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Verapamil Inhibits Proliferation of LNCaP Human Prostate Cancer Cells Influencing K^+ Channel Gating

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ABSTRACT

The mechanisms of verapamil and tetraethylammonium (TEA) inhibition of voltage-gated K^+ channels in LNCaP human prostate cancer cells were studied in whole-cell and outside/inside-out patch-clamp configurations. Rapidly activating outward K^+ currents (I_K) exhibited neither C-type, nor rapid (human ether á go-go-related gene–type) inactivation. With 2 mM $[Mg^{2+}]_o$, I_K activation kinetics was independent of holding potential, suggesting the absence of ether á go-go-type K^+ channels. Extracellular applications of TEA and verapamil (IC_{50} = 11 μM) rapidly (12 s) inhibited I_K in LNCaP cells. Blocking was also rapidly reversible. Intracellular TEA (1 mM), verapamil (1 mM), and membrane-impermeable *N*-methyl-verapamil (25 μM) did not influence whole-cell I_K, although both phenylalkylamines inhibited single-channel currents in inside-out patches. Extracellular application of *N*-methyl-verapamil (25 μM) had no influence on I_K. Our results are compatible with the hypothesis that, in LNCaP cells expressing C-type inactivation-deficient voltage-activated K^+ channels, phenylalkylamines interact with an intracellular binding site, and probably an additional hydrophobic binding site that does not bind charged phenylalkylamines. The inhibiting effects of verapamil and TEA on I_K were additive, suggesting independent K^+-channel blocking mechanisms. Indeed, TEA (1 mM) reduced a single-channel conductance (from 7.3 ± 0.5 to 3.2 ± 0.4 pA at a membrane potential of +50 mV, n = 6), whereas verapamil (10 μM) reduced an open-channel probability (from 0.45 ± 0.1 in control to 0.1 ± 0.09 in verapamil-treated cells, n = 9). The inhibiting effects of verapamil and TEA on LNCaP cell proliferation were not multiplicative, suggesting that both share a common antiproliferative mechanism initiated through a K^+ channel block.

Due to the constant increase in human life expectancy, benign prostate hyperplasia has become a major health problem in men, and prostate cancer is one of the major risk factors in male mortality. It has been clearly established that the growth, differentiation, and apoptosis of prostate cells are regulated by androgens (Isaacs, 1984; Horton, 1992). The main treatment for prostate tumors consists of inhibiting cell growth by suppressing the action of endogenous androgens (Carraro et al., 1996). However, despite this treatment, almost all tumors (especially malignant ones) continue to progress and become hormone-refractory. The background of this clinical phenomenon is poorly understood. Various classes of drugs with antiproliferative properties are considered as possible alternatives to hormone therapy. Verapamil, a phenylalkylamine, has attracted a great deal of attention, due to its capacity to inhibit the proliferation of various cell types effectively. It has also proved capable of reversing the multidrug resistance of cancer cells to a variety of structurally and functionally distinct cytotoxic agents (Theyer et al., 1993). It has previously been shown that LNCaP human prostate cancer cells (derived from a lymph node of a subject with metastatic carcinoma of the prostate) do not express the P-glycoprotein responsible for the multidrug resistance phenotype (Theyer et al., 1993; van Brussel et al., 1999). These cells could, therefore, provide a useful model for studying the pure antiproliferative action of verapamil. There is growing evidence that verapamil's antiproliferative effect in various cell models involves a K^+ channel block (Amigorena et al., 1990; Batra et al., 1991; Pappone and Ortiz-Miranda, 1993; Yao and Kwan, 1999). We have previously shown that LNCaP human prostate cancer cells express voltage-activated K^+ channels and that these are involved in cell proliferation (Skryma et al., 1997, 1999). Moreover, LNCaP cells seem to lack L-type Ca^{2+} channels, another known pharmacological target for verapamil (Skryma et al., 1997, 1999).

It is important to locate the verapamil binding sites on K^+

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ABBREVIATIONS: HERG, human ether à go-go related gene; TEA, tetraethylammonium; I_{vrp}, resting K^+ current in the presence of verapamil; $I_{control}$, control K^+ current; EAG, ether à go-go; I_K, K^+ current; IC_{50}, half-maximal inhibition concentration; γ, index of interaction between two inhibitors; I_{TEA}, potassium current amplitude inhibited by tetraethylammonium; ; I-V, current-voltage; I_{tea}, resting K^+ current in the presence of tetraethylammonium; P_o, open probability; i/I_{max}, normalized resting ion current.

channels in human prostate cancer cells and identify their functional significance, with a view to developing treatments that target these channels. These binding sites could become new targets for potential prostate antiproliferative agents (e.g., synthetic phenylalkylamines). K^+ channels in LNCaP cells are likely to represent a new channel type with a unique combination of biophysical and pharmacological properties (Skryma et al., 1999). These findings raise hopes that these K^+ channels are characterized by high prostate-specific expression [e.g., Slo 3 K^+ channel, specific to mammalian spermatocytes (Schreiber et al., 1998)] and thus, that highly selective antagonists for these channels would have specific antiproliferative properties in prostate tumor tissue. The localization of verapamil binding sites has been addressed in several types of voltage-gated K^+ channels. In most earlier reports, verapamil seemed to cross the membrane in neutral form to block the channel through binding to its internal residue (DeCoursey, 1995; Rauer and Grissmer, 1996; Catacuzzeno et al., 1999; Hanson et al., 1999; Rauer and Grissmer, 1999; Zhang et al., 1999). It is remarkable that, in nearly all previously examined cell types, verapamil-sensitive voltage-activated K^+ channels possessed C-type inactivation, and one of the more prominent effects of verapamil was a dramatic acceleration of K^+-current inactivation. After experiments on villus enterocytes (Tatsuta et al., 1994), human T-lymphocytes (Hanson et al., 1999), and transfected human ether à go-go-related gene (HERG) channels (Zhang et al., 1999), verapamil was assumed to have either direct or allosteric effects on the molecular structures responsible for C-type inactivation. In contrast, a comparison of the effects of verapamil on mutant and wild-type Kv1.3 channels (Rauer and Grissmer, 1999) did not indicate any direct connection between C-type inactivation and the cumulative blocking effect of phenylalkylamines. Such controversial results may originate from different experimental objects, as well as from the multimodal effects of verapamil on K^+ channels. This investigation of verapamil blocking mechanisms in the C-type inactivation-deficient K^+ channels expressed in LNCaP cells (Skryma et al., 1997) may help to isolate the effects of the drug on protein structures that are not involved in channel inactivation. The verapamil binding sites in these K^+ channels may be different from previously described binding sites in other cell types, and modulation of these sites may be used to control prostate tumor cell proliferation.

In this work, we studied for the first time the mechanism of verapamil inhibition of noninactivating K^+ channels and addressed the possible location of verapamil-binding sites on these K^+ channels in LNCaP human prostate cancer cells. Furthermore, we have shown that K^+ channel inhibition by verapamil also inhibits LNCaP cell proliferation.

Materials and Methods

Cell Culture. LNCaP cells from the American Type Culture Collection (Manassas, VA) were grown and prepared for electrophysiological experiments as described previously (Skryma et al., 1999, 2000).

Electrophysiological Recordings. The whole-cell and single-channel modes of the patch-clamp technique were used. This technique has been described in detail in previous publications (Skryma et al., 1999, 2000; Shuba et al., 2000).

Data Analysis and Statistics. Single-channel data were analyzed after elimination of capacity transients and leak current by subtracting recorded averages without channel activity from each current recording. Channel opening and closing was detected using the criterion of a 50% exclusion between fully open and fully closed states to determine the occurrence of the opening or closing event (i.e., crossing the line half-way between zero current level and a level corresponding to the average open-channel amplitude). The open probability was calculated as the open time integral divided by the number of channels in the patch and the duration of the data segment analyzed. The number of channels was estimated by examining the record for multiple openings under conditions of high open probability ($P > 0.75$). Data segments of 8 s (160 ms for 1 episode, 50–100 episodes) were analyzed for open probability estimates.

Results are expressed as the means ± S.D. where appropriate. Each experiment was repeated several times. Student's t test was used for statistical comparison among means, and differences with $P < 0.05$ were considered significant.

Recording Solutions. The extracellular solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4 mM $NaHCO_3$, 5 mM glucose, and 10 mM HEPES. The osmolarity of the external salt solution was adjusted to 310 to 315 mosM with sucrose, and the pH adjusted to 7.3 ± 0.01 using NaOH. The internal solution contained 140 mM KGlu, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 8 mM EGTA, and 5 mM HEPES, pH 7.2 ± 0.01 with KOH; osmolarity, 300 mosM. We have previously shown that the K^+ channel open probability decreased as internal free Ca^{2+} was augmented from 0.01 μM in the standard internal solution to 0.2 to 1 μM (Skryma et al., 1999). Therefore, in all the experiments in this study, the free Ca^{2+} concentration for the solutions applied from the inner side of the membrane (in whole-cell and inside-out experiments) was buffered with 8 mM EGTA to 0.01 μM, calculated using "Maxc Software" (from Chris Patton, Hopkins Marine Station, Stanford University, Stanford, CA).

