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Trifluoperazine enhancement of Ca^{2+}-dependent inactivation of L-type Ca^{2+} currents in *Helix aspersa* neurons

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ABSTRACT The effects of trifluoperazine hydrochloride (TFP), a calmodulin antagonist, on L-type Ca^{2+} currents (L-type ICa^{2+}) and their Ca^{2+}-dependent inactivation, were studied in identified *Helix aspersa* neurons, using two microelectrode voltage clamp. Changes in $[Ca^{2+}]_i$ were measured in unclamped fura-2 loaded neurons. Bath applied TFP produced a reversible and dose-dependent reduction in amplitude of L-type ICa^{2+} (IC_{50} = 28 μM). Using a double-pulse protocol, we found that TFP enhances the efficacy of Ca^{2+}-dependent inactivation of L-type ICa^{2+}. Trifluoperazine sulfoxide (50 μM), a TFP derivative with low calmodulin-antagonist activity, did not have any effects on either amplitude or inactivation of L-type ICa^{2+}. TFP (20 μM) increased basal $[Ca^{2+}]_i$ from 147 ± 37 nM to 650 ± 40 nM (N=7). The increase in $[Ca^{2+}]_i$ was prevented by removal of external Ca^{2+} and curtailed by depletion of caffeine-sensitive intracellular Ca^{2+} stores. Since TFP may also block protein kinase C (PKC), we tested the effect of a PKC activator (12-O-tetradecanoyl-phorbol-13-acetate) on L-type Ca^{2+} currents. This compound produced an increase in L-type ICa^{2+} without enhancing Ca^{2+}-dependent inactivation. The results show that 1) TFP reduces L-type ICa^{2+} while enhancing the efficacy of Ca^{2+}-dependent inactivation. 2) TFP produces an increase in basal $[Ca^{2+}]_i$ which may contribute to the enhancement of Ca^{2+}-dependent inactivation. 3) PKC up-regulates L-type ICa^{2+} without altering the efficacy of Ca^{2+} dependent inactivation. 4) The TFP effects cannot be attributed to its action as PKC blocker.

KEY WORDS: Calcium channel inactivation; calmodulin; protein kinase C; trifluoperazine; phenothiazines; antipsychotics

Introduction

L-type Ca^{2+} channels are a major pathway for Ca^{2+} entry in excitable cells (Reuter, 1996). Intracellular Ca^{2+} acts mainly as a second messenger triggering or modulating a variety of cell functions (Carafoli, 1994). Key amongst these functions is the ability of internal Ca^{2+} to inactivate its own entry into the cell via L- and N-type Ca^{2+} channels (Eckert and Chad, 1984), thereby triggering a negative feedback mechanism for Ca^{2+} influx that prevents cellular Ca^{2+} overload.

Despite its importance, the nature of the mechanism underlying Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels remains controversial. This is in spite of the fact that the molecular structures involved in this inactivation process have already started to be identified (Soldatov *et al.*, 1997). Two major hypotheses have been proposed to explain Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels: 1) direct binding of Ca^{2+} to one or more internal sites of the channel complex (Eckert and Chad, 1984; Imredy and Yue, 1994; Standen and Stanfield, 1982); and 2) dephosphorylation of the Ca^{2+} channel via activation of a Ca^{2+}/calmodulin-dependent phosphatase (Armstrong, 1989; Chad and Eckert, 1986).

Published evidence for or against the dephosphorylation model is controversial. For instance, the

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calmodulin (CaM) antagonist calmidazolium, does not alter Ca^{2+}-dependent inactivation of Ca^{2+} channels in dialyzed myocytes (Imredy and Yue, 1994). Other studies in internally perfused cells in which CaM-antagonists have been externally applied showed a fast decrease (Clapham and Neher, 1984; Doroshenko *et al.*, 1988; Klöckner and Isenberg, 1987) or an increase in peak L-type Ca^{2+} currents (Doroshenko *et al.*, 1988; Mironov and Lux, 1991). A possible explanation for the controversial data is that all of the above studies have been performed on internally perfused cells. This could lead to wash-out of cytoplasmic factors that might modulate Ca^{2+}-dependent inactivation processes. Furthermore, CaM activation requires an increase in $[Ca^{2+}]_i$, and internal Ca^{2+} was chelated in internally perfused cells to prevent run-down of Ca^{2+} currents. In fact, it has been observed that run-down and Ca^{2+}-dependent inactivation of L-type Ca^{2+} currents are tightly correlated processes (Chad and Eckert, 1986; Kostyuk and Lukyanetz, 1993).

In the present work, we assessed the effect of the CaM-antagonist trifluoperazine (TFP) and PKC activators on L-type Ca^{2+} channel inactivation in non-perfused neurons using a two microelectrode voltage clamp technique. We found that although TFP reduces L-type Ca^{2+} currents, it enhances the efficacy of Ca^{2+}-dependent inactivation. In fura-2 loaded cells, TFP significantly increased the basal $[Ca^{2+}]_i$. The latter may contribute to the enhancement of Ca^{2+}-dependent inactivation of L-type Ca^{2+} currents. The PKC activator 12-O-tetradecanoyl-phorbol-13-acetate (TPA), but not the inactive ester 4-α-phorbol, increased the L-type ICa^{2+} without altering the efficacy of the Ca^{2+}-dependent inactivation, suggesting that the effects of TFP on inactivation may not be mediated through its action as a PKC antagonist.

Materials and methods

Preparation

Experiments were done in neurons 1D, 2D, 77F and 78F from the subesophageal ganglia of *Helix aspersa*. The procedure for isolating the ganglia and removing the overlying connective tissue has been described (Alvarez-Leefmans *et al.*, 1992).

