Voltage-Dependent Calcium Channels as Targets for Convulsant and Anticonvulsant Alkyl-Substituted Thiobutyrolactones

ROBERT A. GROSS, DOUGLAS F. COVEY and JAMES A. FERRENDELLI

Departments of Neurology and Pharmacology and Physiology, University of Rochester, Rochester, New York (R.A.G.), Department of Molecular Biology and Pharmacology, Washington University, St. Louis, Missouri (D.F.C.), and Department of Neurology, University of Texas, Houston, Texas (J.A.F.)

Accepted for publication October 18, 1996

ABSTRACT

Alkyl-substituted thiobutyrolactones increase or decrease γ-aminobutyric acid_2 responses at or near the picrotoxin site, but they are structurally similar to ethosuximide, which prompted us to determine the actions of thiobutyrolactones on voltage-dependent Ca_2+ currents. We measured Ca_2+ currents in cultured neonatal rat dorsal root ganglion neurons in the absence and presence of the anticonvulsant α-ethyl,α-methyl-γ-thiobutyrolactone (α-EMTBL) and the convulsant β-ethyl,β-methyl-γ-thiobutyrolactone (β-EMTBL). Low-voltage-activated (T-type) currents were reduced in a concentration-dependent manner, with a maximal reduction of 26% and 30% by α-EMTBL and β-EMTBL, respectively. α-EMTBL reduced high-voltage-activated currents in a concentration- and voltage-dependent manner: maximal responses were 7% when evoked from −80 mV, with more rapid current inactivation; 29% when evoked from −40 mV, with little effect on current inactivation. β-EMTBL increased high-voltage-activated currents ≈20% at 10 to 300 μM, but reduced currents at higher concentrations; the latter action was similar to that of α-EMTBL in its magnitude and voltage dependence. Block of N-type channels with ω-conotoxin GVIA (10 μM) reduced the effect of α-EMTBL and eliminated its voltage dependence. The L-type current component was also reduced by α-EMTBL, with little effect on P- or Q-type current components. The related compound, α-ethyl,α-methyl-γ-butyrolactone, had no effect on Ca_2+ currents. We conclude that thiobutyrolactones affect voltage-dependent Ca_2+ currents in a concentration- and voltage-dependent manner, with greater potency on low-voltage-activated channels. Both the ring structure and the position of its alkyl substitutions determine the identity of the targeted Ca_2+ channel subtypes and the manner of regulation.

The mechanisms of action of antiseizure drugs have been studied intensively, relying on observed drug effects in vitro to make inferences about actions in vivo (Rogawski and Porter, 1990; Macdonald and Meldrum, 1995). For the most part, these drugs act on neuronal ion channels or on the neurotransmitters that regulate their activity. For example, phenytoin, carbamazepine and lamotrigine increase inactivation of voltage-dependent Na_+ channels (McLean and Macdonald, 1983, 1986; Quandt, 1988; Cheung et al., 1992), and barbiturates and benzodiazepines increase GABA_A-mediated inhibition (e.g., Twyman et al., 1989; Macdonald and Meldrum, 1995). The role of Ca_2+ channel blockade as an antiseizure mechanism is less clear, however. The Ca_2+ channel blocker flunarizine and the dihydropyridines have some antiseizure activity (Binnie, 1989; Meyer, et al., 1990), but the effects of the latter are not substantial. Barbiturates, at concentrations similar to those achieved during treatment of status epilepticus, profoundly block high-voltage-activated Ca_2+ currents (Gross and Macdonald, 1988a,b). The best case for the antiseizure effect of Ca_2+ channel blockade is ethosuximide, which blocks the low-voltage-activated T-type channel, likely responsible for its efficacy in primary generalized (absence) epilepsy (Coulter et al., 1989).