In single-channel experiments, test substances were applied to the patches by low-pressure ejection from an additional "puffing" micropipette (tip diameter 5–10 μm). This pipette was filled with the extracellular saline solution used in the bath, with the drug under investigation added in appropriate concentrations. The pipette was brought to a distance of 30 to 60 μm from the investigated cell. All experiments were performed at room temperature (20–22°C).

Chemicals. Tetraethylammonium (TEA) and verapamil were obtained from Sigma (L'Isle d'Abeau, France). *N*-methyl-verapamil was generously provided by Knoll Pharmaceuticals (Ludwigshafen, Germany).

Dose-Response Experiments. K^+ channel inhibitors (verapamil and TEA) were applied in the culture dish using an electric valve-controlled solution application stage (Scientific Instruments, West Palm Beach, FL). The time required for a complete exchange of solutions in the dish was 30 to 40 s. The dose-response dependence of the degree of inhibition of whole-cell K^+ currents by verapamil ($I_{vrp}/I_{control}$) was measured for eight different concentrations of the drug. For each cell, the resting inhibited current was recorded in the presence of a maximal concentration (50 μM) and one of the intermediate concentrations of verapamil. At least four cells were tested at each intermediate concentration. The nonlinear fit to the normalized averaged dose-response points was performed using a function corresponding to eq. 8 (see *Appendix*) incorporated into the Origin 5.0 software (MicroCal Software, Northampton, MA).

[^{3}H]Thymidine Incorporation Assay. For [^{3}H]thymidine incorporation, the cells were seeded in 24-well plates (Nunc, Naperville, CT) precoated with polyornithine (5 mg/1) at 5×10^4 cells per well. K^+ channel inhibitors were added at given concentrations 2 h after plating. Forty-eight hours after the addition of channel inhibitors, 50 nM [^{3}H]methyl-thymidine (specific activity, 60 Ci/mmol; ICN, Orsay, France) were added to each well for 24 h. At the end of this pulse period, the medium was discarded, the cells were rinsed twice in RPMI, and chase was achieved by a 2 h incubation in 50 μM unlabeled thymidine in RPMI. The chase medium was discarded, and the cells were lysed in 0.1 M sodium hydroxide. The lysis medium was

neutralized with 0.1 M hydrochloric acid and transferred into vials containing 6 ml of liquid scintillation counting medium (Ready Safe; Beckman, Gagny, France). The mixture was thoroughly emulsified and counted 24 h later in a Beckman LS6000IC spectrometer (Beckman Coulter, Fullerton, CA). Each concentration was tested in quadruplicate wells, and experiments were performed at least three times.

Determination of Apoptosis. Hoechst staining was used to determine the percentage of apoptotic cells. The detailed procedure has been described previously (Skryma et al., 2000).

Results

Two different voltage-stimulation protocols were tested to investigate whether the K^+ channels in LNCaP cells had an ether à go-go (EAG) channel-like behavior. The first protocol was applied to verify whether these K^+ channels behaved like HERG channels (i.e., fast inactivation developing more rapidly than activation and removing more rapidly than deactivation) (Meyer et al., 1999). This protocol was analogous to the one applied by Zhang et al. (1999), and the family of currents obtained is presented in Fig. 1A. HERG-like behavior (i.e., the large outward tail current corresponding to the repolarizing step) was not observed in LNCaP cells. Furthermore, I_K did not exhibit any tendency to C-type inactivation during depolarizing steps as long as there was 4 s at all membrane potentials tested. Currents obtained under this protocol were strongly inhibited by 10 mM TEA at all potentials tested (Fig. 1A). In accordance with our previous data, neither sodium nor calcium residual voltage-dependent currents were observed in LNCaP cells under these experimental conditions at a wide range of membrane potentials (Skryma et al., 1997, 1999). However, voltage-dependent Ca^{2+} currents can be rather small in nonexcitable cells (Skryma et al., 1994) and could be masked by the outward current that remains after TEA application. We therefore checked the potential voltage-dependent Ca^{2+} channel activity in LNCaP cells under optimal experimental conditions for Ca^{2+} channel recording [i.e., equimolar substitution of Cs^+ ions for intracellular K^+ ions and inclusion of Ba^{2+} ions (10 mM) as charge carrier in Ca^{2+}-deprived extracellular solution containing 10 mM TEA]. We did not observe any inward current under these recording conditions in either whole-cell ($n = 7$) or single-channel ($n = 9$) patch-clamp technique configurations, at any membrane potential (data not shown). A second protocol was applied to verify whether the activation kinetics of I_K in LNCaP cells slowed down in the presence of physiological $[Mg^{2+}]_o$, when currents were activated from deeply hyperpolarizing holding potentials. This is the case in several types of human melanoma cell lines, expressing noninactivating EAG-type K^+ channels (Meyer et al., 1999). LNCaP cells were held for 7 s at membrane potentials varying from −40 mV to −120 mV, then depolarized to +50 mV (Fig. 1B). We found that the activation kinetics of currents evoked from different holding potentials was strictly reproducible ($n = 15$). This series of experiments demonstrated that the noninactivating voltage-activated K^+ channels expressed in LNCaP cells do not exhibit the typical functional properties of EAG channels.

Verapamil Block of I_K. I_K in LNCaP cells was shown to be blocked by extracellular TEA, α-dendrotoxin, and mast-cell degranulating peptide. It was insensitive to 4-aminopyridine, charybdotoxin, and iberiotoxin (Skryma et al., 1997, 1999). To study verapamil's K^+ channel inhibition mechanisms, we compared its effects on I_K with those produced by TEA, a well known, positively-charged K^+ channel pore blocker. Both TEA and verapamil, applied extracellularly, effectively inhibited I_K (Fig. 2A).

The inhibition of K^+ channels by TEA and verapamil applied at concentrations producing equivalent suppression (by 70–80%) of I_K (2.5 mM and 25 μM, respectively) was rapid (dozens of seconds), comparable with the time course of bath solution exchange. I_K amplitude was restored in the same short time interval after washout of either of the drugs (Fig. 2B). Our observations suggested that the TEA and verapamil binding sites on K^+ channels expressed in LNCaP cells were easily accessible from the outer part of the cell membrane.

We therefore investigated whether the phenylalkylamine binding site in LNCaP cells was located on the extracellular residue of the K^+ channel. For this purpose, the charged membrane-impermeable verapamil analog *N*-methyl-verapamil was applied extracellularly at a concentration of 20

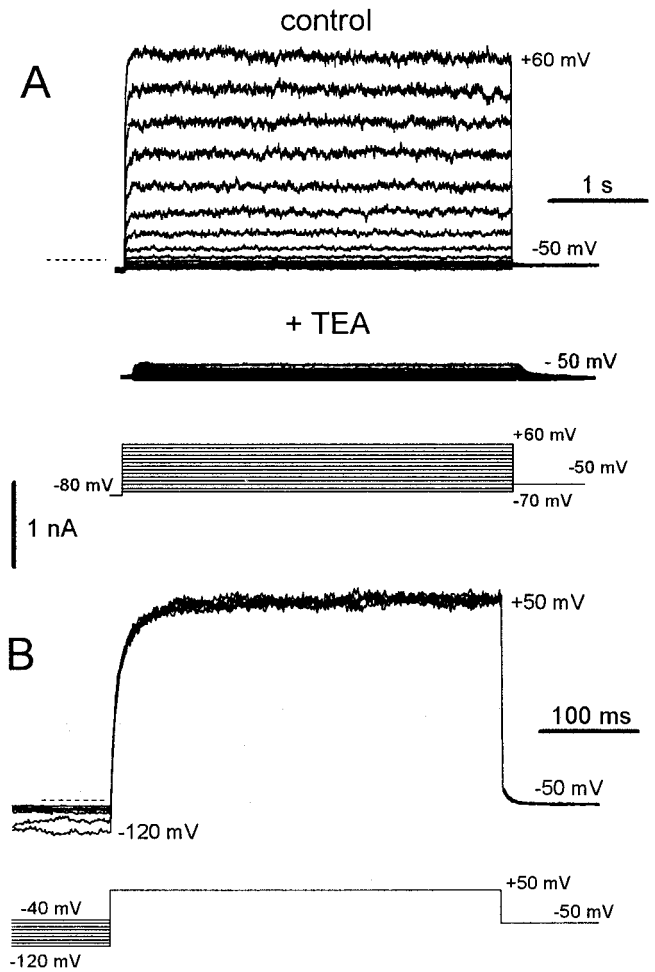


Fig. 1. LNCaP voltage-activated K^+ channels do not possess C-type or EAG-type inactivation. A, family of K^+ currents (I_K) obtained before (top) and after (bottom) 10 mM TEA application, evoked by 4 s depolarizing pulses from a holding potential of $V_h = -80$ mV to various membrane potentials (−70 to +60 mV; $\Delta V = 10$ mV), followed by repolarizing steps to $V_m = -50$ mV. B, family of I_K evoked by 400 ms depolarizing pulses from different holding potentials (−40 to 120 mV; $\Delta V = -10$ mV) to $V_m = +50$ mV, followed by repolarizing pulses to $V_m = -50$ mV. Currents in A and B, recorded in main extracellular solution (2 mM $[Mg^{2+}]_o$), were nonleakage-subtracted.