Pharmacological isolation of the inward Ca^{2+} current

The inward ICa^{2+} in the cell bodies of *Helix* neurons flows mainly through L-type Ca^{2+} channels (Chad and Eckert, 1986; Nishi *et al.*, 1983). The Ca^{2+} inward current was isolated from the Na^+ inward current by replacing the external Na^+ with tetraethylammonium chloride (see below). To suppress outward K^+ currents, intracellular K^+ was replaced with Cs^+ by bathing the neurons for 10 min with a Cs^+/nystatin-containing solution (Russell *et al.*, 1977). During the recording period, the neurons were continuously superfused (3 ml/min; chamber volume 700 µl) with a solution containing (in mM): 70 tetraethylammonium chloride, 5 4-aminopyridine, 5 CsCl, 20 $CaCl_2$, 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 5 dextrose. The pH was adjusted to 7.5 with CsOH. This solution was designed to completely eliminate any remnant outward K^+ and inward Na^+ currents, leaving only Ca^{2+} inward currents in isolation, as tested by their sensitivity to Cd^{2+}. *Helix* neurons are also endowed with a voltage-dependent outward H^+ current (Thomas and Meech, 1982) which, in the absence of external K^+ channel blockers, could modify the kinetics of the ICa^{2+} relaxation (Byerly and Suen, 1989). Under our experimental conditions, this H^+ current cannot contaminate the relaxation of the Ca^{2+} currents since external TEA and 4-AP, at the concentrations used in our recording solution, are effective blockers of the outward H^+ current (Byerly *et al.*, 1984).

Recording of inward Ca^{2+} currents

Ca^{2+} inward currents were recorded with a standard two-microelectrode voltage clamp circuit (Dagan 8500, Dagan Corp., Minneapolis, MN). Command pulses (100 ms, 0.025 Hz) were applied in steps of 10 mV from a holding potential of -50 mV. Transmembrane current records were digitized to a resolution of 12 bits through a LabMaster interface (Lab Master, Scientific Solutions Inc., Solon, OH) that also generated the command pulses. Data were analyzed with customized software and SigmaPlot (Jandel Scientific, San Rafael, CA). Linear leakage currents and capacitative transients were subtracted following the P-P/4 protocol. Measured series resistance was estimated to be about 20 KΩ. Since the largest recorded currents (100 nA) produced an offset of 2 mV, series resistance was not compensated.

$[Ca^{2+}]_i$ measurement

In a separate set of experiments $[Ca^{2+}]_i$ was measured in neurons loaded by pressure injection with the fluorescent dye fura-2, as described previously (Alvarez-Leefmans *et al.*, 1994). For these measurements the ganglia were attached to a partitioned Leyden chamber (200 µl) placed on the stage of an inverted, epi-fluorescence microscope (Diaphot-TMD, Nikon, Tokyo, Japan). The microscope was attached to a microspectrophotometry system (Deltascan, Photon Technology International, Brunswick, NJ). Fura-2 was excited at 340 and 380 nm and emission was recorded at 510 nm. Assessment of background fluorescence

and calibration of the dye were as described by Negulescu and Machen (1990).

To rule out possible optical artifacts induced by TFP on fura-2 measurements, we tested the effect of TFP (10 and 100 μM) on fura-2 spectral properties. In addition we determined the excitation and emission spectra of 10 mM TFP solutions without fura-2 in 0.1M KCl, 10% DMSO and 5 mM HEPES. Peak excitation and emission of TFP was at 390 nm and 588 nm respectively. At concentrations used in the biological experiments (10 to 100 μM), TFP had no measurable effect on either fura-2 excitation or emission spectra, or on the K_d for Ca^{2+}, determined with Ca^{2+}-calibration buffers, in the range between 0 and 39.8 μM [Ca^{2+}], prepared as described by Tsien and Pozzan (1989). The fluorescence emission signal of TFP (100 μM) was less than 1% the signals for fura-2 even at the lowest [Ca^{2+}]. These tests ensured that the recorded fura-2 signals were a result of changes in intracellular Ca^{2+} and not of interference from TFP on the fluorescence signals.

In some fura-2 experiments, a nominally Ca^{2+}-free external Ringer solution was used, having the following composition (in mM): 72.5 NaCl, 4 KCl, 7 $MgCl_2$, 5 HEPES, 5 EGTA, 5 dextrose. The pH was adjusted to 7.5 with NaOH.

All measurements are expressed as mean ± SE. The Student's t test was used to establish the significance of differences between means at the 95% confidence interval.

Chemicals and drugs

Stock solutions of drugs were prepared using dimethylsulfoxide (DMSO) as a solvent. The final concentration of DMSO in the recording solution never exceeded 0.05%, a concentration that by itself did not affect the Ca^{2+} currents. Trifluoperazine-sulfoxide (TFP-S) was kindly provided by Smith Kline & French Laboratories, Philadelphia, PA. Trifluoperazine hydrochloride (TFP), 12-O-tetradecanoyl-phorbol-13-acetate (TPA), 4-α-phorbol, Nystatin, DMSO and all salts for preparing solutions came from Sigma Chemicals Co. (St. Louis, MO). Fura-2 free acid came from Molecular Probes (Eugene, OR).

Results

Effects of trifluoperazine on peak Ca^{2+} currents

Helix neurons are endowed with an L-type Ca^{2+} current sensitive to dihydropyridines and Cd^{2+} (Chad and Eckert, 1986; Gutnick *et al.*, 1989; Nishi *et al.*, 1983). Trifluoperazine (TFP) produced a reversible decrease in peak amplitude of this L-type ICa^{2+} at all membrane potentials tested (-40 to +50 mV). This effect was not accompanied by shifts in the current/voltage relation along the voltage axis or by changes in the transmembrane voltage of activation of the ICa^{2+}. An example is shown in Fig. 1.