Although newer pharmaceuticals are being developed with increasingly specific and selective actions, some affect more than one ion channel or neurotransmitter system; it may be difficult, therefore, to determine which action is most desirable for clinical efficacy (or which produces undesirable toxicity) and whether more than one therapeutic action may be advantageous. The alkyl-substituted butyro- and thiobutyrolactones are a novel group of compounds with either anticonvulsant or convulsant activity, depending on the alkyl substitution.

ABBREVIATIONS: α-EMGBl, α-ethyl,α-methyl-γ-butyrolactone; α-EMTBL, α-ethyl,α-methyl-γ-thiobutyrolactone; β-EMTBL, β-ethyl,β-methyl-γ-thiobutyrolactone; ATP, adenosine triphosphate; DRG, dorsal root ganglion; GABA_A, γ-aminobutyric acid; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid.
vulsant or convulsant activity, dependent on the location and size of their alkyl groups. Compounds with small $\alpha$-substitutions are anticonvulsant, whereas $\beta$-substituted compounds are convulsant (Klunk et al., 1982a,b; Holland et al., 1990). Both groups act at or near the picrotoxin site of the GABA $\alpha$ receptor complex (Weissman et al., 1984; Levine et al., 1985; Canney et al., 1991; Xu et al., 1995), exhibiting either picrotoxin agonist (convulsant) or inverse agonist (anticonvulsant) properties. These compounds do not appear to gate the GABA $\alpha$ receptor directly, but the actions of some are complex, with differing quantitative effects in the presence of varying GABA concentrations (Yoon et al., 1993).

The structural similarities of the butyrolactone analogs to succinimides, and to ethosuximide in particular, suggested that these compounds may regulate voltage-dependent Ca $^{2+}$ channels as well (fig. 1). If this were to be the case, then butyrolactone analogs may afford the opportunity to make inferences about the relative merits of GABA or Ca $^{2+}$ channel effects vis-a-vis convulsant or anticonvulsant action. We therefore sought to characterize the actions of a selected group of alkyl-substituted butyrolactones on voltage-dependent Ca $^{2+}$ currents in cultured neurons. The effects of the anticonvulsant compound $\alpha$-EMTBL were compared with those of the convulsant $\beta$-EMTBL and with $\alpha$-EMGBL, an anticonvulsant compound that blocks the action of $\alpha$-EMTBL on GABA responses in cultured hippocampal neurons (Holland et al., 1990). Our studies suggest that GABA $\alpha$ receptor blockade is the major action of the convulsant compounds but that blockade of Ca $^{2+}$ channels, particularly T-type channels, may participate in the anticonvulsant actions of the $\alpha$-substituted thiobutyrolactones.

**Methods**

**Cell Culture**

Primary DRG cultures were prepared from day 7 to 10 neonatal Sprague-Dawley rats (Harlan) as described previously (Gross et al., 1990). The animals were sacrificed after CO$_2$ narcosis by decapitation. The DRGs were dissected away from the spinal cord and placed in ice-cold Hanks' buffer (pH 7.4 with HEPES; Sigma Chemical Company, St. Louis, MO). After enzymatic digestion with trypsin (1 mg/ml; Sigma) and mechanical trituration, the cells were spun down and resuspended in plating medium (MEM containing bicarbonate (GIBCO, Grand Island, NY), 50 ng/ml neural growth factor (Collaborative Biomedical Research, Bedford, MA), 5% equine serum and 5% fetal bovine serum (Hyclone Laboratories, Logan UT)). Cells were then plated on 35-mm dishes with collagen (Sigma) as substrate. The medium was changed within several hours and replaced with MEM containing 10% equine serum, without fetal bovine serum. Ara-C (1–5 $\mu$M; Sigma) was used, if needed, to inhibit the growth of non-neuronal cells within the first week of longer term cultures. The cultures were maintained at 37°C under a 95% air-5% CO$_2$ atmosphere and fed on a twice-weekly basis with 50% exchanges of growth medium. These cultures were used for experiments as early as day 2, and for up to 10 to 12 weeks. The vast majority of experiments were performed on neurons in culture for 2 to 4 days.

