at the voltages indicated by asterisks ($P < 0.05$, student's *t*-test).

half-maximal voltages for activation (−39 mV, a +8 mV change) and inactivation (−57 mV, a +17 mV change) shifted significantly ($P < 0.05$). [The statistical significance of such shifts in voltage dependence was tested for data in Figs. 1, 2 and 3 at voltages on the steep slopes of the activation (−30 and −50 mV) and inactivation curves (−50 to −80 mV).] The calculated magnitude of the window current increased by 2.3-fold (844 pA without ATP *vs.* 1,981 pA with ATP), mainly because the h_∞-*V* curve shifted further positive than the *g*-*V* curve. The voltage at which channel activity is expected to be maximal (''crossover'' voltage in Fig. 1) shifted from about −65 mV without ATP to −50 mV with ATP. Consistent with these effects of ATP, intracellular application of 5 mM ATPγS (an hydrolysis-resistant analogue), caused similar positive shifts in the voltage dependence (*figure not shown*). The voltages for half-maximal activation shifted by +7 mV (to −40 mV) and by +18 mV (to −56 mV) for inactivation. There was no significant increase in G_{max} (4.6 ± 0.8 nS, n = 5); however, due to the voltage shifts the window current increased to 1,835 pA. Further control experiments—in which the ATP in the pipette solutions was degraded by storage at room temperature for several hours—showed no shifts in *g*-*V* or h_∞-*V* curves (*data not shown*).

The same intracellular free Mg^{2+} and Ca^{2+} concentrations were maintained in the presence or presence of ATP, thus, the observed shifts in voltage dependence cannot be attributed to surface charge screening by these divalent cations. In separate experiments (*not shown*) we found that increasing free Mg^{2+} from 0.08 to 0.38 mM in the absence of added ATP had no effect on G_{max}, but produced a −2.7 mV shift in the *g*-*V* curve and a +1.7 mV shift in the h_∞-*V* curve. Such opposite voltage shifts,

which cannot easily be explained by surface charge screening, underscore the importance of experimentally controlling Mg^{2+} concentrations.

Even after a prolonged diffusional exchange of cytoplasm with pipette solution, it is possible that ATP is generated in the cell because mitochondria and glycolytic enzymes may still be present. If so, a continual production of ATP could maintain phosphorylation of the channels, especially since the concentration of ATP necessary to affect channel function may be quite low—only 10–100 μM ATP was needed to maximally stimulate the delayed-rectifier K^+ channel in squid axons (Perozo et al., 1989). To test whether basal levels of intracellular ATP were sufficient to affect the current, we compared cells in which no ATP was added to the pipette solution with cells in which ''death brew'' (Frace & Hartzell, 1993) was included in the pipette solution.That is, CCCP was used to block oxidative phosphorylation, 2-deoxyglucose was used to substitute for glucose and reduce glycolytic ATP production, and ATP was replaced with the poorly hydrolyzable AMP-PNP.

In the presence of death brew (Fig. 2*A*) the Kv1.3 current decreased slowly with time. Compared with control cells (no added ATP) G_{max} decreased by 41 ± 2% (to 2.4 ± 0.3 nS, $n = 3$) after 25 min, after which it remained constant. [This time course is consistent with a previous study using antimycin on rat thymocytes, wherein ATP was depleted by 90% after 20 min and 100% after 45 min (Grinstein et al., 1985).] Death brew also affected the inactivation rate of the current (*see* Fig. 2*A*, inset). In control cells (no added ATP) the time constant (τ_j) at +30 mV was 281 ± 15 ($n = 11$) msec, compared with 219 ± 22 ($n = 3$) msec for cells treated with death brew ($P < 0.05$). We also examined the h_∞-*V* relation in the presence of death brew (Fig. 2*B*), since ATP had markedly shifted the voltage dependence of inactivation (*see above*). After 30 min was allowed for death brew to diffuse into the cell and deplete ATP, the voltage for half-maximal inactivation was −74 mV ($n = 3$), which is identical to the value without added ATP.