μM. In our experiments, *N*-methyl-verapamil failed to block I_K (n = 14, not shown), suggesting that verapamil diffused into the membrane, or through it, to reach its binding site.

To verify whether there were verapamil and/or TEA binding sites on the inner part of the K^+ channel in LNCaP cells, we examined a possible blocking effect of both drugs applied intracellularly. For this purpose, 1 mM TEA or 25 μM verapamil were added to the patch-pipette solution. If verapamil or TEA binding sites were accessible from the cytoplasmic part of the membrane, we would expect to observe a gradual decrease in I_K amplitude after the time course of cell perfusion with verapamil and TEA. However, neither 1 mM TEA (Fig. 3A) nor 25 μM verapamil (not shown) added to the intracellular solutions produced any noticeable inhibition of I_K during a 10-min recording (7 cells with TEA and 5 cells with verapamil). To verify the rate of intracellular perfusion, we used a Cs^+-based patch-pipette solution. The equimolar substitution of nonpermeable Cs^+ ions (140 mM) for intracellular K^+ ions (140 mM) resulted in the gradual disappearance of outward I_K. It took about 3 min to replace half the cell cytoplasm solution with pipette solution (Fig. 3A). Cs^+, applied extracellularly at millimolar concentrations, did not influence I_K in our tests. We further augmented the verapamil concentration in the pipette solution to 1 mM. Applied intracellularly at such a high concentration, verapamil did not produce any visible blocking effect on K^+ channels (n = 6; an example is presented in Fig. 3A). The absence of a blocking effect of internally applied verapamil has been reported in dialyzed basophilic leukemia cells containing recombinant Kv1.3 K^+ channels where the inclusion of 25 μM *N*-methyl-verapamil in the pipette solution inhibited K^+ currents (Rauer and Grissmer, 1996). We therefore tested the effect of internally applied *N*-methyl-verapamil on K^+ channels in LNCaP cells. In our experiments (11 cells), the addition of 25 μM of *N*-methyl-verapamil to the patch-pipette solution had no effect on I_K (Fig. 3A). Our results may suggest the absence of phenylalkylamine or TEA binding sites on the inner part of K^+ channels in LNCaP cells. However, when K^+ single-channel currents were studied in inside-out configuration (Fig. 3B), the application of both verapamil and *N*-methyl-verapamil clearly decreased the open channel probability (Fig. 3C). This experiment revealed the presence of a phenylalkylamine binding site on the intracellular residue of a K^+ channel in LNCaP cells analogous to sites detected in other cell types. Note that substituting half *N*-methyl-verapamil for verapamil considerably reduced the K^+ channel open channel probability (Fig. 3B). This may indicate 1) different binding sites, preferentially targeted by verapamil or *N*-methyl-verapamil and/or 2) a more effective interaction of charged forms of phenylalkylamines with the K^+ channel intracellular binding site.

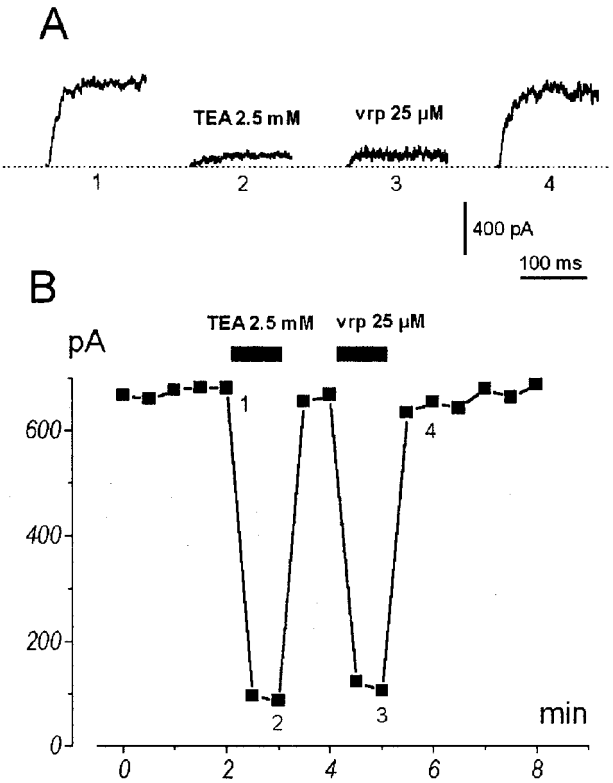


Fig. 2. Extracellular TEA and verapamil block outward K^+ currents in LNCaP cells. A, current recordings from the same cell in the normal extracellular solution: (1) before, (2) during the application of 2.5 mM TEA, or (3) 25 μM verapamil, and (4) after washout of the cell. Currents were evoked by membrane depolarizing steps from a holding potential of V_h = −50 mV to +40 mV. B, time/amplitude protocol of the experiment, for which the current traces are presented in A.

A rapid rate of K^+ channel blocking by extracellular application of verapamil and TEA may indicate the existence of another LNCaP cell K^+ channel binding site that is more easily accessible for verapamil than the intracellular site. It was, therefore, probable that extracellular applications of verapamil and TEA produced their inhibitory effects by targeting the same K^+ channel function. To verify this hypothesis, we examined a possible competition between the blocking effects of extracellularly applied verapamil and TEA on K^+ channels. Drug concentrations that inhibited half the K^+ current (IC_{50}) were used for these competition assays. We have previously shown that the TEA IC_{50} value for I_K in LNCaP cells is on the order of 1 to 2 mM (Skryma et al., 1997). To determine the IC_{50} value of verapamil, we established the dose-response curve for K^+ channel blocking by externally applied drug. The normalized verapamil inhibition of I_K [the amplitude of resting current in the presence of verapamil (I_{vrp}), divided by the control amplitude ($I_{control}$) of I_K recorded in normal solution], was measured at eight different verapamil concentrations. At least four cells were tested at each concentration. The average verapamil dose-response points are presented in Fig. 4 on the linear concentration scale. The nonlinear regression fit to dose-response points presented in Fig. 4 yielded an IC_{50} value of 11 μM for I_K inhibition by verapamil.

Verapamil-TEA Competition Assays. We used 1 mM TEA and 10 μM verapamil for the competition assays. A typical example of a competition experiment is presented in Fig. 5. We applied two different approaches to evaluate the type of competition between TEA and verapamil during their simultaneous inhibition of K^+ channels. The first approach relied on the multiplication of the inhibitory effects of two drugs on I_K if their inhibition is produced via independent mechanisms (Rauer and Grissmer, 1996, 1999; Hanson et al., 1999). We compared the normalized amplitudes of resting currents recorded in the presence of 1 mM TEA, 10 μM verapamil, and both drugs applied together (1 mM TEA + 10 μM verapamil). In the majority of cells tested, 1 mM TEA and 10 μM verapamil reduced the I_K amplitude to approximately

half the control, as expected from dose-response data (Fig. 5A). We found that the resting current, $(I_{TEA+vrp})_{norm}$, resulting from the cumulative action of these two blockers, was always close to the multiplication product of resting currents recorded in the presence of TEA and verapamil applied separately $[(I_{TEA})_{norm} \cdot (I_{vp})_{norm}]$. The value of $(I_{TEA+vrp})_{norm} = 0.37$ for the cell presented in Fig. 5 was slightly higher (+7%) than would be predicted from the multiplication of separate inhibitory effects of TEA and verapamil $[(I_{TEA})_{norm} \cdot (I_{vrp})_{norm} = 0.60 \cdot 0.57 = 0.342]$. A slight positive deviation (+7 ± 1%) of $(I_{TEA+vrp})_{norm}$ from the product of $(I_{TEA})_{norm} \cdot (I_{vrp})_{norm}$ was reproduced in two other cells. The small discrepancy between $(I_{TEA+vrp})_{norm}$ and $(I_{TEA})_{norm} \cdot (I_{vrp})_{norm}$ ruled out a significant competition between TEA and verapamil, suggesting that these drugs block K^+ channels in LNCaP cells via independent mechanisms. However, a small but persistent deviation of the cumulative blocking effect from a pure multiplication of the individual blocking effects of verapamil and TEA may suggest that there is some degree of negative cooperation (probably due to allosteric effects) between the two blockers' inhibitory mechanisms.

The second approach used for competition tests was based on the general scheme of double enzyme inhibition (Keleti and Fajszi, 1971), applied, in this case, to TEA-verapamil blocking of K^+ channels (see *Appendix*: Double Inhibition Scheme). An index of interaction between two inhibitors (γ) was introduced, which would be close to 1 in the case of purely independent inhibition, but tended toward infinity (∞) in the case of purely competitive inhibition. The averaged γ values of 1.75 ± 0.11 obtained from 3 cells were close but not equal to 1, a theoretical value for two inhibitors with purely independent K^+ channel blocking mechanisms.