**Electrophysiology**

**Preparation of solutions.** Butyrolactones were dissolved in external solution (below) on the day of the experiment. Nifedipine and $\omega$-conotoxin GVIA (both from Sigma) and $\omega$-agatoxin IVA (Peptides International, Louisville KY) were made fresh on the day of the experiment. Nifedipine was dissolved in dimethyl sulfoxide (Sigma) at a concentration of 10 mM and was diluted in external solution to a final concentration of 10 $\mu$M. $\omega$-Conotoxin GVIA and $\omega$-agatoxin IVA were stored frozen in water at a concentration of 10 mM and were used for experiments within 2 to 3 months. On the day of the experiment, the stock solutions were diluted in external solution.

**Whole-cell patch-clamp recordings.** Whole-cell voltage-clamp recordings were obtained with the whole-cell variation of the patch-clamp technique. Cells were bathed in a solution containing (in mM): CaCl$_2$, 5.0; choline Cl, 67; MgCl$_2$, 0.8; TEA, 100; glucose, 5.6; KCl, 5.3; HEPES, 10 (pH 7.3–7.4, 310–330 mOsm; all reagents from Sigma). Glass recording patch pipettes were fashioned from Fisher-brand microhematocrit tubes with a Sutter Instruments Brown-Flaming P-87 pipette puller. These electrodes had resistances of 1.5 to 3.0 meqohm when filled with a recording solution consisting of the following (in mM): CsMeSO$_3$, 140; HEPES, 10; ethyleneglycol-bis(β-aminomethyl ether)-N,N,N',N'-tetraacetic acid, 10; ATP-Mg$,^{2+}$, 5; GTP-Na, 0.1 (all reagents from Sigma). The pH (7.3–7.4) was adjusted with 1 N CsOH after the addition of ATP. The osmolality was adjusted to 10 to 15% less than that of the bath solution, 280 to 300 mOsm.

Recordings were made at room temperature by the Axopatch 200 patch-clamp amplifier (Axon Instruments, Foster City, CA). Pipette and whole-cell capacitance and series resistance were corrected by compensation circuitry on the amplifier. Series resistance was estimated by cancellation of the capacitance-charging current transient after patch rupture; typical values for the series resistance were 2.5 to 4 meqohm. In most cases, series resistance compensation of 80 to 90% was possible without significant noise or oscillation. Voltage step commands were generated, and currents were digitized (5 kHz), stored and analyzed by a microcomputer (CompuAdd 325, 425; Zeos Pentium; or clones) with the program pClamp 5.6 and 6.0 (Axon Instruments). The current traces were filtered with a Bessel filter at 2 kHz (~3 dB). Data were accepted only if space-clamp was adequate, determined by well-controlled incremental current activation over a series of voltage steps (usually, ~65 to +10 mV), and if tail currents deactivated rapidly. Furthermore, with 10-mV test pulses, we required settling of the capacitance-charging transient within 2 msec.

Leak current was determined by a P/P4 or P/P6 protocol. This current was digitally subtracted from the relevant inward current to obtain the calcium current.

Butyrolactones, nifedipine, $\omega$-agatoxin IVA and $\omega$-conotoxin GVIA were applied to the cell under study by pressure ejection (6–10 kPa) from separate blunt-tipped (internal diameter, 12–15 $\mu$m) glass micropipettes positioned ~30 $\mu$m from the cell. Applications of these compounds were 10 to 15 sec in duration, just before currents were evoked and the puffer pipettes were removed from the bath when not in use. In some experiments, drug applications were accomplished by a “U-tube” microperfusion system; the results were similar to those.
obtained with puffer application. Diluents had no effect on evoked
currents. Control experiments showed that dimethyl sulfoxide (nif-
fipine) at concentrations up to 1% had no effect on Ca$^{++}$ currents.
In all experiments, the culture was perfused continuously with bath
(external) solution by a gravity-fed, vacuum-removed system oper-
atting at about 0.3 ml/min; thus, drug concentrations were minimized
in the remainder of the culture dish.