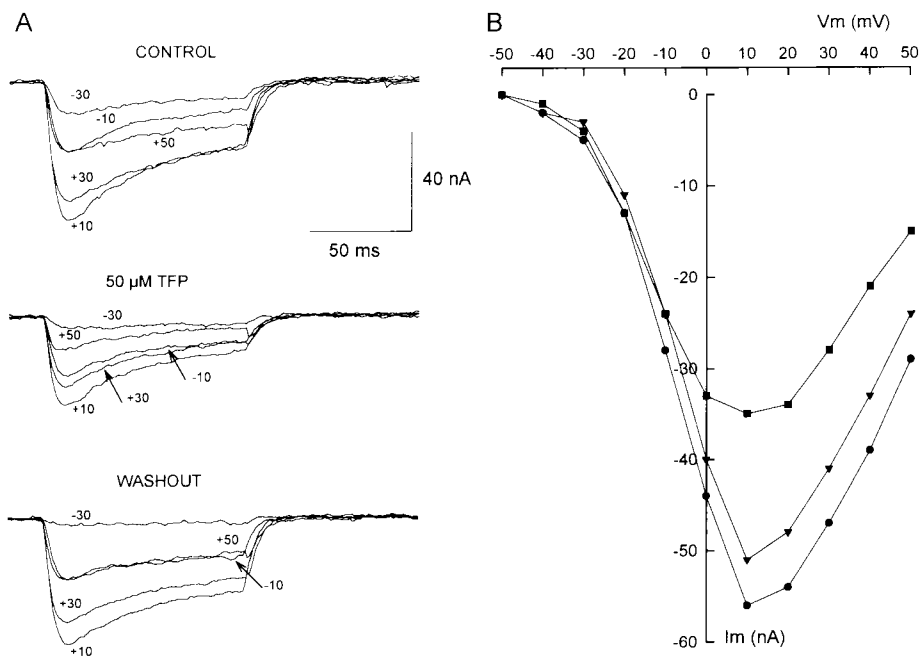


Fig. 1. Effect of TFP on L-type Ca^{2+} currents recorded from a neuron 77F. A: *transmembrane current records obtained before (CONTROL), 25 min after onset of continuous superfusion of 50 μM TFP, and 35 min after TFP washout. Numbers on each current record indicates the value of the transmembrane voltage at which Ca^{2+} currents were elicited from a holding voltage of -50 mV.* **B:** *current/voltage relations obtained from data of the experiment partly shown in A: Control (circles), 50 μM of TFP (squares) and TFP washout (triangles). Measurement of the peak inward currents at +10 mV voltage steps indicates that in this experiment TFP reduced the peak inward Ca^{2+} current by 37.5% and recovered to 91%, 30 min after washout.*

The effect of TFP was dose-dependent with an IC_{50} of 28 μM. This value was obtained from measurements of peak amplitudes of L-type Ca^{2+} inward currents elicited by depolarizing steps to +10 mV, for various concentrations of TFP in the range between 5 to 100 μM. TFP did not produce a complete block of the current even at concentrations of 100 μM. At this concentration the current was maximally reduced by 60 ± 6 % with respect to control (n=5).

Most experiments were done using a concentration of 50 μM TFP. In 7 neurons, this concentration produced a maximal reduction of 37 ± 2 % in the peak L-type ICa^{2+}. The maximal effect was reached 27 ± 5 min after onset of TFP superfusion. The peak ICa^{2+} amplitude recovered to 87 ± 13% of its control value, 30 ± 7 min after TFP washout.

To assess the pharmacological specificity of the TFP effects on L-type ICa^{2+}, we tested the action of a sulfoxide derivative of TFP with low affinity for CaM (Levin and Weiss, 1979) and poor CaM-antagonist action (Crimaldi *et al.*, 1980; Levin and Weiss, 1980; Weiss *et al.*, 1980). Several reports indicate that the sulfoxide derivative of TFP, TFP-S, is unable to block various CaM-dependent cellular functions at doses equal to or several fold higher than the parent compound (Marone *et al.*, 1986; Pierce *et al.*, 1989; Ruben *et al.*, 1983; Veldhuis *et al.*, 1984). We found that L-type ICa^{2+} was not affected by exposing the neurons to 50 μM TFP-S (n=3), as shown in Fig. 2.

Effect of trifluoperazine on Ca^{2+}-dependent inactivation

It is known that inactivation of L-type ICa^{2+} entails both voltage- and Ca^{2+}-dependent mechanisms (Gutnick *et al.*, 1989; Lee *et al.*, 1985). To disclose only the Ca^{2+}-dependent component of inactivation, a two sequential command pulse protocol was applied, as described by Eckert and Tillotson (Eckert and Tillotson, 1981). In this protocol, that is illustrated in the inset of Fig. 3, a conditioning pulse (P_1) was varied in amplitude in 10 mV steps from a holding voltage of -50 mV, producing a series of Ca^{2+} currents (I_1). A test pulse (P_2), having a fixed amplitude (+10 mV), was delivered 200 ms after the end of P_1. Both pulses had a duration of 100 ms. By increasing the amplitude of P_1 in stepwise manner, the total amount of electric charge carried by Ca^{2+} (Q_1), that is entering the cell with respect to time (i.e. the time integral of I_1), can be systematically controlled and its effect on the peak amplitude of I_2 can be determined quantitatively. The decrease of I_2 in this protocol reflects Ca^{2+}-dependent inactivation. The latter was assessed from plots of normalized I_2 amplitude against Q_1. Normalized I_2 was calculated as the ratio of the peak amplitude of I_2 generated after a given P_1, divided by the peak amplitude of I_2 elicited by P_2 without being preceded by a conditioning P_1. These plots yield a linear relation with negative slope i.e. an inverse relation between I_2 and Q_1 (cf. Eckert and Tillotson, 1981). The relative value