Effect of Verapamil and TEA on Single K^+ Channels. We examined the effect of both drugs on single-channel K^+ currents in outside-out membrane patches excised from LNCaP cells. The general characteristics of these currents in LNCaP cells (conductance of ~80 pS, inhibition by $[Ca^{2+}]_i$) have already been described (Skryma et al., 1999). The pharmacological effect of TEA on the single-channel current is illustrated in Fig. 6A. Extracellular application of 200 μM TEA resulted in an immediate, marked reduction in the single-channel current amplitude from 7.3 pA to 3.2 pA at a membrane potential of +50 mV ($n = 6$) (Fig. 6D), although the channel opening probability was unchanged (Fig. 6C). The amplitude of single-channel K^+ currents was restored after washout of TEA.

The effect of verapamil on single-channel K^+ currents was clearly different from that produced by TEA (Fig. 6B). Shortly after the application of 50 μM verapamil, the channel opening probability (P_o) decreased considerably (from 0.45 ± 0.10 in control to 0.1 ± 0.09 in the presence of verapamil, $n =$

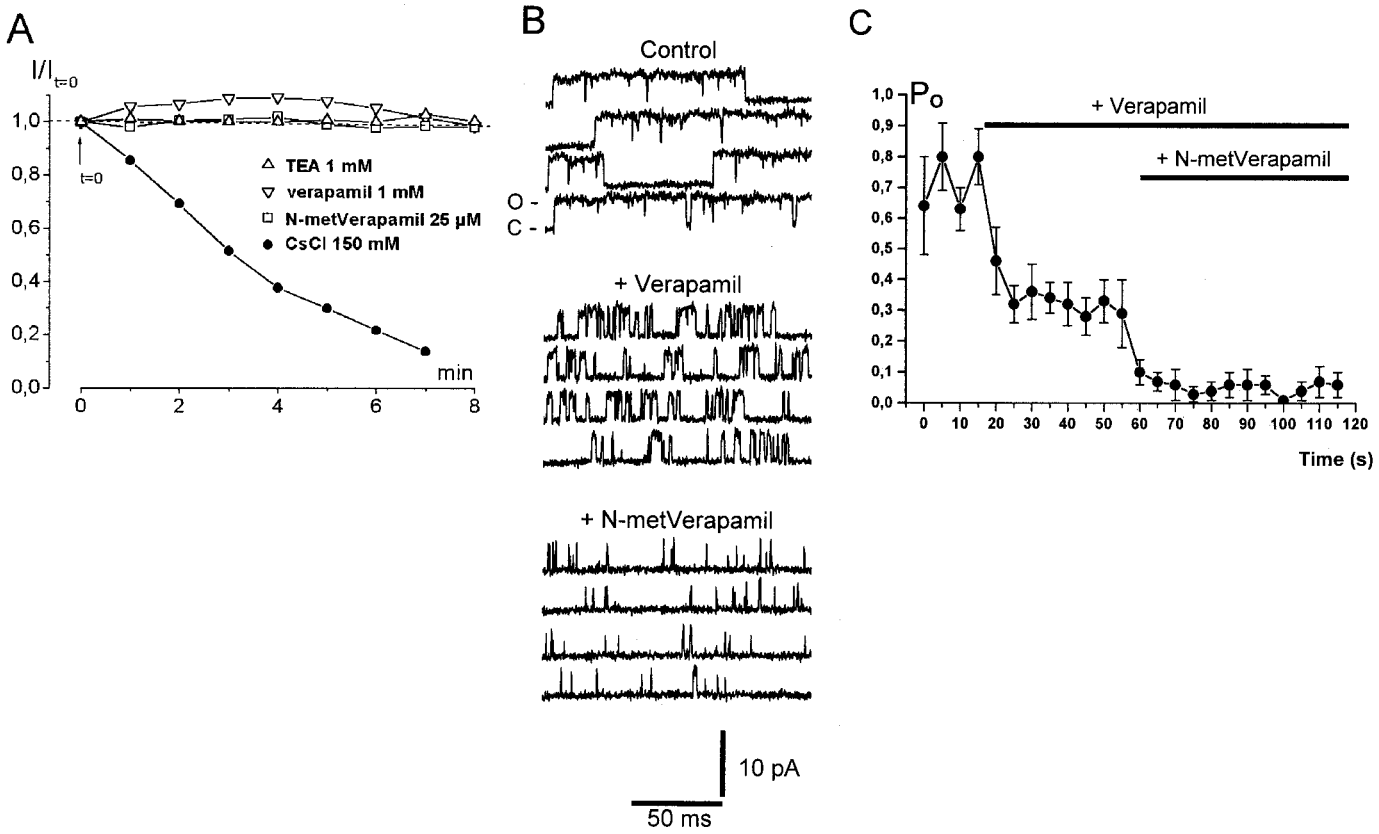


Fig. 3. Intracellular TEA, verapamil, and *N*-methyl-verapamil do not influence whole-cell I_K, but both phenylalkylamines inhibit single-channel K^+ currents in inside-out patches. A, In four different cells perfused with main intracellular solution with the addition of: 1) 1 mM TEA (△); 2) 1 mM verapamil (▽); 3) 25 μM *N*-methyl-verapamil (□); and 4) 140 Cs (substitution for 140 KCl, ●). I_K amplitudes were measured just after the rupture of the membrane patch, and then several times, at 1-min intervals. Amplitudes of currents evoked by depolarizing steps from a holding potential of $V_h = -50$ mV to +40 mV are presented in normalized form (divided by the amplitude of the initial current recorded at $t = 0$ min). B, representative traces of K^+ single-channel currents recorded in the same inside-out patch under control conditions (top) and after application of intracellular solutions containing 50 μM verapamil (middle) and 25 μM verapamil + 25 μM *N*-methyl-verapamil (bottom). C, time course of the open probability (P_o) of K^+ channels in the control and in the presence of verapamil and *N*-methyl-verapamil.

9; Fig. 6C). The reduction of K^+ channel open probability due to verapamil was reversible (Fig. 6C).

I-V Characteristics of Verapamil- and TEA-Inhibited I_K. To further analyze the K^+ channel blocking mechanisms of TEA and verapamil, we examined whether these drugs modified the current-voltage (I-V) dependence of I_K. An analysis of possible electrostatic effects of TEA and verapamil on the steady-state I-V relationship of I_K is presented in Fig. 7. The I-V curve of TEA-inhibited currents (I_{tea}) was multiplied by a normalizing coefficient of 2.155, which corresponded to the $I_{control}/I_{tea}$ ratio in this cell, calculated at a membrane potential of +60 mV. The resulting normalized curve ($I_{tea} \times 2.155$) demonstrated a clear right-shift lag on the scale of potentials relative to the control I-V curve ($I_{control}$). This effect of TEA was observed in all cells studied ($n = 7$). The original traces of the control and TEA-inhibited currents, recorded at +60 mV and multiplied by a normalizing coefficient of 2.155 (Fig. 7A), are superimposed in the inset in Fig. 7B. The TEA-inhibited current was observed to have slightly slower activation kinetics. An analogous examination of the possible electrostatic nature of the K^+ channel inhibition by verapamil is presented in Fig. 7, C and D. The I-V curve of verapamil-inhibited currents in this experiment (I_{vrp}, ■) multiplied by a normalizing coefficient of 1.946, corresponding to the $I_{control}/I_{vrp}$ ratio in this cell, calculated at a membrane potential of +70 mV, is shown in Fig. 7D by □ ($I_{vrp} \times 1.946$). This curve matched the control I-V curve ($I_{control}$, ○) closely in the range of membrane potentials higher than +40 mV, at which the open probability of K^+ channels is already stable (maximal). A slight leftward shift of the ($I_{vrp} \times 1.946$) curve relative to the control I-V curve was observed in the local range of membrane potentials (−20 to +30 mV), where K^+ channels start to sense membrane depolarization. The voltage threshold for activation of verapamil-inhibited I_K was apparently unchanged, compared with the threshold of activation of control I_K. The modulation of steady-state I-V curves by verapamil was reproducible in the five cells tested. Superimposed individual traces of the control and normalized verapamil-inhibited current recorded at membrane potential of +70 mV (Fig. 7C) are shown in the inset in Fig. 7D. No significant changes were observed in the activation kinetics of verapamil-inhibited current relative to the control current at this high depolarizing potential ($n = 5$).