Data Analysis

Data are expressed as means ± S.E.M., and statistical compari-
sions between group means were made with Student's two-tailed t
test.

Results

Effects on low-voltage-activated whole-cell Ca$^{++}$ currents. The effects of thiobutyrolactones were tested first
on low-voltage-activated (T-type) Ca$^{++}$ currents because of
their structural similarity to ethosuximide, an antiabsence
seizure drug that reduces T-type currents in thalamic and
sensory neurons (fig. 2). T-type currents, which require very
negative $V_h$ to remove steady-state inactivation, can be stud-
ed in isolation by evoking currents at relatively negative $V_c$
(negative to −20 mV), at which little if any high-voltage-
activated currents are evoked. For these experiments, cur-
rents were evoked from $V_h$ = −90 mV at a series of poten-
tials, ranging from −65 to −5 mV (see figure). Peak current
magnitudes were measured, and the effect of each compound
was assessed on all currents uncontaminated with the more
slowly inactivating high-voltage-activated current compo-
nents.

The anticonvulsant compound α-EMTBL reduced T-type
currents, with a mean peak current reduction of 26 ± 3%
($n = 12 ± \text{SEM}; P ≤ .001$). There was no apparent effect on
the voltage dependence of current activation as evidenced in
the current-voltage plots (although unequivocal determina-
tion of the potential at which the maximal T-type current
occurred was difficult because of contamination with high-
voltage-activated current components), nor any effect on cur-
rent kinetics. The convulsant compound β-EMTBL had a
similar action. Low-voltage-activated currents were reduced
with a maximal mean reduction of 30 ± 1% ($n = 4; P ≤ .011$).
By contrast, the anticonvulsant butyrolactone α-EMGBL
(500 μM) had no effect on T-type currents (see also below). These
actions were compared with those of 500 μM ethosux-
imide applied to neurons from the same culture groups. Etho-
suximide reduced T-type currents, but was less efficacious
than either thiobutyrolactone, producing a current reduction
of 16 ± 4% ($n = 8; P ≤ .002$).

Thiobutyrolactones reduced T-type currents in a concen-
tration-dependent manner but with slightly differing poten-
ties and efficacies. T-type currents were not significantly
affected by 10 μM α-EMTBL. At greater concentrations, T-
type currents were reduced 9 ± 2% (50 μM, $n = 6$), 18 ± 2%
(100 μM, $n = 8$), 25 ± 3% (300 μM, $n = 5$) and 26 ± 3% (500
μM, $n = 13$). Higher concentrations did not have a greater
effect. The concentration-response plot (fig. 3) was used to
estimate by visual inspection the half-maximal concentration
(EC$_{50}$; 75 μM). β-EMTBL had similar concentration-
response parameters, slightly more efficacious but slightly less
potent. Currents were reduced 1.5% (50 μM, $n = 2$), 2 ± 1%
(100 μM, $n = 5$), 7 ± 1% (300 μM, $n = 4$), 30 ± 5% (500 μM,
$n = 9$) and 30 ± 1% (1000 μM, $n = 4$), yielding an apparent
EC$_{50}$; 350 μM.

Fig. 2. Reduction of low-voltage-activated (T-type) Ca$^{++}$ currents by α-EMTBL, β-EMTBL and ethosuximide. (A) Currents were evoked in
different cells from $V_h$ = −90 mV at the potentials shown in the absence (Control) or presence of α-EMTBL (500 μM) or β-EMTBL (1000 μM).
Current traces are shown above peak current-voltage plots obtained from the same cells. (B) Currents evoked in the absence or presence of 500
μM ethosuximide (left) and the peak current-voltage plot from the same cell (right).
Effects on high-voltage-activated whole-cell Ca\textsuperscript{2+} currents. The effects of α-EMTBL and β-EMTBL were assessed next on high-threshold-activated current components. The thiobutyrolactones had complicated actions on these current components, with both concentration- and voltage-dependent effects. Across a wide concentration range (10–1000 μM), α-EMTBL reduced high-voltage-activated Ca\textsuperscript{2+} current components in all neurons tested, but did so in a voltage-dependent manner (fig. 4). By contrast, β-EMTBL increased currents at concentrations ≤300 μM, without a clear voltage dependence, and reduced currents at greater concentrations in a voltage-dependent manner similar to that of α-EMTBL (fig. 5).