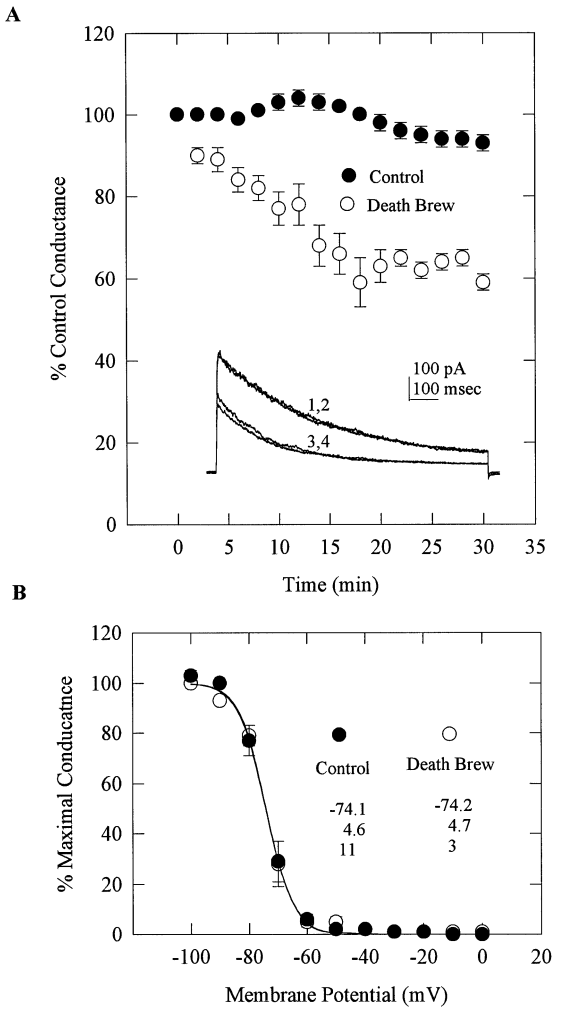

Fig. 2. Depleting intracellular ATP reduces the Kv1.3 conductance. The bathing medium contained standard NaCl saline. The pipette contained standard solution (*no added ATP*), without (control) or with ''death brew'' (5 mM AMP-PNP, 2 mM 2-deoxyglucose, 10 μM CCCP). The free Mg^{2+} concentration in both pipette solutions was 0.08 mM. (*A*) Normalized peak K^+ conductances (calculated at +30 mV) as a function of time after establishing whole-cell recordings. Inset shows actual Kv1.3 currents 4 to 5 min (traces 1, 2) and 30 to 31 min (traces 3, 4) after beginning a recording with death brew in the pipette. Pairs of currents are shown to illustrate the stability of the currents at these times. (*B*) Voltage dependence of inactivation with or without death brew in the pipette. Relative G_K, Boltzmann curves and parameters were determined as in Fig. 1*C*.

PKC Activation Increases G_{max} and Affects the Voltage Dependence

In normal T cells, phorbol esters activate both PKCα and β isozymes, with peak PKC activity lasting for 4 hr without downregulation (Berry et al., 1990). To examine how PKC affects Kv1.3 function, we used the phorbol ester, 4β-PDBu, as a specific PKC activator, with 4α-PDBu as a negative control (*see* Fig. 3). In all such experiments, the cells were preincubated for 30 min at 37°C with the phorbol ester before a whole-cell recording was established with a pipette solution containing ATP. Control recordings were made from the same batches of cells without phorbol ester treatment. G_{max} was not affected (P>0.05) by the inactive phorbol ester analogue—the value was 3.9 ± 0.4 nS ($n = 8$) for control cells and 3.6 ± 0.4 nS ($n = 7$) after treatment with 4α-PDBu. In contrast, the active phorbol ester, 4β-PDBu increased G_{max} by 60% to 6.0 ± 0.4 nS ($n = 22$, $P < 0.05$, Fig. 3, right). Moreover, the active phorbol ester shifted the voltage dependence of the current. For control currents (4α-PDBu, Fig. 3*B* left) half-maximal conductance was at −47 mV, inactivation was half-