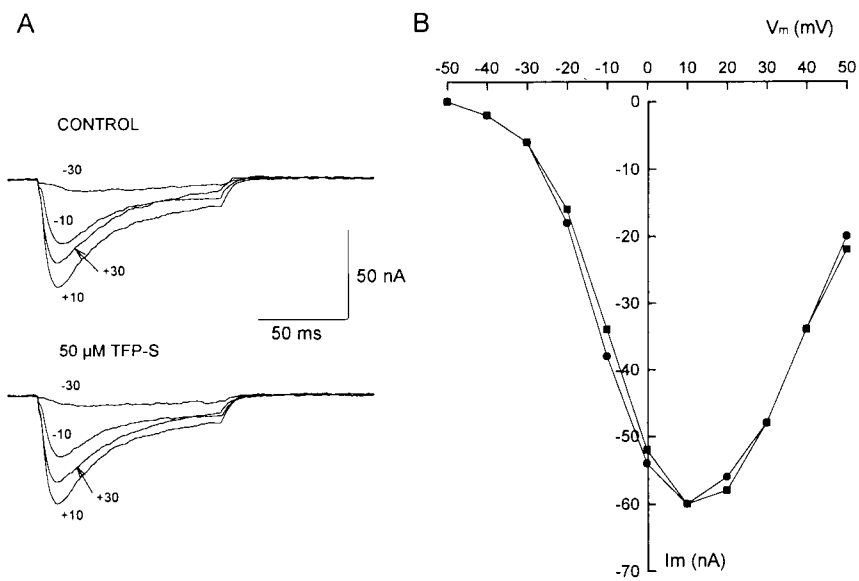


Fig. 2. Lack of effect of trifluoperazine-sulfoxide, TFP-S, on L-type Ca^{2+} inward currents. A: *transmembrane current records obtained before (CONTROL) and 30 min after onset of superfusion with 50 μM TFP-S. Numbers on each current record indicates the value of the transmembrane voltage at which Ca^{2+} currents were elicited from a holding voltage of -50 mV.* **B:** *current/voltage relations plotted from the data of the experiment partly shown in A. Control (circles) and during 50 μM of TFP-S (squares). Cell 1F.*

of the slope reflects the efficacy of Ca^{2+}-dependent inactivation. An example of one of these plots for the control condition is shown in Fig. 3B (circles). In the presence of TFP (50 μM), Q_1 was reduced for all P_1 values tested. However, this smaller Ca^{2+} entry produced a significantly bigger reduction of I_2 than that observed in the control series (squares in Fig. 3B). This enhancement in the efficacy of inactivation was evidenced by the increment in slope of the fitted linear regressions (compare the regression fitted to the circles versus the squares in Fig. 3B). The enhanced Ca^{2+}-dependent inactivation reversed upon TFP washout (bottom traces in Fig. 3A and triangles in Fig. 3B).

In a total of 6 double-pulse protocol experiments like the one illustrated in Fig. 3, linear regressions were fitted to the data points describing the I_2 versus Q_1 relations. In these experiments the slope increased from -0.079 ± 0.012 to -0.263 ± 0.045, 28 ± 6 min after 50 μM TFP and recovered to -0.123 ± 0.042, after 46 ± 10 min of TFP washout.

The enhancement of Ca^{2+}-dependent inactivation produced by TFP was also suggested by the effect that this phenothiazine had on the time course of the ICa^{2+} relaxation (Fig. 4). By scaling up the ICa^{2+} generated during the TFP effect, to match the control current peak amplitude, it was possible to demonstrate that TFP decreased the relaxation time constant i.e. it makes the relaxation faster than control (Fig. 4A and B). This effect was more pronounced at transmembrane voltages more positive than 0 mV (Fig. 4 C). However, it must be kept in mind that this experimental protocol does not allow discrimination between the relative contribution of voltage- versus Ca^{2+}-dependent inactivation to the current relaxation (Eckert and Chad, 1984). Hence, the TFP-induced changes in relaxation kinetics could be attributed to either or both mechanism of Ca^{2+} channel inactivation. Therefore experiments were done in which Ba^{2+} was used as charge carrier through the Ca^{2+} channels. Since Ba^{2+} is not able to inactivate Ca^{2+} channels, the relaxation of the Ba^{2+} currents should reflect purely voltage-dependent inactivation (Zong & Hofmann, 1996). TFP reduced the amplitude of the Ba^{2+} currents but the effect was less reversible than when Ca^{2+} was the charge carrier. Interestingly, TFP (50 μM) decreased the relaxation time constant of the Ba^{2+} current (Fig. 4D). This shows that TFP is able to produce its effects on both amplitude and relaxation of the inward Ca^{2+} currents even if the channels are not permeated by Ca^{2+}. This could be due to an effect of TFP on voltage-dependent inactivation or to a TFP-induced increase in intracellular $[Ca^{2+}]$ producing inactivation of Ca^{2+} channels permeated by Ba^{2+}, or both.