High-voltage-activated Ca\textsuperscript{2+} current components were smaller in the presence of α-EMTBL, with maximal peak current reductions at a concentration of 500 μM. When currents were evoked from \( V_h = -80 \) mV at +10 mV, there was no apparent effect of 10 μM α-EMTBL. At higher concentra-

**Fig. 3.** Concentration-response relationships for α-EMTBL and β-EMTBL for T-type currents. The peak current magnitude was measured for the maximal T-type current of each cell, evoked from \( V_v = -90 \) mV at -20 or -15 mV; and the percent reduction was plotted for both α-EMTBL (●) and β-EMTBL (■) over the stated ranges of concentrations. See text for details.

**Fig. 4.** Effect of α-EMTBL on high-voltage-activated Ca\textsuperscript{2+} currents. (A) Currents were evoked from either -80 mV (■) or -40 mV (●) at +10 mV in the absence or presence of 500 μM α-EMTBL. Traces are shown above the peak current-voltage plot obtained from the same cell. (B) Concentration-response relationship plotted as percent reduction in peak current magnitude for currents evoked from \( V_h = -80 \) mV (■) or -40 mV (■) at \( V_c = +10 \) mV.
produced higher concentrations, peak current magnitudes were reduced from \(V_h\) when currents were evoked from \(V_h = -40\) mV at \(+10\) mV, the peak reductions were greater, \(4 \pm 1\% (50 \mu M, P \leq .01), 8 \pm 1\% (100 \mu M, P \leq .001), 16 \pm 2\% (300 \mu M, P \leq .001), 28 \pm 1\% (500 \mu M, P \leq .001)\) and \(30 \pm 4\% (1000 \mu M, P \leq .001)\). Although the reduction in peak current was modest, currents inactivated more rapidly (see fig. 4). When currents were evoked from \(V_h = -40\) mV at \(+10\) mV, the peak reductions were greater, \(4 \pm 1\% (50 \mu M, P \leq .01), 8 \pm 1\% (100 \mu M, P \leq .001), 16 \pm 2\% (300 \mu M, P \leq .001), 28 \pm 1\% (500 \mu M, P \leq .001)\) and \(30 \pm 4\% (1000 \mu M, P \leq .001)\). When currents were evoked from these more positive potentials in the presence of \(\alpha\)-EMTBL, the rate of current inactivation was slightly greater than control, but not nearly so rapid as when evoked from \(-80\) mV. The apparent \(EC_{50}\) was \(-300\) \(\mu M\).