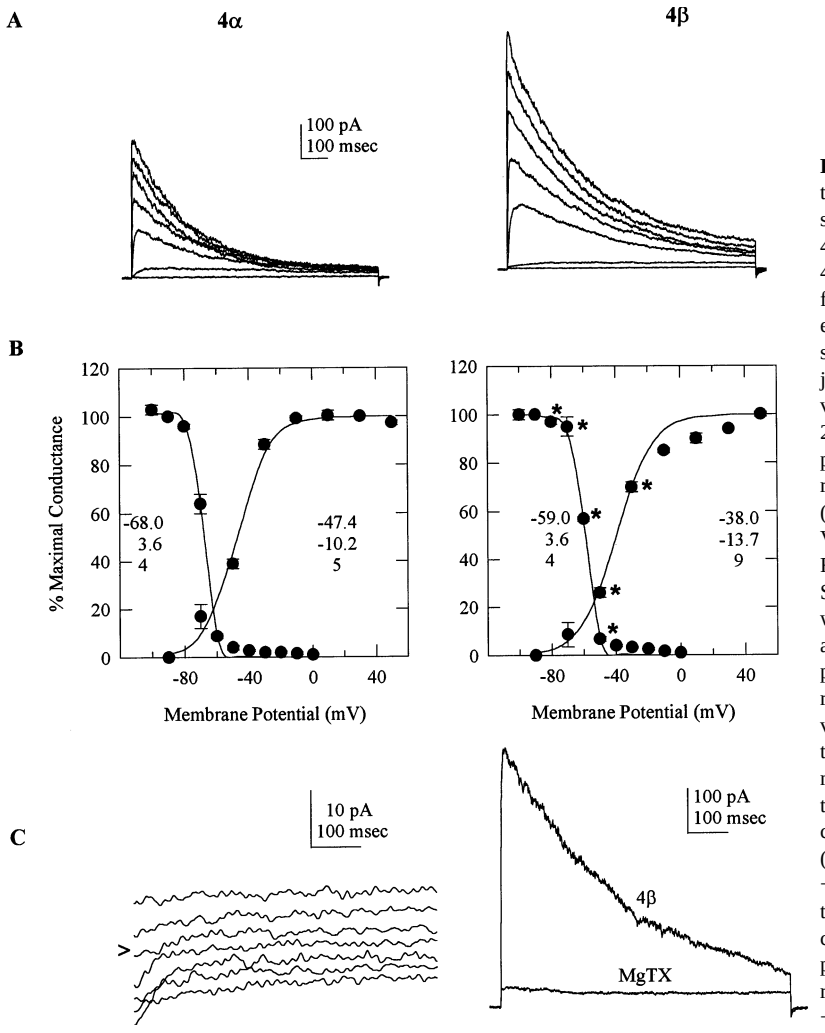

Fig. 3. A PKC-activating phorbol ester increases the Kv1.3 conductance and alters its voltage sensitivity. Cells were preincubated with 1 μM 4α-PDBu (inactive analogue, left panel) or 4β-PDBu (active analogue, right panel) at 37°C for 15–30 min, then whole-cell recordings were established. The bath and pipette contained standard solutions with ATP added to the pipettes just before use. (*A*) K^+ currents were elicited by voltage steps from −70 to +50 mV applied in 20-mV increments every 1–2 min from a holding potential of −90 mV. Note the outward current remaining at the end of each voltage step (analyzed in more detail in part C, below). (*B*) Voltage dependence of activation and inactivation. For parameters and curve fits, see Fig. 1*C*. Significant differences in relative conductance were observed at the voltages indicated by asterisks ($P < 0.05$, student's *t*-test). (*C*) Left panel. Reversal potential of the outward current remaining at the end of a 1,150 msec-long voltage-clamp step to +30 mV after 4β-PDBu treatment, as in part A. Tail currents were measured beginning 1 msec after stepping to each test potential (−60 to −120 mV) i.e., after the capacitance current had settled. The zero current (arrowhead) indicates a reversal potential close to −80 mV. Right panel. After 1 μM 4β-PDBu treatment, the time-dependent outward current was completely blocked by 5 nM margatoxin (MgTX) perfused into the bathing medium. Current was recorded at +30 mV from a holding potential of −90 mV.

maximal at −68 mV and complete at holding potentials above −60 mV. The resulting window current (calculated as the area under the ''crossover'' of the *g-V* and h_∞-*V* curves, times the maximal conductance) was 950 pA. After treatment with the active analogue (4β-PDBu, Fig. 3*B* right), the voltages for half-maximal activation (−38 mV) and inactivation (−59 mV) were shifted positive by 9 mV (compare values at −30 and −50 mV for activation and −60 and −70 mV for inactivation), as was the crossover voltage (to ~−52 mV). Most importantly, these shifts in voltage dependence and increase in G_{max} significantly increased the window current—by 2.7-fold to 2,567 pA.

After phorbol ester treatment the outward current did not completely inactivate, even at the end of a 1-sec long depolarizing pulse. To test whether the remaining current was Kv1.3 or some other outward current (e.g., Cl^-, K_{Ca}) we determined the reversal potential for the residual current and examined its sensitivity to the Kv1.3 specific toxin, margatoxin (MgTX). Since the residual current reversed at −80 mV (Fig. 3*C*, right), which is very close to the Nernst potential for K^+ in this solution, and was completely blocked by 5 nM MgTX (Fig. 3*C*, left), we conclude that the Kv1.3 current was specifically increased by the PKC-activating phorbol ester. The PKC-activating phorbol ester affected the rate of current inactivation. As opposed to death brew, which increased this rate (*see above*), 4β-PDBu significantly slowed the inactivation (τ_j, 287 ± 9 msec, $n = 22$) compared with the inactive analogue, 4α-PDBu (258 ± 40 msec, $n = 7$, $P < 0.05$).

PKC Inhibitors Reduce G_{max}

We further investigated the dependence of Kv1.3 on PKC by exploiting inhibitors that act at the PKC catalytic (C) domain (staurosporine, H-7) or regulatory (R) domain (inhibitory peptides, calphostin C). Drugs that bind to the C domain are not highly specific compounds, be-

ing only 2 to 10 times more specific for PKC than PKA (Matsumoto & Sakai, 1989), whereas drugs that bind to the R domain can be very specific. Binding of cofactors such as Ca^{2+}, phospholipid, diacyglycerol, or phorbol ester to the R domain exposes the C domain, thus activating the enzyme (Huang, 1989). Conversely, the R domain can inhibit the C domain via a sequence called the pseudosubstrate site. This sequence is highly conserved in most PKC isoforms, including PKCα, β, and γ, and can be used to produce potent, specific synthetic peptide inhibitors, two of which we used. One—which we call PKC (19-31)—contains the pseudosubstrate sequence of PKC (amino acids 19–31) but no phosphorylatable residue. The second is the same peptide but with alanine substituted by serine (we call it PKC (19-31), A25S). This substitution produces a competitive inhibitor by making the peptide a good substrate for PKC. Both peptides have proven to be potent and specific inhibitors of PKC (House & Kemp, 1990). Calphostin C, a microbial compound which interacts with the R domain, is 1,000 times more specific for PKC (IC_{50} 50 nM) than for PKA or tyrosine protein kinases (IC_{50} 50 μM), and inhibits the α, β, and γ isoforms of PKC to the same degree (Kobayashi et al., 1989).