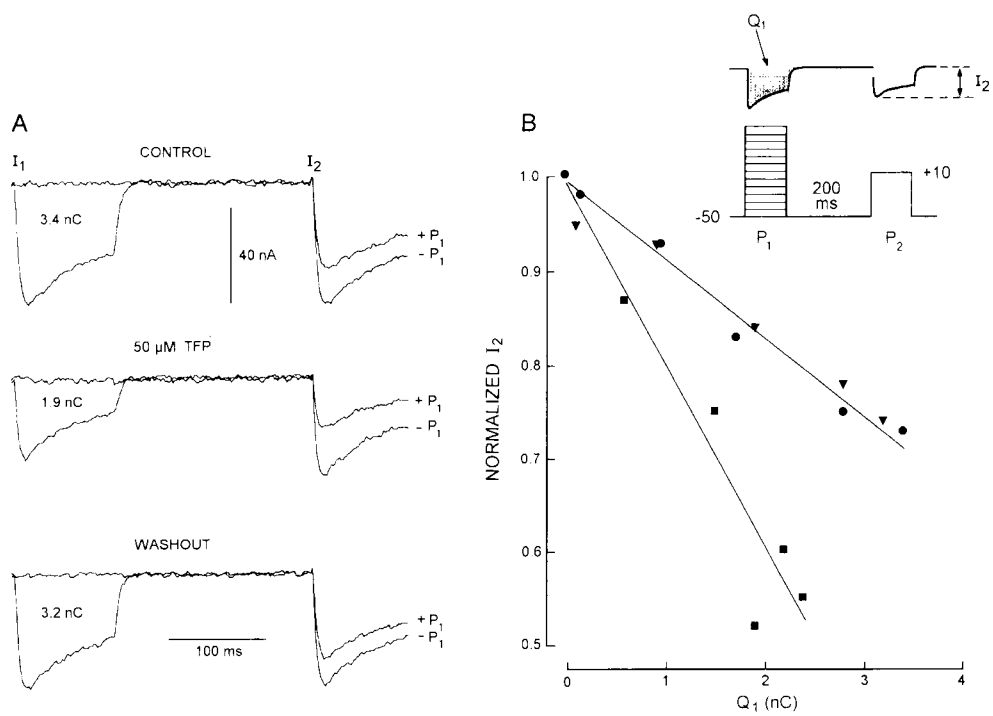


Fig. 3. Effect of TFP on Ca^{2+}-dependent inactivation of L-type Ca^{2+} currents. *Inset in B illustrates the double pulse protocol used. In this protocol a 100 ms duration conditioning pulse (P_1), whose amplitude was varied in 10 mV steps from a holding voltage of -50 mV, was followed 200 ms later by a test pulse (P_2) of a constant amplitude (+10 mV) and duration (100 ms).* **A**: *sample traces of I_1 and I_2 recorded at +10 mV, before (CONTROL), 22 min after 50 μM TFP superfusion (TFP) and 30 min after washout. Note that, when preceded by a P_1 pulse (+P_1), I_2 peak amplitude was reduced compared to the I_2 elicited without a conditioning P_1 pulse (-P_1). The value, in nC, of the total amount of charge carried by Ca^{2+} during I_1 is indicated at the corresponding traces.* **B**: *normalized I_2 peak amplitude plotted as a function of Q_1. Data obtained during control conditions (circles), 22 min after onset of superfusion with 50 μM TFP (squares) and 30 min after TFP washout (triangles). Cell 78F.*

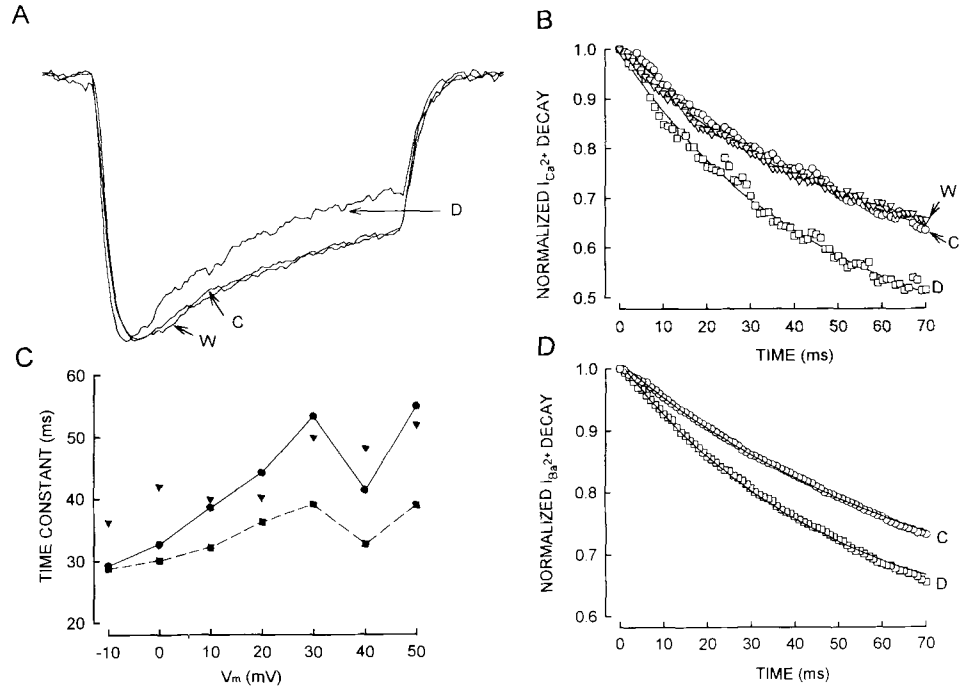


Fig. 4. Effect of TFP on L-type Ca^{2+} and Ba^{2+} current relaxations. **A**: *superposition of ICa^{2+} records elicited before (C), 20 min after 50 μM TFP (D) and 35 min after TFP washout (W). The ICa^{2+} record was digitally scaled up by a factor of 1.5 to match the control (C) peak amplitude.* **B**: *single exponential fits to the ICa^{2+} decays from the records shown in A. The decay time constant decreased from 37.8 ms to 31.3 ms with TFP and returned to 39.8 ms after TFP washout. All relaxations were measured at +10 mV transmembrane voltage steps.* **C**: *plot of relaxation time constants against transmembrane voltage (V_m) for currents recorded in control conditions (circles), with 50 μM TFP (squares) and during TFP washout (triangles).* **D**: *single exponential fits to Ba^{2+} current decays. The decay time constant decreased from 55 ms to 38 ms with TFP. Cell 77F.*

Trifluoperazine increases basal $[Ca^{2+}]_i$

In addition to Ca^{2+} entry through voltage activated Ca^{2+} channels, the basal levels of $[Ca^{2+}]_i$ modulate Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels (Gutnick *et al.*, 1989; Plant *et al.*, 1983). *Helix* neurons, like many other cells, are endowed with an ATP-dependent Ca^{2+} pump in their plasma membrane, which is activated by CaM and contributes to the maintenance of basal $[Ca^{2+}]_i$ (Carafoli, 1994; Kennedy and Thomas, 1995). Since TFP blocks the Ca^{2+} pump by virtue of its binding to CaM (Levin and Weiss, 1980), it is conceivable that the effects of this phenothiazine on Ca^{2+} currents and Ca^{2+}-dependent inactivation are a result of, at least in part, an increase in basal $[Ca^{2+}]_i$ resulting from inhibition of CaM-dependent intracellular Ca^{2+} regulatory mechanisms. Hence we studied the effect of TFP on basal intracellular Ca^{2+} levels.