In the presence of \(\beta\)-EMTBL, currents were also affected in a concentration- and voltage-dependent manner, but an additional effect was seen, enhancement of \(Ca^{2+}\) currents at relatively low concentrations (fig. 5). As with \(\alpha\)-EMTBL, there was no effect of \(10 \mu M\) \(\beta\)-EMTBL. When currents were evoked from \(V_h = -80\) mV at \(+10\) mV, peak current magnitudes were increased \(10 \pm 4\% (50 \mu M, n = 9, P \leq .02), 13 \pm 5\% (100 \mu M, n = 7, P \leq .05)\) and \(7 \pm 5\% (300 \mu M, n = 4)\); at higher concentrations, peak current magnitudes were reduced \(7 \pm 2\% (500 \mu M, n = 10, P \leq .02)\) and \(7 \pm 2\% (1000 \mu M, n = 6, P \leq .005)\). When currents were evoked from \(V_h = -40\) mV at \(+10\) mV, currents were increased \(10 \pm 4\% (50 \mu M, P \leq .02), 10 \pm 5\% (100 \mu M, P \leq .05)\) and \(5 \pm 9\% (300 \mu M)\); at higher concentrations, peak current magnitudes were reduced \(23 \pm 4\% (300 \mu M, P \leq .001)\) and \(29 \pm 3\% (1000 \mu M, P \leq .001)\). The current enhancements seen with \(\beta\)-EMTBL were not voltage dependent, i.e., the percent increase was similar when currents were evoked from either \(-80\) mV or \(-40\) mV. Current reductions seen in the presence of \(\beta\)-EMTBL, by contrast, were voltage dependent: lesser peak current reductions and faster current inactivation were evident in currents evoked from \(V_h = -80\) mV; greater peak current reductions and more normal current inactivation were evident in currents evoked from \(V_h = -40\) mV. For current enhancement, the \(EC_{50}\) could not be determined unequivocally, because we could not determine, at any given concentration, whether \(\beta\)-EMTBL was acting solely to increase currents. Assuming that the maximal enhancement was \(13\%\) at \(100 \mu M\), and given that \(10 \mu M\) had no effect, a rough estimate of \(EC_{50}\) is \(50 \mu M\). With the same limitation, the \(EC_{50}\) for current reduction was \(-400\) \(\mu M\).

At the higher concentrations tested, current-voltage plots showed that the maximal high-voltage-activated currents occurred at similar potentials in the absence and presence of thiobutyrolactones and that \(EC_{50}\) did not appear shifted (see also fig. 2).

We next examined whether \(\alpha\)-EMGBL, an anticonvulsant compound that blocks the effects of \(\alpha\)-EMTBL on \(GABA_A\) receptors, affected \(Ca^{2+}\) currents in a similar manner. \(\alpha\)-EMGBL was tested in the absence and presence of \(\alpha\)-EMTBL, with both compounds applied at a concentration of \(500 \mu M\) (fig. 6). When applied alone, \(\alpha\)-EMGBL had no effect on high-voltage-activated \(Ca^{2+}\) currents, evoked from \(V_h = -80\) at \(-40\) mV; this lack of effect was observed across the entire physiological range of current activation, as shown in the current-voltage plot (\(V_h = -90\) mV). In the next series of experiments, \(\alpha\)-EMGBL was applied first to verify its lack of effect. Next, \(\alpha\)-EMGBL and \(\alpha\)-EMTBL were applied simultaneously from another puffer pipette; compared with the effect of \(\alpha\)-EMTBL alone, obtained at the end of the experiment, \(\alpha\)-EMGBL failed to alter the action of \(\alpha\)-EMTBL. In a series of control experiments, \(\alpha\)-EMGBL was applied contin-
Fig. 6. The effect on Ca\(^{2+}\) currents of \(\alpha\)-EMGBL alone and in the presence of \(\alpha\)-EMTBBL. (A) The effect of 500 \(\mu\)M \(\alpha\)-EMGBL (top traces, peak current-voltage plot) on high-voltage-activated currents, evoked from either \(-80\) mV (○) or \(-40\) mV (•) at \(+10\) mV, compared with the effect of 500 \(\mu\)M \(\alpha\)-EMTBBL (bottom traces). (B) Currents were evoked from \(-80\) mV at \(+10\) mV in the absence and presence of \(\alpha\)-EMGBL (left) or in the presence of \(\alpha\)-EMGBL and \(\alpha\)-EMTBBL (middle). The effect of \(\alpha\)-EMTBBL in this cell is shown on the right for comparison.