For experiments in which either the pseudosubstrate or substrate peptide was added to the pipette solution and allowed to diffuse into the cells, ATP was omitted from the pipette. When ATP-free solutions were used for control recordings from the same batches of cells, the mean maximal K^+ conductance (G_{max}) was 4.0 ± 0.3 nS (n = 11). When the peptides were used (MW ~ 2,000, 5 μM in the pipette), their diffusion into the cell began after breakthrough to the whole-cell configuration and G_{max} reached a final, stable value within 10–15 min. To ensure that the current amplitude had stabilized we recorded currents at the same voltage every minute. As shown in Fig. 4, pairs of currents recorded about 5 min apart were the same. Both peptide inhibitors reduced G_{max} by 35–40% (*see* Fig. 5). The pseudosubstrate peptide (PKC 19–31) reduced G_{max} to 2.5 ± 0.3 nS (n = 4, $P < 0.05$), and the substrate peptide (PKC 19-31, A25S) to 2.3 ± 0.2 nS (n = 4, $P < 0.05$). We did not attempt to activate PKC after the current was reduced by PKC-inhibitory peptides because we had found that 4β-PDBu was no longer effective several minutes after establishing a whole-cell recording (*see* Discussion). Further evidence for phosphorylation-dependent regulation of Kv1.3 was that staurosporine (250 nM) and H-7 (100 μM)—concentrations that inhibit both PKC and PKA—reduced the K^+ conductance by ~50% (*data not shown*).

Calphostin C is activated by light (Kobayashi et al., 1989; Bruns et al., 1991), so it can be added in the dark for control recordings, then illuminated to inhibit PKC. Because calphostin C contains several perylene quinone groups, it is possible that free radicals will be produced

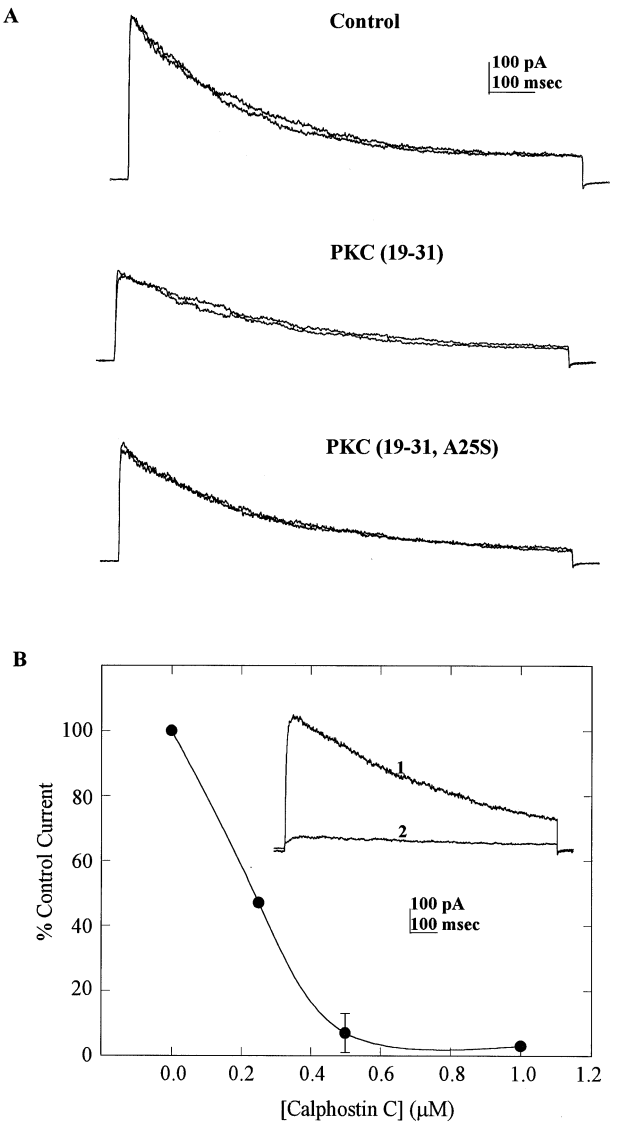

Fig. 4. PKC inhibitors reduce the Kv1.3 current. The bath and pipette contained standard solutions—no ATP was added to the pipette. Currents were recorded at +30 mV from a holding potential of −90 mV. (*A*) Representative currents from a control cell, and from cells into which either 5 μM PKC pseudosubstrate peptide (PKC 19-31) or 5 μM PKC substrate peptide (PKC 19-31, A25S) had diffused from the pipette. All cells were from the same batch. For each treatment, the illustrated currents were obtained 10 to 20 min after recordings began. A pair of current traces at the same voltage was recorded about 5 min apart to show that the effects of the inhibitory peptides on current amplitude had reached a steady level, and that the control currents were stable at this time without ATP in the pipette (*see also* Fig. 2*A*). (*B*) Calphostin C inhibits Kv1.3 dose-dependently. Since calphostin C is light activated (*see* Results) currents were recorded before (control) and after a 2-min illumination. Relative peak current was calculated as a percent of the current without illumination and plotted as a function of the calphostin C concentration. One mM ascorbic acid was added to each bath and pipette solution as a free radical scavenger (*see text*). Inset. Whole-cell Kv1.3 currents after bath perfusion of 500 nM calphostin C in the dark (trace 1) and after the cell was illuminated for 2 min with a 50-watt mercury lamp (trace 2).