We examined the types of changes caused by TEA and verapamil in the instantaneous I-V curves, which reflect the properties of open ion-transmission pores. Recordings of control and 1 mM TEA-inhibited I_K evoked in the same cell by depolarizing steps to +40 mV followed by repolarizing steps to various membrane potentials are presented in Fig. 8A. Tail current amplitudes at the beginning of the repolarizing pulse are presented as instantaneous I-V relationships in Fig. 8B. The I-V curve obtained for TEA-inhibited currents (I_{tea}) was multiplied by a normalizing coefficient of 2.324, corresponding to the $I_{control}/I_{tea}$ ratio calculated at +40 mV. The resulting normalized curve ($I_{tea} \times 2.324$) was compared

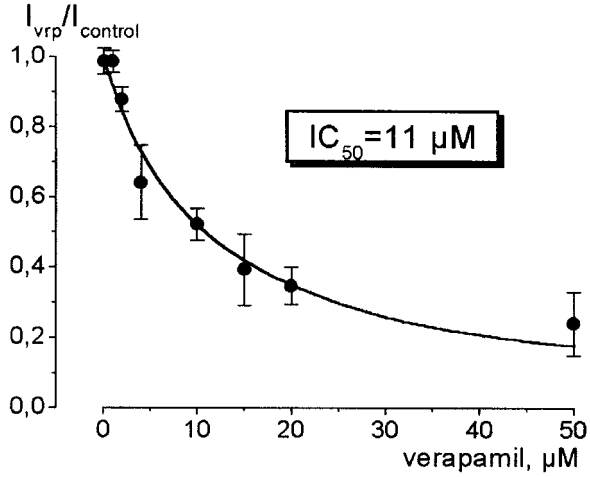


Fig. 4. Dose-response dependence for I_K inhibition by extracellular verapamil. The ratio of the resting current amplitude recorded in the presence of verapamil to the control current amplitude ($I_{vrp}/I_{control}$) was measured at concentrations of 0.1, 1, 2, 4, 10, 15, 20, and 50 μM. For each cell tested ($n = 30$), the $I_{vp}/I_{control}$ ratio was measured at 50 μM and at one of the intermediate verapamil concentrations. Each dose-response point represents a value (±S.D.) averaged from at least four measurements. The smooth line represents a nonlinear fit to the points with a function of $y = 1/(1 + [I]/IC_{50})$, where [I] is the verapamil concentration and IC_{50} is the apparent inhibition constant.

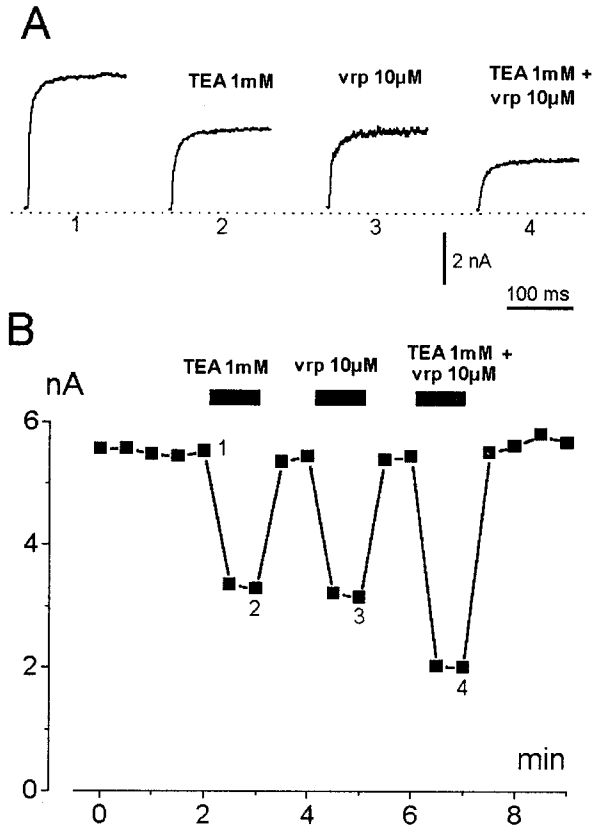


Fig. 5. Verapamil (vrp)-TEA competition test by means of separate and cumulative inhibition of K^+ channels: after rupture of the membrane patch and stabilization of the amplitude of I_K, 1 mM TEA, and 10 μM verapamil were applied to the cell in turn (then washed out), and the resting K^+ current in the presence of each blocker was recorded. The extracellular solution containing both 1 mM TEA and 10 μM verapamil was then applied to the cell, and resting K^+ current in the presence of both antagonists was recorded. A, Traces showing: 1) control current, 2) (1 mM TEA)-inhibited current, 3) (10 μM verapamil)-inhibited current, and 4) (1 mM TEA + 10 μM verapamil)-inhibited current recorded in the same cell at times indicated by the appropriate numbers in B. Amplitudes of 1 mM TEA-inhibited current and (10 μM verapamil)-inhibited current are very close (both ≈50% of $I_{control}$). The amplitude of the 1 mM TEA + 10 μM verapamil-inhibited current is about one-half that of currents inhibited by TEA/verapamil alone. I_K values were evoked by membrane depolarizing steps from a holding potential of $V_h = -40$ mV to +40 mV. B, time/amplitude protocol of the experiment presented in A.

with the control instantaneous I-V curve ($I_{control}$). A clear rightward shift of the curve was observed for TEA-inhibited tail currents. The rightward shift varied from +8 to +25 mV ($\Delta V = +12 \pm 4$ mV; $n = 4$). These results indicate that TEA binds to the outer part of K^+ channel pores and represents a strong electrostatic barrier for intracellular K^+ ions. Superimposed traces of the control and normalized TEA-inhibited currents (repolarizing steps to +10 mV) are presented in the inset in Fig. 8B.

An analogous examination of the instantaneous I-V relationships of verapamil-inhibited currents is presented in Fig. 8, C and D. The instantaneous I-V curve (I_{vrp}; Fig. 8D) for the verapamil-inhibited tail currents from Fig. 8C was multiplied by a normalizing coefficient of 2.071, corresponding to the $I_{control}/I_{vrp}$ ratio obtained at +30 mV. The resulting ($I_{vrp} \times 2.071$) curve perfectly overlapped the control instantaneous I-V characteristics ($I_{control}$; Fig. 8D). These results strongly indicate that verapamil binding does not influence the ion-transmission pore properties of K^+ channels in LNCaP cells ($n = 5$). However, significant changes were observed in the kinetics of the repolarizing phase of verapamil-inhibited tail currents relative to control tail currents. The tail current relaxation phase recorded at higher repolarizing potentials (≥0 mV) was abolished by verapamil (Fig. 8, C and D, inset), suggesting that it causes a significant modulation of voltage-sensor and/or K^+ channel gate compartments ($n = 5$).

Verapamil-TEA Cross-Inhibition of LNCaP Cell Proliferation. We tested whether verapamil was able to inhibit LNCaP cell proliferation. In view of the probable multimodal pharmacological effects of verapamil, we also addressed the question whether the antiproliferative effect of verapamil was really mediated by blocking the K^+ flux. The competition between verapamil and TEA during inhibition of LNCaP cell proliferation was again examined. In the case of independent inhibition mechanisms, the antiproliferative effects of verapamil and TEA would be additive, and the resting level of LNCaP cell proliferation activity in the presence of both drugs would be close to the product of those levels measured for verapamil and TEA alone.

We carried out the competition tests using 25 μM verapamil and 1 mM TEA (Fig. 9). The degree of inhibition of