In fura-2 loaded cells, TFP produced a dose-dependent increase in $[Ca^{2+}]_i$ (Fig. 5A). This increment in $[Ca^{2+}]_i$ could be prevented by removal of external Ca^{2+} (Fig. 5B) or curtailed by depletion of caffeine sensitive intracellular Ca^{2+} stores in the presence of external Ca^{2+} (Alvarez-Leefmans *et al.*, 1994; Kostyuk *et al.*, 1989), even when the concentration of TFP was 100 μM (Fig. 5C).

In seven experiments, 20 μM TFP produced a progressive increase in $[Ca^{2+}]_i$ from an estimated resting level of 147 ± 37 nM to 650 ± 40 nM. The latter value was reached 25 min after onset of TFP superfusion. The latency and time course of this effect was similar to that seen for the Ca^{2+}-dependent inactivation.

Effects of the protein kinase C activator 12-O-tetradecanoyl-phorbol-13-acetate, on Ca^{2+} currents and Ca^{2+}-dependent inactivation

It has long been known that TFP and other CaM-antagonists also inhibit PKC at concentrations similar to those that inhibit CaM (DeRiemer *et al.*, 1985; Norman *et al.*, 1987). Electrophysiological studies in neurons have shown that phorbol esters, which are activators of PKC, can either depress (Hammond *et al.*, 1987; Hockberger *et al.*, 1989; Rane and Dunlap, 1986) or increase (Strong *et al.*, 1987; Yang and Tsien, 1993) L-type Ca^{2+} currents. Since the TFP effects described above may be mediated by PKC inhibition, we assessed the possible modulation of L-type Ca^{2+} currents and Ca^{2+}-dependent inactivation by the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA). We found that TPA (10 to 30 nM), increased the peak L-type ICa^{2+} at all transmembrane potentials tested (Fig. 6A-B). However, in spite of the fact that TPA increased voltage-sensitive Ca^{2+} entry, it did not alter the efficacy of Ca^{2+}-dependent inactivation

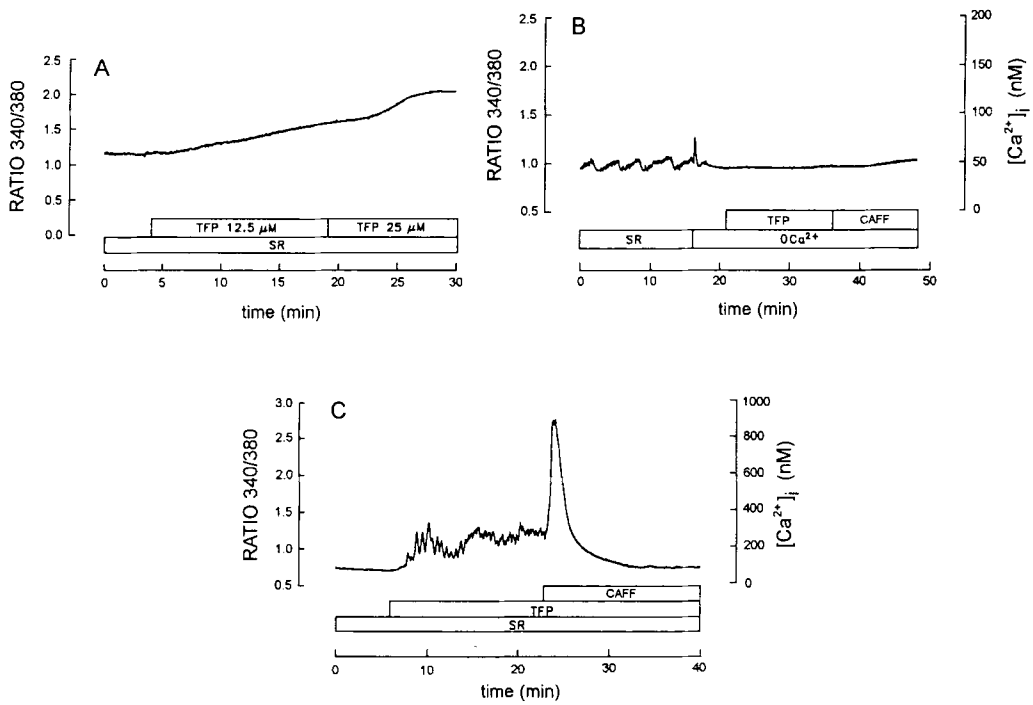
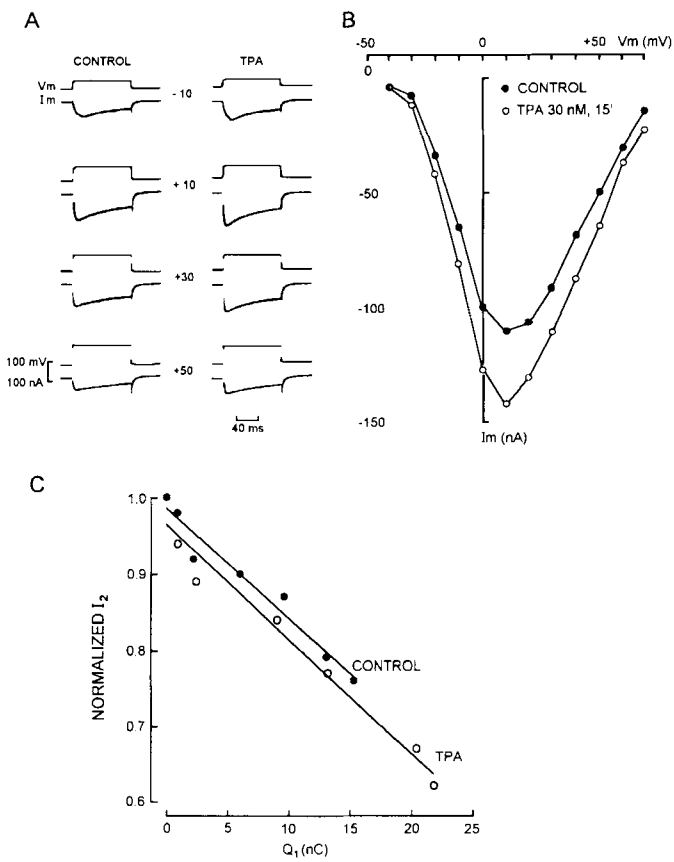