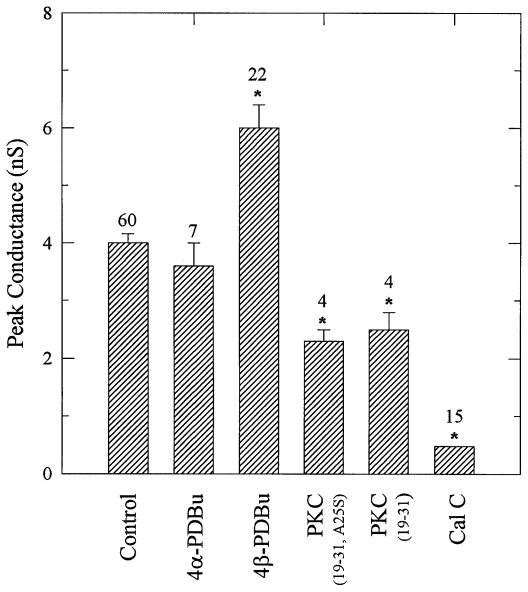

Fig. 5. Summary of effects on the Kv1.3 conductance of activating or inhibiting PKC. Mean maximal G_K values (±SEM) for the number of cells indicated above each bar. Conductances that differ significantly from control values are indicated by asterisks (student's *t*-test, $P < 0.05$). The pipette contained standard saline for Control, Cal C, 4α- or 4β-PDBu treatments, or standard saline plus 5 μM PKC pseudosubstrate (PKC (19-31)) or 5 μM PKC substrate peptide (PKC (19-31, A25S)). For Cal C treatment, 500 nM calphostin C was perfused into the bath followed by 2-min illumination. For phorbol ester treatments, cells were preincubated at 37°C for 15–30 min with 1 μM 4α-PDBu or 4β-PDBu.

when it is light-activated. Although free radical production is only considered to be a concern if very high calphostin C concentrations are used (Kobayashi et al., 1989; Bruns et al., 1991), as a precaution we added a free radical scavenger (1 mM ascorbic acid) to the bath and pipette solutions. Ascorbic acid itself did not affect G_{max}, the current kinetics or the current's sensitivity to calphostin C (*data not shown*). After establishing a whole-cell recording, when calphostin C was perfused into the bathing medium in the dark, it did not affect the K^+ current at any concentration tested (200 to 1000 nM). Then when the cells were illuminated for 1 to 2 min using the normal 50 watt mercury lamp of the inverted microscope, 500 nM calphostin C abolished the current (Fig. 4*B*, inset). Lower calphostin C concentrations dose-dependently inhibited the current; for instance, with 2-min illumination at a constant light intensity, the IC_{50} was 250 nM (Fig. 4*B*). At submaximal doses the current was inhibited more by longer or higher intensities of illumination (*data not shown*). Figure 5 summarizes the effects on G_{max} of the specific PKC inhibitors and activators. The PKC-activating phorbol ester, 4β-PDBu (but not the inactive analogue, 4α-PDBu) significantly increased G_{max}. Conversely, G_{max} was significantly reduced by all the PKC inhibitors tested (calphostin C, PKC pseudosubstrate and substrate peptides, staurosporine, H-7)—calphostin C being the most potent and effective.

Discussion

Regulation of Kv1.3

Because of the importance of the Kv1.3 channel in human lymphocyte function, post-translational modulation of the current has received increasing attention. Early studies of physiologically relevant factors showed that the current is reduced by high intracellular Ca^{2+} (1–10 μM, Bregestovski, Redkozubov & Alexeev, 1986) or H^+ concentrations (pH 6.2, Deutsch & Lee, 1989), or by a reduction in temperature below 37°C (Pahapill & Schlichter, 1990). Effects on current kinetics were also observed; for instance, elevated internal or external Ca^{2+} accelerates current inactivation (Bregestovski et al., 1986; Grissmer & Cahalan, 1989), whereas elevated external K^+ slows the rate of channel closing (Cahalan et al., 1985). All of these modulatory effects were taken as physical interactions with the channel pore or gates, except the effects of temperature. Results of our temperature study (Pahapill & Schlichter, 1990) were not consistent with simple physical effects on channel activity and suggested to us that second messenger/biochemical regulation may be important. We showed that Kv1.3 channel behavior changes with temperature in ways that optimize its activity at physiological temperatures—through changes in the number of activatable channels and single-channel conductance, which increased the whole-cell conductance (G_{max}), and in the voltage dependence of activation and inactivation, which increased the number of tonically activated channels (window current). In our subsequent studies we have focused especially on regulatory pathways that affect the amount of current near the resting potential. We found (Pahapill & Schlichter, 1992) that a number of agents that raise cAMP in T cells (8-Br cAMP, forskolin, isobutylmethyl xanthine, isoproterenol, prostaglandin E_2, histamine) stimulate K^+ channel activity around the resting potential in intact cells.