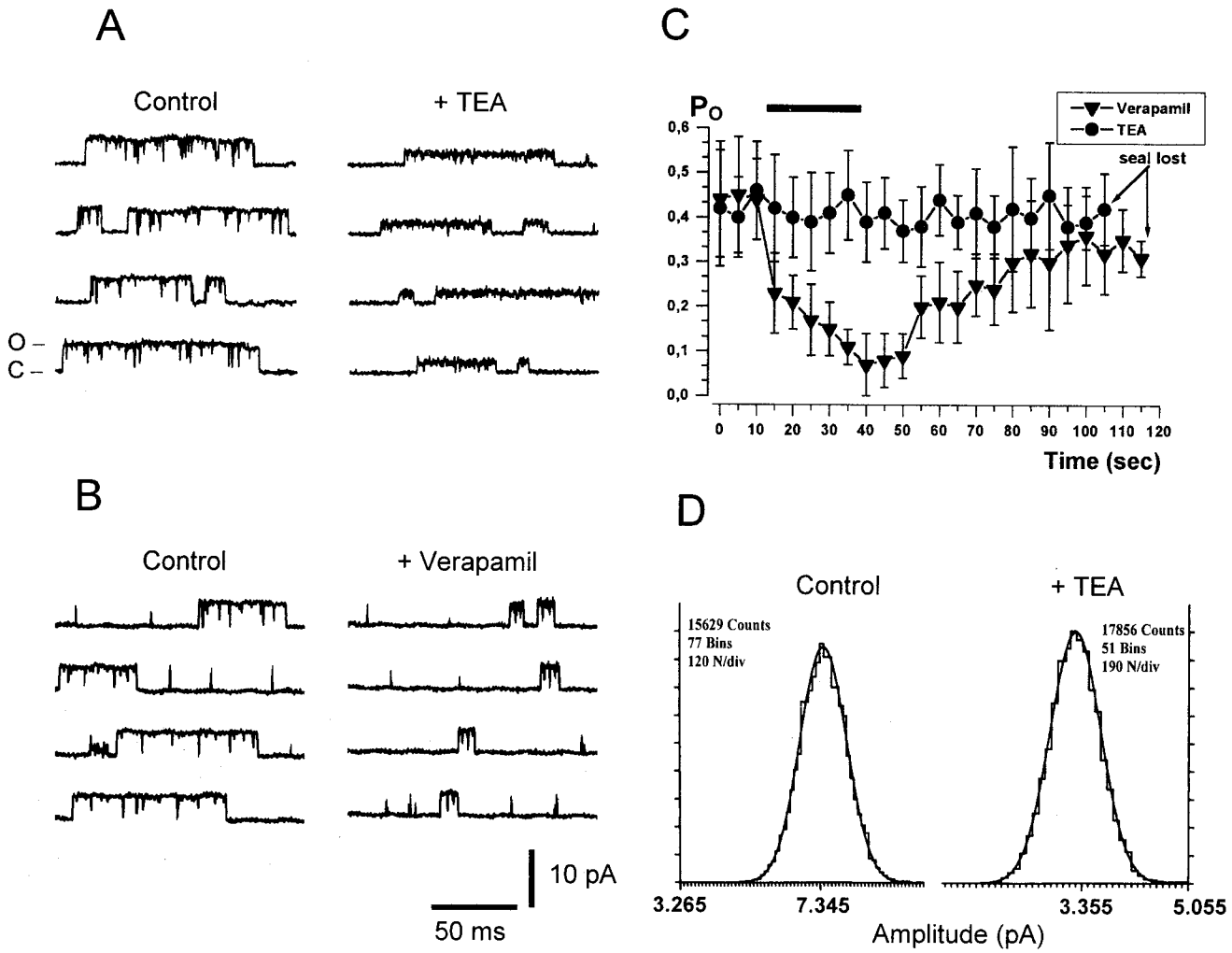


Fig. 6. Effect of TEA and verapamil on single-channel K^+ currents in excised (outside-out) membrane patches. Currents were recorded at 5 mM external and 140 mM internal [K^+]. A, representative traces of single-channel K^+ currents recorded at a membrane potential of +50 mV in control solution (left) and 1 min after 200 μM application of TEA (right). B, representative traces of single-channel K^+ currents recorded (in a different patch from A) at a membrane potential of +40 mV in the control solution (left) and 40 s after 50 μM application of verapamil (right). C, comparative time course of K^+ channel open probability in external solutions containing 50 μM verapamil (▼) and 200 μM TEA (●). Bar indicates drug application time. D, amplitude histograms for control (left) and 200 μM TEA-inhibited (right) single-channel K^+ currents recorded from the patch shown in A.

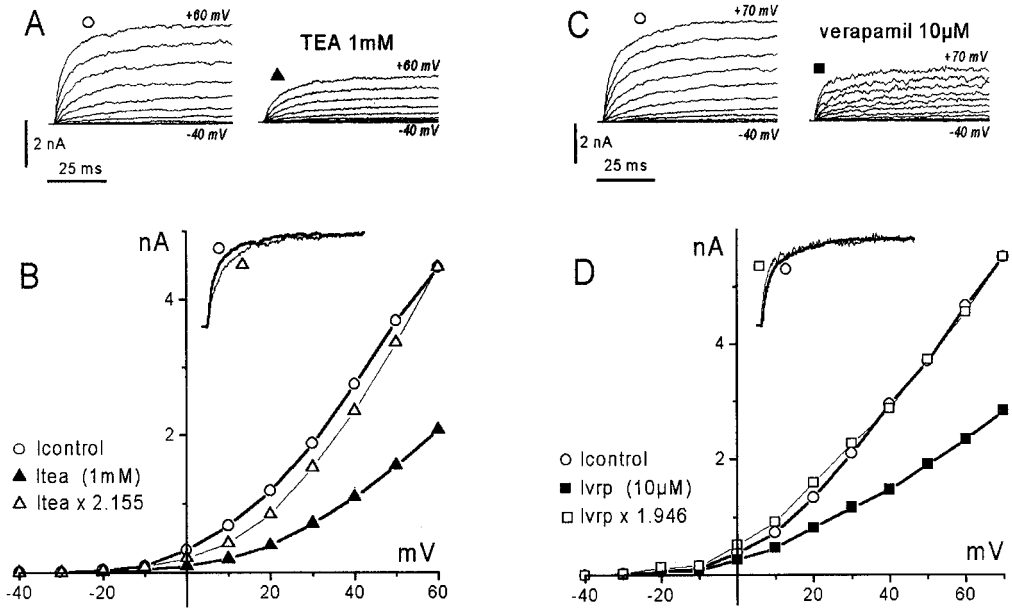


Fig. 7. Steady-state I-V characteristics of TEA- and verapamil-inhibited I_K. A, family of control I_K (left, ○) and 1 mM TEA-inhibited currents (right, ▲) recorded in the same cell, evoked by 100 ms depolarizing pulses (at 10 s intervals) from $V_h = -40$ to various, gradually incremented (+10 mV steps) membrane potentials. B, steady-state I-V curves for currents presented in A: control I_K ($I_{control}$, ○) and 1 mM TEA-inhibited currents (I_{tea}, ▲). Thin line ($I_{tea} \times 2.155$, △) represents the I_{tea} curve multiplied by 2.155, a normalizing coefficient for TEA-inhibited current recorded at a membrane potential of +60 mV. Inset: superimposed traces of control (bold) and normalized TEA-inhibited (thin) currents (from A) recorded at a membrane potential of +60 mV. C, family of control I_K (left, ○) and 10 μM verapamil-inhibited (right, ■) currents recorded in the same cell (not the cell in A) using a similar experimental protocol to that used in A. D, steady-state I-V curves for currents presented in C: control I_K ($I_{control}$, ○); (10 μM verapamil)-inhibited currents (I_{vrp}, ■). The thin continuous line ($I_{vrp} \times 1.946$, □) represents the I_{vrp} curve multiplied by 1.946, a normalizing coefficient for verapamil-inhibited current recorded at a membrane potential of +70 mV. Inset: superimposed traces of control (bold) and normalized verapamil-inhibited (thin) currents (from C) recorded at a membrane potential of +70 mV.

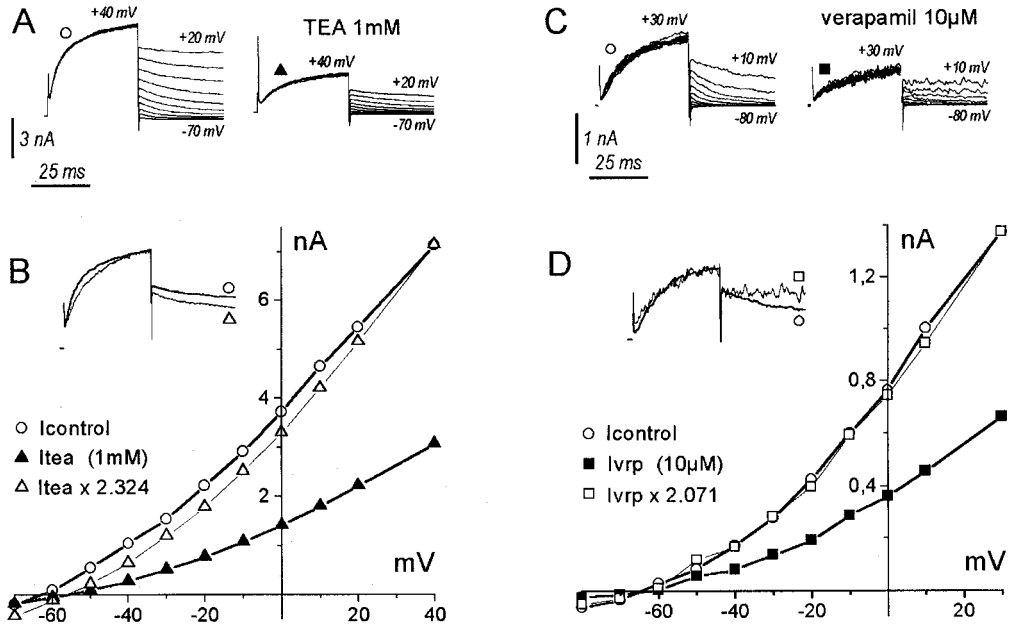


Fig. 8. Instantaneous I-V characteristics of the TEA- and verapamil-inhibited K^+ currents. A, family of control I_K (○) and 1 mM TEA-inhibited currents (▲) recorded in the same cell, evoked by 40 ms depolarizing conditioning pulses from $V_h = -40$ to +40 mV, followed by 80 ms repolarizing test pulses to various membrane potentials. (From +20 mV to −70 mV, decrement −10 mV). B, instantaneous I-V curves plotted by the amplitudes of tail currents (from A), measured during the initial stage of the repolarizing pulse in: control I_K ($I_{control}$, ○) and 1 mM TEA-inhibited currents (I_{tea}, ▲). The thin continuous line ($I_{tea} \times 2.324$, △) represents the I_{tea} curve multiplied by 2.324, a normalizing coefficient for TEA-inhibited current recorded at the end of the depolarizing pulse (+40 mV). Inset: superimposed traces of control (bold) and normalized (thin) TEA-inhibited currents (from A), corresponding to a repolarizing level of +10 mV. C, Family of control (○) and 10 μM verapamil-inhibited (■) currents recorded in the same cell (not the cell in part A) with a similar experimental protocol to that used in A. (Depolarizing pulse to +30 mV; repolarizing pulses varied from +10 mV to −80 mV). D, Instantaneous I-V curves plotted by tail current amplitudes (from C) in: control I_K ($I_{control}$, ○) and 10 μM verapamil-inhibited currents (I_{vrp}, ■). Thin continuous line ($I_{vrp} \times 2.071$, □) represents the I_{vrp} curve multiplied by 2.071, a normalizing coefficient for verapamil-inhibited current recorded at the end of the depolarizing pulse (+30 mV). Inset: superimposed traces of control (bold) and normalized (thin) verapamil-inhibited currents (from C), corresponding to a repolarizing level of +10 mV.