Fig. 5. Effect of TFP on basal $[Ca^{2+}]_i$ in fura-2 loaded neurons. A: *increase in $[Ca^{2+}]_i$ produced by 12.5 and 25 μM of TFP, as indicated by the bottom bars, in a neuron 1F.* **B**: *removal of external Ca^{2+} and its replacement with a nominally Ca^{2+}-free snail Ringer containing 7 mM Mg^{2+} plus 5 mM EGTA, prevented the increase in Ca^{2+} that would be produced by 100 μM TFP. Caffeine (5mM, CAFF) after a long (>10 min) exposure to $0Ca^{2+}$ produced only a modest and slow increase in $[Ca^{2+}]_i$.* **C**: *The increase in basal $[Ca^{2+}]_i$ produced by a high concentration of TFP (100 μM) could be curtailed by depletion of caffeine-sensitive intracellular Ca^{2+} pools with 5 mM caffeine in the presence of normal external Ca^{2+}. SR: snail Ringer solution.*



(Fig. 6C). The effect of TPA on Ca^{2+}-dependent inactivation was studied using the two pulse protocol described above. TPA produced a significant increment in Q_1, that led to a reduction of I_2 similar to that expected for the control condition. However, plots of normalized I_2 against Q_1, showed that the slope of the regression line fitting the data was not altered with respect to control, indicating that the efficacy of Ca^{2+}-dependent inactivation was unchanged by TPA (Fig. 6C). In four different experiments, slopes of the straight lines fitted to the data of I_2 versus Q_1 relations were -0.0374 ± 0.002 and -0.0342 ± 0.002 before and during TPA superfusion, respectively. The differences between the means were not statistically significant. In these experiments TPA increased peak L-type Ca^{2+} currents by 22.1 ± 2.2%. The effect was irreversible after 60 min TPA washout. Interestingly, TPA (30 nM) had no effect on Ba^{2+} currents, suggesting that the up-regulation of the Ca^{2+} currents produced by TPA requires permeation of the channels by Ca^{2+}.

Fig. 6. Effects of the protein kinase C activator TPA on L-type Ca^{2+} currents and on Ca^{2+}-dependent inactivation. **A**: *Ca^{2+} current records at indicated transmembrane voltages obtained before (CONTROL) and 15 min after onset of exposure to 30 nM TPA (right column).* **B**: *current/voltage relations obtained from the same neuron 77F shown in A.* **C**: *normalized I_2 plotted as a function of Q_1 before (filled circles) and during TPA exposure (open circles). The data were obtained from a neuron 1D. The holding voltage in all cases was -50 mV.*

To assess if the TPA effects on the ICa^{2+} were mediated through PKC activation, we studied the action of 4-α-phorbol, an ester that is a relatively poor PKC activator (DeRiemer *et al.*, 1985). 4-α-phorbol (30 nM) had no effect on Ca^{2+} currents (not shown). These results suggest that in *Helix* neurons, PKC up-regulates L-type ICa^{2+} without affecting the efficacy of Ca^{2+}-dependent inactivation and that for this effect to ensue, Ca^{2+} needs to be the charge carrier entering the cell.

Discussion

Pharmacological specificity of trifluoperazine hydrochloride

The present results show that in non-internally perfused neurons, TFP produces a decrease in peak L-type Ca^{2+} currents and an increase in the efficacy of Ca^{2+}-dependent inactivation. Both effects were dose-dependent, reversible, had a latency of ~10 min and were maximal at ~20 minutes upon continuous superfusion of TFP. This long-latency and time course were not due to restricted diffusion access since Cd^{2+} (400 μM), at the same fluid exchange rate, completely blocked the Ca^{2+} current within less than 2 min.

The TFP-induced changes in L-type Ca^{2+} currents are probably mediated, at least in part, through inhibition of a CaM-dependent process since TFP-S, a derivative of TFP with much weaker action as CaM antagonist (Crimaldi *et al.*, 1980; Levin and Weiss, 1980; Weiss *et al.*, 1980), has no effects on L-type ICa^{2+} at the same concentrations as TFP does. The lack of effect of TFP-S cannot simply be attributed to a relatively low plasma membrane permeability of this compound compared with that of TFP. This is supported by previous work in which the sulfoxide has been used extensively on intact cells as an experimental control of TFP-induced inhibition of various CaM-mediated cellular responses (Marone *et al.*, 1986; Pierce *et al.*, 1989; Ruben *et al.*, 1983; Veldhuis *et al.*, 1984). Moreover, although the octanol/water partition coefficients for TFP and TFP-S have not been reported, those for chlorpromazine and chlorpromazine sulfoxide, which are closely related phenothiazines sharing the same pharmacological properties as TFP and TFP-S (Levin and Weiss, 1979), are available (Leo *et al.*, 1971). The values reported are consistent with the sulfoxides being permeable through lipid bilayers, although they are expected to cross them more slowly than the parent compounds.

On the other hand, it is well known that TFP blocks CaM as effectively as it does block PKC (DeRiemer *et al.*, 1985). However, we show that the PKC activator TPA increased the amplitude of L-type Ca^{2+} currents but had no effect on the efficacy of Ca^{2+}-dependent inactivation, suggesting that the latter effect does not involve PKC.