Results of the present study suggest that there is a resting level of PKC activity and phosphorylation that can be reduced by ATP depletion or PKC inhibitors, and that further phosphorylation can occur with added ATP or phorbol esters. We found that depleting intracellular ATP—by diffusional loss with or without ''death brew'' in the pipette—reduced the maximal conductance (G_{max}) and shifted the voltage dependence of activation and steady-state inactivation. Conversely, adding intracellular ATP (or ATPγS) produced positive voltage shifts in the activation and inactivation curves. As a result of these ATP-dependent changes in voltage dependence,

tonic channel activity (window current) increased around the resting potential, and maximal activity occurred at significantly less negative voltages. In contrast with these changes around the resting potential, G_{max} was not affected by adding ATP or ATPγS, compared with ATP-free solutions. Thus, levels of ATP remaining during intracellular diffusion of an ATP-free solution appear to allow the same G_{max} to be evoked by large voltage pulses, but ATP depletion below this level reduces the evoked current. The shifts in voltage dependence that most affected the window current were more sensitive to ATP depletion, since it was necessary to add ATP or ATPγS to the pipette solution to prevent these shifts. One prediction is that the cell's metabolic state (ATP levels) will strongly influence how much Kv1.3 current is activated or recruitable to contribute to cell function (e.g., during cell depolarization).

We believe that the observed ATP dependence is due, at least in part, to phosphorylation, as indicated by our results using a PKC-activating phorbol ester and several PKC inhibitors. Both the voltage dependence and G_{max}—thus, tonically activated channels—were affected. PKC activation caused a 270% increase in window current, which resulted from a 70% increase in G_{max} and significant shifts in the voltage dependence. Conversely, all the PKC inhibitors tested significantly reduced G_{max} in resting (unstimulated) cells. We did not attempt to activate PKC after allowing the inhibitory peptides to exert their effect because we had found that phorbol ester treatment was no longer effective if added several minutes after establishing a whole-cell recording. One possibility is that components of the second-messenger pathway (e.g., a specific PKC isozyme) are disrupted or washed out during whole-cell recording (*see below*). Since such cytoplasmic disruption will take place over a variable time course, we did not attempt to determine whether there is a brief time window during the first few minutes when significant phorbol ester effects could be seen. Instead, all phorbol ester treatments were done by preincubating intact cells before establishing a whole-cell recording.

Although we noted a slowing of current inactivation by the PKC-activating phorbol ester (compared with the inactive analogue), we do not wish to speculate about its cause since we have not made a detailed study (e.g., with or without ATP, with PKC inhibitors). The inactivation rate is not central to the purpose of this study. Other studies that have analyzed Kv1.3 inactivation (e.g., Cahalan et al., 1985; Grissmer & Cahalan, 1989; DeCoursey, 1990; Pahapill & Schlichter, 1990; Kupper et al., 1995) have found that it is affected by many factors, including temperature, Ca^{2+}, anion species in the pipette, patch excision, yet the mechanisms accounting for channel inactivation are unclear. Most important for our purposes was to show that the maintained current, even after a 1-sec long pulse, is incompletely inactivated Kv1.3, not some other current.

Some of our previous studies had already suggested that biochemical processes can enhance the current at physiologically relevant membrane potentials—temperature (Pahapill & Schlichter, 1990), and protein kinase A (Pahapill & Schlichter, 1992). Several other studies have addressed the biochemical regulation of Kv1.3 current—both of native channels in immune cells and of channels exogenously expressed in *Xenopus* oocytes. Some comparisons can be made between our results on normal (nontransformed) human T lymphocytes and previous studies of PKC dependence. Overall, PKC effects on G_{max} appear to depend on cell type and the pathway used to stimulate the kinase. Since none of the previous studies examined details of voltage dependence and window current amplitudes, or the effects of ATP addition or depletion, these cannot be compared. In this study, we focused on short-term effects on Kv1.3, since PKC is activated within seconds to minutes after T-cell stimulation, rather than on long-term changes that could involve regulation of protein expression.

Whereas we observed a significant increase in G_{max} following PKC activation, a previous report on normal T cells (Deutsch et al., 1986) reported no effect of short-term PKC activation (minutes) but an increase in G_{max} several hours after treatment. Both of these results on normal (nontransformed) T cells differ from a report on Jurkat T cells (Payet & Dupuis, 1992), and from two studies of Kv1.3 channels expressed in *Xenopus* oocytes (Attali et al., 1992*a;* Aiyar, Grissmer & Chandy, 1993). Acute phorbol ester treatment reduced G_{max} in Jurkat T cells (Payet & Dupuis, 1992), in splenic B cells (Partiseti et al., 1992), and in *Xenopus* oocytes (Attali et al., 1992*a;* Aiyar et al., 1993). We found that inhibiting PKC with calphostin C, H-7, staurosporine, and pseudosubstrate and substrate peptides all reduced G_{max}, whereas staurosporine prevented the suppression of current by phorbol ester in *Xenopus* oocytes (Aiyar et al., 1993).

The PKC dependence of Kv1.3 has also been addressed as part of the pathway regulating responses to the neurotransmitter, serotonin (5-HT). In a pre-B cell line, serotonin—evidently via endogenous 5-HT_1- and 5-HT_3-like receptors—increased G_{max} and accelerated the channel inactivation rate within minutes (Choquet & Korn, 1988). More recently, Kv1.3 channels have been co-expressed in *Xenopus* oocytes with specific serotonin receptors (5-HT_{1C}, 5-HT_2) to study the pathway coupling these receptors to K^+ channel function (Kavanaugh et al., 1991; Attali et al., 1992*b;* Aiyar et al., 1993). In contrast to the enhancement of current in the B-cell line, serotonin profoundly inhibited Kv1.3 current (within 10–20 min) in all three studies on *Xenopus* oocytes. Because 5-HT_{1C} and 5-HT_2 receptors are thought to be coupled

via G proteins to phospholipase C (thus to PKC and intracellular Ca^{2+}) the roles of G proteins, PKC and Ca^{2+} in mediating the serotonin effects have been investigated. Consistent with a direct effect of PKC in regulating Kv1.3, phorbol esters or a diacylglycerol analogue decreased the K^+ current in *Xenopus* oocytes in much the same way as did 5-HT (Attali et al., 1992*a;* Aiyar et al., 1993). However, the role of PKC in the 5-HT-induced suppression of current is still uncertain—staurosporine blocked the 5-HT effect in one study (Attali et al., 1992*a*), but had no effect in another (Aiyar et al., 1993).