LNCaP cell proliferation was measured in the presence of 25 μM verapamil and 1 mM TEA separately, and with both inhibitors applied together (25 μM verapamil + 1 mM TEA). In our experiments, both verapamil and TEA markedly inhibited LNCaP cell proliferation after 3 days of culture. The average resting cell proliferation activity in the presence of 25 μM verapamil (0.373 of control, Fig. 9) closely matched the resting I_K in at the same concentration (see Fig. 4). This observation suggested that verapamil's antiproliferative action mechanism resulted directly from its blocking effect on K^+ channels. However, 1 mM TEA inhibited cell proliferation much more markedly (0.109 of control, Fig. 9) than would be expected from ~50% level of inhibition of K^+ currents by the same concentration (Fig. 5, 7A, and 8A). The cumulative inhibition produced by 25 μM verapamil + 1 mM TEA resulted in a resting cell proliferation level of 0.092 (Fig. 9), more than twice (~225%) the value predicted from the product of the individual effects of these drugs (0.373 × 0.109 ≈0.041), and only 16% less than the effect produced by TEA alone (0.109). This result implied that the antiproliferative actions of verapamil and TEA were highly "competitive" and that both effects shared the same intrinsic cell mechanism. Using the values obtained for resting levels of proliferation activity in the presence of verapamil and TEA, we calculated (for evaluation purposes) the interaction index γ (see *Appendix*) for the antiproliferative effects of these two drugs. The calculated values of γ ~900 (compared with ≈1.7 for the two drugs' independent K^+ channel blocking mechanisms, see above) corresponded to mainly competitive mechanisms. In accordance with this result, the simultaneous application of 50 μM verapamil and 1 mM TEA did not significantly modify the level of resting LNCaP cell proliferation activity measured in the presence of 1 mM TEA alone (Fig. 9). Our results strongly suggested that verapamil's LNCaP cell proliferation inhibition mechanism is shared with that of TEA.

Alteration in cell-growth kinetics by TEA and verapamil was not due to cytotoxicity, because the percentage of cells extruding Trypan blue was not affected by over 72 h incubation with the blockers in the range of concentrations used.

Because tumor-cell populations may simultaneously undergo proliferation and programmed (apoptotic) death, we investigated whether K^+ channel blockers induced apoptosis in LNCaP cells. Hoechst staining was used to determine apoptosis induced by TEA (1 mM) and verapamil (50 μM). The percentage of apoptotic cells was identical (<1%) in control populations and those treated with drugs for over 72 h (data not shown).

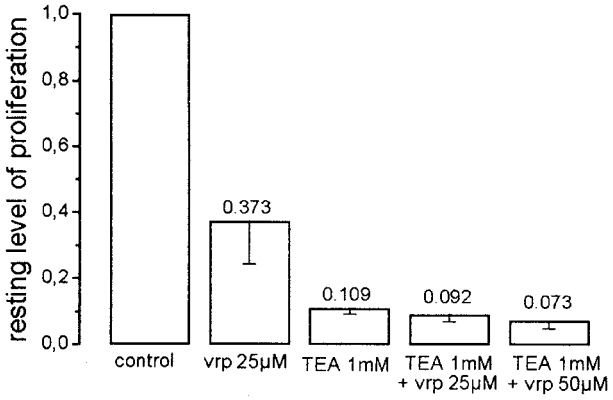


Fig. 9. Inhibition of LNCaP cell proliferation by verapamil (vrp) and TEA. Cell proliferation rate was measured under various experimental conditions (marked below the columns) when verapamil and TEA were applied alone and together, then cultured for 3 days (see *Results*). The average cell proliferation rate values, obtained under each experimental conditions from four trials, were normalized by the value obtained under control conditions and presented as columns (±S.E.) with numerical values marked above them.

Discussion

It is known that various types of K^+ channels play a key role in cancer cell proliferation. A high expression of EAG channels inhibited by physiological $[Ca^{2+}]_i$ has been demonstrated in several human melanoma (Meyer et al., 1999) and somatic cancer cell lines (Pardo et al., 1999). In LNCaP cells, the K^+ channels controlling proliferation are also $[Ca^{2+}]_i$-inhibited (Skryma et al., 1999). We therefore verified whether the K^+ channels in LNCaP cells could demonstrate the functional properties of human EAG (HERG) channels. The result was negative (Fig. 1). Furthermore, no indication of the C-type inactivation, characteristic of other types of verapamil-sensitive K^+ channels, has been observed in LNCaP cell I_K. Verapamil inhibited I_K in LNCaP cells with an IC_{50} value of 11 μM. This value is close to those previously reported for transfected Kv1.3 (Rauer and Grissmer, 1996) and Kv1.1 (Waldegger et al., 1999) channels, as well as for delayed K^+ channels in rat intracardiac neurons (Hogg et al., 1999).

To identify the blocking location and K^+ channel inhibition molecular mechanism of verapamil in LNCaP cells, we performed a comparative analysis of the pharmacological action of verapamil and TEA, a well known K^+ channel pore blocker. The characteristics of verapamil inhibition of K^+ channels differed from those described in other cell types. First, verapamil reduced I_K without changing its macroscopic kinetics. Verapamil did not produce the expected apparent "inactivation" of I_K in LNCaP cells analogous to the previously reported acceleration of I_K inactivation in other cell models (DeCoursey, 1995; Rauer and Grissmer, 1996; Trequattrini et al., 1998; Waldegger et al., 1999; Zhang et al., 1999), considered as a time-dependent interaction of verapamil with open K^+ channels. Second, the verapamil-produced inhibition of K^+ channels was not use-dependent: the initial I_K evoked after ≈30 s preincubation with verapamil solution was inhibited to its steady-state level, indicating that verapamil interacted with the resting form of the channel. Verapamil and other phenylalkylamines were previously considered to be mainly state-dependent open-channel blockers (DeCoursey, 1995; Trequattrini et al., 1998; Rauer and Grissmer, 1999) and K^+ channel-inactivated state blockers (Tatsuta et al., 1994; Hanson et al., 1999; Zhang et al., 1999). Third, K^+ channel inhibition by externally applied verapamil in LNCaP cells was rapid and rapidly reversible (≈30 s), suggesting an easy association/dissociation with its binding site. The rapid on- and off-rate of verapamil action was new relative to inhibition mechanisms for inactivating K^+ channels described previously, which were blocked by verapamil diffused through the membrane and bound to the inner residue on the channel (DeCoursey, 1995; Rauer and Grissmer, 1996, 1999; Catacuzzeno et al., 1999; Zhang et al., 1999). This difference could be explained if the verapamil binding site were located on the extracellular K^+ channel residue in con-

tact with the outer solution. However, the absence of a blocking effect when membrane-impermeable *N*-methyl-verapamil was applied extracellularly challenged this hypothesis. This failure of *N*-methyl-verapamil to produce any blocking effect was in line with several other recent reports on the weak or nonexistent blocking ability of extracellularly applied charged verapamil analogs on K^+ channels in cells where the neutral form of extracellularly applied verapamil produced an effective K^+ channel block (DeCoursey, 1995; Rauer and Grissmer, 1996, 1999; Catacuzzeno et al., 1999; Zhang et al., 1999). This could mean that, in LNCaP cells, as in other cell types, verapamil diffuses into or through the membrane to inhibit K^+ channels. In inside-out patches, we detected an intracellular phenylalkylamine binding site (Fig. 3B), as previously found in other types of K^+ channels (see introduction). Similarly, the failure of extracellular and intracellular applications of phenylalkylamine quaternary derivatives to block these channels was previously reported for L-type Ca^{2+} channels in rat ventricular myocytes (Wegener and Nawrath, 1995), in which a phenylalkylamine binding site on the outer surface has been suggested. We checked if verapamil could bind to the outer channel pore compartment. In this case, the competition between simultaneously applied verapamil and TEA, could be expected. Our experiments revealed a very low (≈7%) level of competition between verapamil and TEA when they inhibited I_K simultaneously. On the contrary, the antagonist effects of both drugs were multiplicative, suggesting that they have different K^+ channel inhibition mechanisms in LNCaP cells.