Trifluoperazine-induced changes in Ca^{2+} current relaxation reflect an effect on Ca^{2+} channel inactivation

For single depolarizing voltage steps the TFP-induced decrease in peak amplitude of the L-type ICa^{2+} was accompanied by a decrease in its relaxation time constant. The latter could reflect an effect of TFP on Ca^{2+}-dependent inactivation, but we cannot rule out a parallel effect on the voltage-dependent inactivation component of the relaxation. This is because TFP also decreased the relaxation time constant of the Ba^{2+} currents. However, the latter effect could result from a TFP-induced increase in $[Ca^{2+}]_i$ that could produce Ca^{2+}-dependent inactivation of Ca^{2+} channels permeated by Ba^{2+} (see below). In contrast, the double pulse experiments clearly demonstrate that TFP enhances Ca^{2+}-dependent inactivation independently of any possible action on voltage-dependent inactivation.

Possible mechanisms of trifluoperazine-induced enhancement of Ca^{2+}-dependent inactivation

The main result of the present work is that TFP enhances the efficacy of Ca^{2+}-dependent inactivation. The question arises as to the mechanism underlying this effect. There are at least two main possibilities: 1) TFP could be increasing the affinity for Ca^{2+} of the putative Ca^{2+} binding site directly associated with the inner mouth of the Ca^{2+} channel that promotes inactivation (Imredy and Yue, 1994; Zong and Hofmann, 1996); or 2) TFP could be blocking a CaM-dependent process that may be directly or indirectly involved in Ca^{2+}-dependent inactivation. The first possibility can only be tested with techniques that allow direct control and access to the intracellular environment. One of the aims of the present work was to use methods that preserved naturally occurring cytoplasmic factors which may be the key players of Ca^{2+}-dependent inactivation under more physiological conditions. The latter notion may explain why Imredy and Yue (1994) did not find any effect of the CaM-antagonist calmidazolium on Ca^{2+}-dependent inactivation in dialyzed myocytes.

The second possibility encompasses two aspects: a) TFP could be blocking an intermediary involved in Ca^{2+}-dependent inactivation. Under normal conditions, this intermediary would be decreasing the efficacy of inactivation. When the intermediary is blocked, inactivation should be released and enhanced. Clearly this intermediary cannot be the Ca^{2+}/CaM-activated phosphatase calcineurin, as proposed by Chad and Eckert (Chad and Eckert, 1986), because inhibition of this enzymatic pathway should have opposite effects to the ones we found. In

agreement with this interpretation, it was found that phosphatase inhibitors, at concentrations sufficient to inhibit calcineurin, had no effects on either Ca^{2+} current relaxation (Fryer and Zucker, 1993) or on Ba^{2+} current peak amplitude (Golowasch *et al.*, 1995); b) TFP could be blocking the CaM-activated plasma membrane Ca^{2+} pump (Carafoli, 1994) leading to an increase in $[Ca^{2+}]_i$ which would contribute to inactivation. In *Helix* neurons the plasma membrane Ca^{2+} pump is the main mechanism responsible for $[Ca^{2+}]_i$ regulation (Alvarez-Leefmans *et al.*, 1981; Kennedy and Thomas, 1995). In addition, TFP could be releasing Ca^{2+} from internal stores (Schlatterer and Schaloske, 1996; Wyskovsky *et al.*, 1988). Our results, in fura-loaded cells, revealed that indeed TFP produces a slow and progressive increase in $[Ca^{2+}]_i$. The source of this Ca^{2+} appears to be both extracellular and intracellular since it could be prevented by removal of external Ca^{2+} to a sufficient extent to deplete the internal stores (eg. Fig. 5B), or curtailed by depletion of caffeine sensitive internal stores (eg. Fig. 5C). Altogether these findings suggest that elevation of basal $[Ca^{2+}]_i$ contributes to the enhancement of inactivation of L-type Ca^{2+} currents. In agreement with this explanation are previous results showing that buffering intracellular Ca^{2+} reduces Ca^{2+}-dependent inactivation and increasing $[Ca^{2+}]i$ increases the inactivation (Gutnick *et al.*, 1989; Plant *et al.*, 1983). Moreover, Ca^{2+} released from intracellular organelles has been shown to produce Ca^{2+}-dependent inactivation of L-type Ca^{2+} currents (Kramer *et al.*, 1991).

Protein kinase C up-regulates Ca^{2+} currents

The effects of TPA, a direct activator of PKC on Ca^{2+} currents, deserve further comment. We found that L-type Ca^{2+} currents were increased by TPA. This result is opposite to that reported by Hammond *et al.* (Hammond *et al.*, 1987) working in the same identified *Helix* neurons used in the present study. Using TPA and direct injection of PKC they concluded that PKC down-regulates Ca^{2+} currents. However, their experiments were done using Ba^{2+} as charge carrier through Ca^{2+} channels, in cells that had been injected with the Ca^{2+} chelator EGTA, which is known to decrease basal intracellular $[Ca^{2+}]$ effectively or damp transient changes in this cation (Alvarez-Leefmans *et al.*, 1981; Ahmed and Connor, 1988). Moreover, it is known that PKC does not interact directly with diacylglycerol or phorbol esters in the absence of Ca^{2+} (Huang, 1989). In agreement with this view, we found that TPA had no effect on Ba^{2+} currents. This result was expected considering the well known fact that Ba^{2+} cannot replace Ca^{2+} as an intracellular messenger.

Implications for the antipsychotic action of trifluoperazine

Trifluoperazine and other phenothiazines are widely used compounds to treat nearly all forms of psychosis, including schizophrenia (Marder and Van Putten, 1995). It is not known which of their various pharmacological and biochemical actions explain their therapeutic properties. Besides antagonizing calmodulin and PKC, phenothiazines block, with relative high affinity, many receptor types including dopamine D_2, α_1-adrenoceptors, Histamine H_1, and 5-HT_2 (Reynolds, 1992). To all these actions we now have to add the so far unique property of TFP to enhance Ca^{2+}-dependent inactivation.

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