There are several possible explanations for the differences noted above, including differences in the means used to activate PKC, and more fundamental differences between the cell types in which Kv1.3 regulation has been studied. For instance, there may be different expression or activation of PKC isoforms in different cell types, or stage-specific expression and downregulation of PKC isoforms. Of the ten isoforms of PKC described so far—each regulated by different combinations of cofactors, including Ca^{2+}, phospholipids, diacylglycerol and phorbol esters (Huang, 1989)—nine have been found in T cells and T-cell lines (*for review*, Hug & Sarre, 1993). Regulated expression and downregulation of PKC isoforms may occur during cell differentiation: PKCβ is present in all T-cell subsets (immature and mature) whereas PKCα is present in mature T cells (both CD4+ and CD8+ classes) (Taranito, Debre & Korner, 1994). Normal T lymphocytes express higher levels of PKCβ than α, whereas Jurkat T cells express more PKCα and β (Lucas et al., 1990). When Jurkat cells are activated with concanavalin A, PKCα appears to be translocated to a greater extent and for a longer duration than PKCβ (Kvanta, Jondal & Fredholm, 1991). Moreover, in normal T cells, phorbol ester (TPA) treatment increases the expression of PKCβ by 2-3-fold, with no effect on PKCα or γ (Altman, Mally & Isakov, 1992). Thus, discrepancies between results on Jurkat cells and normal T cells could reflect a specific isoform or coordination of differentially expressed isoforms. As an example, when K^+ and Na^+ channels from chick brain were expressed in *Xenopus* oocytes, different PKC isoforms affected the two channels differently. PKCγ reduced the fast-inactivating K_A current without changing Na^+ current, whereas PKCα and β reduced both K_A and Na^+ currents (Lotan et al., 1990). A complex interplay of PKC isoforms was apparent, since a mixture of α, β, and γ reduced K_A current to about the same extent as each individual isoform, but did not reduce Na^+ current. At this stage, regulation of Kv1.3 by different PKC isoforms has not been reported.

Potential Phosphorylation Sites

Although we do not know where the PKC-dependent phosphorylation occurs in our experiments, the amino acid sequence of Kv1.3 provides some clues. Kv1.3 clones from mouse (MK3), rat (RGK5, RCK3, Kv3), and human (HLK3) (Grissmer et al., 1990; Douglass et al., 1990; Stühmer et al., 1990; Swanson et al., 1990; Attali et al., 1992*b*) are highly homologous (>98% homology between human and rat clones). Most important for the present work, all but one of the consensus sites for phosphorylation by PKC are conserved in the sequences of mouse, rat, and human Kv1.3 clones. Rat and human Kv1.3 channels contain four PKC phosphorylation sites, two strong sites on the S4–S5 cytoplasmic loop, one mild site on the S2–S3 cytoplasmic loop, and one mild site on the C-terminal cytoplasmic portion. Biochemical analysis of phosphorylated amino acids *in vivo* and *in vitro* suggests that PKC phosphorylates the channel exclusively at serine residues in the Jurkat T-cell line (Cai & Douglass, 1993). There are two strong, serine-containing PKC sites on the S4–S5 cytoplasmic loop (Cai & Douglass, 1993). We speculate that one or both of these sites are involved in the changes in voltage dependence we have observed, because both sites are very close to the putative voltage sensor (positive charges in the S4 transmembrane segment), and we found that PKC activation produced positive shifts in the activation and inactivation curves.

The PKC-dependent increase in G_{max} we observed could have resulted from both longer channel openings and more functioning channels. Since the increase occurred within 15 to 30 min of treatment, it is unlikely that new channels were assembled and inserted into the membrane. We have previously reported that a similar increase in whole-cell Kv1.3 current at physiological temperatures arose, in part, from recruitment of inactive channels within minutes (Pahapill & Schlichter, 1990). Because of the short time scales of both of these effects, we believe that recruitment of silent channels preexisting in the membrane occurred—perhaps through phosphorylation at a critical PKC site. Any one of the four potential PKC sites in the Kv1.3 sequence could be involved in regulating the amplitude of G_{max}. There is a recent report that replacement of serine at one of the two PKC sites near the S4–S5 cytoplasmic loop led to dramatically less current when Kv1.3 was expressed in *Xenopus* ooyctes (Kupper et al., 1995). However, as current in such studies is measured days after mRNA injection, it is unclear whether fewer channels were expressed in the membrane or whether reduced phosphorylation of existing channels occurred.