Indeed, open channel unit conductance was significantly reduced by TEA without noticeably influencing open-channel probability P_o (Fig. 6, A, C, and D). In keeping with our earlier report (Skryma et al., 1999) and similar effects obtained in cardiocytes (Benz and Kohlhardt, 1994) and dorsal root ganglion neurons (Safronov et al., 1996), the reduction of single-channel unitary conductance indicated that TEA molecules represent a significant electrostatic and/or mechanical barrier for K^+ ions passing through the channel, and that TEA blocks a K^+ channel pore in LNCaP cells. On the contrary, verapamil did not affect single-channel conductance, but markedly reduced the P_o of K^+ channels (Fig. 6, B and C). This reduction in P_o due to verapamil has also been reported for outwardly rectifying K^+ channels in aortic (Pavenstadt et al., 1991) and cardiac (Benz and Kohlhardt, 1994) cells.

The K^+ channel functional compartment affected by verapamil (pore vs. voltage sensor) and the type of forces exerted (electrostatic vs. chemical) were not clear. To address these questions, we compared the effects produced by TEA and verapamil on the steady-state and instantaneous I-V characteristics of I_K. Normalized steady-state characteristics of TEA-inhibited currents were rightward shifted relative to control I-V over the entire range of membrane potentials (Fig. 7B). This could result from: 1) an electrostatic barrier for outwardly passing K^+ ions, produced by TEA; or 2) a local electrostatic field induced by TEA in the voltage sensor compartment. The first mechanism was undoubtedly confirmed by the rightward shift of the normalized instantaneous I-V characteristics of TEA-inhibited I_K (Fig. 8B). The contribution of the second mechanism did not seem to be significant, because there were no marked changes in P_o and minimal influence (slow down) on the activation kinetics of I_K produced by TEA.

The normalized steady-state I-V characteristics of verapamil-inhibited I_K overlapped with control I-V curves at high membrane potentials, with a slight leftward shift at potentials within the range of reactivity of K^+ channel voltage sensors (from about the threshold to open state saturation, −20 to +30 mV), although the voltage threshold of activation of verapamil-inhibited I_K was apparently the same as in control currents (Fig. 7D). This "facilitation" of K^+ channel opening gates without influencing the reactivity of the voltage sensor that unlocks these channels may reasonably be explained by the fact that verapamil loosens a basic functional link between those supposed channel structures. Another observation supporting this hypothesis was the fact that verapamil eliminated current relaxation during the repolarizing phase at high membrane potentials (inset in Fig. 8D). A similar effect of verapamil on the relaxation phase of potassium currents mediated by transfected HERG K^+ channels was observed by Zhang et al. (1999). If this really occurs, the functional disconnection between voltage sensor and channel gates of K^+ channels could result in a drastic reduction in P_o in the presence of verapamil (Fig. 6, B and C). The influence of verapamil on the ion pore of open K^+ channels seems to be minimal, as indicated by the close match between control and normalized verapamil-inhibited instantaneous I-V curves (Fig. 8D) and the unchanged unitary conductance of channels influenced by verapamil (Fig. 6B).

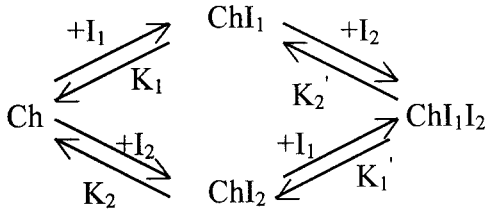
It has been suggested that TEA (Nilius and Wohlrab, 1992; Wang et al., 1992; Pancrazio et al., 1993; Lepple-Wienhues et al., 1996; Skryma et al., 1997) and verapamil (Batra et al., 1991; Yao and Kwan, 1999) inhibit the cancer cell proliferation by suppressing their K^+ fluxes. In this work, we show that verapamil in micromolar concentrations inhibits LNCaP cell proliferation as well. We obtained a good correlation with verapamil concentrations producing an equivalent extent of K^+ channel blocking and inhibition of LNCaP cell proliferation (Figs. 4 and 9). This result, analogous to those previously observed in other cell types (Amigorena et al., 1990; Batra et al., 1991; Pappone and Ortiz-Miranda, 1993; Yao and Kwan, 1999), was a good indication, but not sufficient proof, of a causal link between K^+ channel blocking and inhibition of LNCaP proliferation. More convincing arguments were obtained from TEA-verapamil competition experiments (Fig. 9). Deep inhibition (by ≈90%) of LNCaP cell proliferation with TEA was not reinforced by additional application of verapamil at the concentrations used, although it had reduced the LNCaP cell proliferation rate by more than 60% in control experiments. This result strongly argued that verapamil and TEA had a common antiproliferative mechanism. Given that both drugs blocked K^+ channels effectively, we may conclude that the main antiproliferative action of verapamil in LNCaP cells is initiated by its inhibition of K^+ channels.

In conclusion, our results suggest that, in LNCaP cells, verapamil binds to the intracellular residue of K^+ channels as well as, probably, to an additional intramembrane hydrophobic site. It loosens a functional link between voltage sensor and activation gate structures, reducing the effectiveness of channel triggering. The resulting reduction in K^+ ion efflux leads to the disruption of the chain of biochemical processes required for LNCaP cell proliferation. If the new intramembrane verapamil-binding site indicated by the results of our work is specific to K^+ channels in prostate cells,

it could be a target for new synthetic pharmacological agents directed against prostate cancer cell proliferation.

Appendix

Double Inhibition Scheme. Quantitative analysis of ion channel (Ch) inhibition in the presence of two inhibitors (I_1 and I_2) was performed in a similar way to the general approach for enzyme double inhibition systems (Keleti and Fajszi, 1971).



The scheme represents reversible binding/dissociation steps involving I_1 and I_2 and Ch, leading to the formation of blocked channel-inhibitor complexes ChI_1, ChI_2, and ChI_1I_2. (The latter complex is formed if I_1 and I_2 bind to different binding sites on the Ch protein.) K_1, K_2, K_1', and K_2' are dissociation constants of the appropriate reaction steps. At the state of equilibrium, the next elementary molecular reactions and corresponding molecular balance equations may be considered:

$$Ch + I_1 \rightleftarrows ChI_1 \qquad K_1 = [Ch][I_1]/[ChI_1] \tag{1}$$

$$Ch + I_2 \rightleftarrows ChI_2 \qquad K_2 = [Ch][I_2]/[ChI_2] \tag{2}$$

$$ChI_1 + I_2 \rightleftarrows ChI_1I_2 \qquad K_2' = [ChI_1][I_2]/[ChI_1I_2] \tag{3}$$

$$ChI_2 + I_1 \rightleftarrows ChI_1I_2 \qquad K_1' = [ChI_2][I_1]/[ChI_1I_2]. \tag{4}$$

From eqs. 1 to 4, it follows that

$$K_1K_2' = K_2K_1'. \tag{5}$$

There may be three general cases of interactions between the two inhibitors and channel protein: 1) both inhibitors compete although they bind with a single common binding site (i.e., complex ChI_1I_2 does not exist); 2) two inhibitors bind to different binding sites without influencing the binding (or inhibitory effects) of each other; 3) two inhibitors bind to different binding sites and the binding of one inhibitor has an allosteric influence on the binding (or blocking) action of the other inhibitor. To distinguish between these three cases, the interaction index (γ) is introduced as $K_1' = \gamma K_1$. One can see from eq. 5 that symmetrical expression of $K_2' = \gamma K_2$ is automatically valid. In physical terms, γ equals 1 in the case of purely independent binding/(blocking) mechanisms of I_1 and I_2 [i.e., case (ii)]. In this case $K_1 = K_1$ and $K_2' = K_2$. In the opposite case of pure competition (i), $\gamma \to \infty$ because, according to eqs. 3 and 4, $[ChI_1I_2] = 0$ and K_1', $K_2' \to \infty$. Intermediate γ values varying between 1 and ∞ would reflect various levels of negative (or, if $0 < \gamma < 1$, then positive) cooperation between inhibitors.

The experimentally measured normalized resting ion current (i/I_{max}) in the presence of two inhibitors would be proportional to the fraction of nonbound channels ([Ch]):

$$i/I_{max} = [Ch]/[Ch]_{total} = [Ch]/([Ch] + [ChI_1] + [ChI_2] + [ChI_1I_2]). \tag{6}$$

Substituting expressions from eqs. 1 to 4 containing only [Ch] concentrations for $[ChI_1]$, $[ChI_2]$, and $[ChI_1I_2]$ in eq. 6 gives, after cancellation:

$$i/I_{max} = 1/(1 + [I_1]/K_1 + [I_2]/K_2 + [I_1][I_2]/\gamma K_1K_2). \tag{7}$$

If only one inhibitor is used (i.e., $[I_2] = 0$), eq. 7 is transformed into the conventional form:

$$i/I_{max} = 1/(1 + [I]/K_I). \tag{8}$$

Equations 7 and 8 may be used to obtain the value of γ in competition experiments and assess the type of interaction between two inhibitors. Initially, the i/I_{max} values are measured separately at fixed concentrations of each inhibitor ($[I_1]'$ and $[I_2]'$) and values of $[I_1]'/K_1$ and $[I_2]'/K_2$ for these concentrations are calculated from eq. 8. Then, both inhibitors are applied together at previously used concentrations, and the i/I_{max} value is measured in the presence of both inhibitors. The numerical value of γ is obtained from eq. 7 by substituting the measured value for i/I_{max} and the calculated values for $[I_i]/K_i$.

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