In principle, the phosphorylated sites may not be on the channel protein itself, but on an associated regulatory molecule, such as a β subunit. Kv1.3, like other Shaker-related K^+ channels, is formed from four identical α subunit monomers that associate reversibly with a β subunit. At least three isoforms of β subunits of K^+ channels have been identified and sequenced (*for review*,

Isom, DeJongh & Catterall, 1994), and in some cases they modulate rates of current inactivation (Rettig et al., 1994). When the Kv1.3 channel was immunoprecipitated from Jurkat T cells, a^{40} kDa protein (about the same size as known β subunits) coprecipitated. Both the channel and this associated protein served as substrates for phosphorylation *in vivo* and *in vitro* (Cai & Douglass, 1993). It is possible that phosphorylation of this protein—or β subunits, if they are not the same protein—modulates Kv1.3 channel activity differently according to cell type. At present however, the functional modulation of the Kv1.3 channel by association/dissociation of β subunits is poorly understood.

Implications for T-Cell Biology

From the earliest discovery of the *n*-type K^+ channel (Kv1.3), functional studies have implicated it in a variety of immune-cell functions. The consistent finding is that inhibitors of this channel inhibit functions of T lymphocytes. In lymphocytes, Kv1.3 is involved in several Ca^{2+}-dependent processes: interleukin 2 (IL-2) secretion (Freedman, Price & Deutsch, 1992), cell proliferation (Chandy et al., 1984; Cahalan et al., 1985), Ca^{2+} signaling (Lin et al., 1993), and cytotoxic killing (Schlichter, Sidell & Hagiwara, 1986). These studies (*see* Lewis & Cahalan, 1995 *for review*) have been based on pharmacological blockers of the channel, originally using compounds that are membrane permeant and not very selective, then using much more potent and selective toxins, such as charybdotoxin and margatoxin. Kv1.3 channels are also involved in the K^+ efflux during the regulatory volume decrease (RVD) that follows lymphocyte swelling (Deutsch & Chen, 1993; Lewis & Cahalan, 1995). Several drugs that block Kv1.3 block RVD, and—not only does the ability of mouse T-cell lines to volume regulate correlate with the level of endogenous Kv1.3 expression—but transfecting Kv1.3 channels into a line that lacks them confers an ability to volume regulate (Deutsch & Chen, 1993).

During T-cell activation, a prolonged elevation of intracellular Ca^{2+} is necessary (*see* Lin et al., 1993; Lewis & Cahalan, 1995, and references therein). Both Ca^{2+} and PKC activation are required for T-cell activation, IL-2 secretion, and proliferation. The principal route of Ca^{2+} entry in lymphocytes—as in many nonexcitable cells—is through channels activated secondarily to store depletion (*see* Lewis & Cahalan, 1995). Since the gating of these channels is not voltage dependent, the role of membrane potential and K^+ channels in Ca^{2+} influx is different from excitable cells. Ca^{2+} influx, which is electrogenic, tends to depolarize the cell and reduce the driving force for Ca^{2+} influx. By maintaining the negative membrane potential in resting T cells (Leonard et al., 1992) Kv1.3 channels establish the electrochemical driving force for Ca^{2+} influx. Consequently, depolarizing T cells with high external K^+ concentrations, directly by using a voltage clamp, or by reducing the membrane K^+ conductance with channel blockers reduces both Ca^{2+} influx and the resulting rise in Ca^{2+} (*see* Lewis & Cahalan, 1995; Lin et al., 1993). A similar role for Kv1.3 in promoting Ca^{2+} entry appears to exist in human natural killer cells, where Ca^{2+}-dependent vesicular secretion of cytotoxic molecules depends on Kv1.3 channels (Schlichter et al., 1986). Thus, the PKC-induced increase in Kv1.3 conductance and window current that we observed should increase the ability of lymphocytes to volume regulate, to maintain a driving force for Ca^{2+} entry in the presence of activating, depolarizing stimuli, and to undergo exocytosis of cytotoxic molecules.

The regulation of Kv1.3 current observed in the present study is likely to have broader implications. Since the original cloning from a rat brain library, Kv1.3 transcripts have been found in a variety of tissues (*see* Chandy & Gutman, 1995; Lewis & Cahalan, 1995), most abundantly in spleen and thymus (organs that produce immune cells), and in lymphocytes and lymphocytic cell lines. Low levels of Kv1.3 mRNA are also found in rat and mouse brain, lung, pancreatic β cells. It is unlikely that the expression in brain reflects widespread expression in neurons. A more likely contributor is the endogenous brain microglia cell, which expresses both Kv1.3 mRNA and currents (Nörenberg, Gebicke-Haerter & Illes, 1993; Schlichter et al., 1996). Currents with essentially identical properties—assumed to arise from Kv1.3 channels—exist in various immune cells (macrophages, T cells, B cells, and their cell lines; for review, Gallin, 1991), lung type II alveolar cells (DeCoursey, 1990), melanocytes and melanoma cells (Nilius, Bohm & Wohlrab, 1992). Among the functions exhibited by these cells are fluid and electrolyte transport, proliferation, secretion of lymphokines, antibodies, surfactants, and killing of foreign and infected-cells. Thus, the biochemical regulation of Kv1.3 current in general, and by PKC in particular, may have widespread influences on the biology of both nonexcitable and excitable cells.

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