Discussion

This study investigated the regulation of voltage-dependent Ca\(^{2+}\) currents in cultured DRG neurons by alkyl-substituted thiobutyrolactones. The results show for the first time that these compounds can block high-voltage-activated Ca\(^{2+}\) currents nonselectively, but that its voltage-dependent effect was selective for N-type channels. As seen in the derived difference currents, the effect of \(\alpha\)-EMTBBL, when applied at \(-80\) mV, was slow to develop, requiring most of the 100-msec voltage step to achieve maximal block. When applied at \(-40\) mV, the effect was greatest at the current peak, with a parallel reduction in current persisting throughout the remainder of the voltage step. After application of \(\omega\)-conotoxin GIVA, the rapidly inactivating N-type current component was lost. The subsequent application of \(\alpha\)-EMTBBL showed that the effect of this compound was reduced by 51 \(\pm\) 7\% \((n = 9)\). An interesting finding was that the voltage-dependent effect was lost, with equal current reductions from both \(V_h\) values; the current reductions developed rapidly, with a parallel current reduction throughout the voltage step. Elimination of P- and Q-type current components by continued applications of the toxins reduced the control currents further, with little current inactivation evident during the voltage step. Replication of \(\alpha\)-EMTBBL produced a similar current reduction after block of P- and Q-type currents: in 5 of 8 neurons, the effect of \(\alpha\)-EMTBBL was the same, whether \(\omega\)-agatoxin IVA was applied alone or in combination with \(\omega\)-conotoxin GIVA; in the remaining 3 neurons, \(\omega\)-agatoxin IVA reduced the effect of \(\alpha\)-EMTBBL by no more than \(10\%\). These data show that the greatest effect of \(\alpha\)-EMTBBL on high-voltage-activated Ca\(^{2+}\) current components was caused by an action on N- and L-type channels, with the former occurring in a voltage-dependent manner.

This study investigated the regulatory mechanisms underlying voltage-dependent Ca\(^{2+}\) currents in cultured DRG neurons by alkyl-substituted thiobutyrolactones. The results show for the first time that these compounds can block high-voltage-activated Ca\(^{2+}\) currents nonselectively, but that its voltage-dependent effect was selective for N-type channels. As seen in the derived difference currents, the effect of \(\alpha\)-EMTBBL, when applied at \(-80\) mV, was slow to develop, requiring most of the 100-msec voltage step to achieve maximal block. When applied at \(-40\) mV, the effect was greatest at the current peak, with a parallel reduction in current persisting throughout the remainder of the voltage step. After application of \(\omega\)-conotoxin GIVA, the rapidly inactivating N-type current component was lost. The subsequent application of \(\alpha\)-EMTBBL showed that the effect of this compound was reduced by 51 \(\pm\) 7\% \((n = 9)\). An interesting finding was that the voltage-dependent effect was lost, with equal current reductions from both \(V_h\) values; the current reductions developed rapidly, with a parallel current reduction throughout the voltage step. Elimination of P- and Q-type current components by continued applications of the toxins reduced the control currents further, with little current inactivation evident during the voltage step. Replication of \(\alpha\)-EMTBBL produced a similar current reduction after block of P- and Q-type currents: in 5 of 8 neurons, the effect of \(\alpha\)-EMTBBL was the same, whether \(\omega\)-agatoxin IVA was applied alone or in combination with \(\omega\)-conotoxin GIVA; in the remaining 3 neurons, \(\omega\)-agatoxin IVA reduced the effect of \(\alpha\)-EMTBBL by no more than \(10\%\). These data show that the greatest effect of \(\alpha\)-EMTBBL on high-voltage-activated Ca\(^{2+}\) current components was caused by an action on N- and L-type channels, with the former occurring in a voltage-dependent manner.

Discussion

This study investigated the regulation of voltage-dependent Ca\(^{2+}\) currents in cultured DRG neurons by alkyl-substituted thiobutyrolactones. The results show for the first
time that these compounds affected both low-voltage- and high-voltage-activated Ca\(^{2+}\) currents in a concentration-dependent manner and in the presumed active range of 200 to 400 \(\mu\)M (Canney et al., 1991). A particularly novel result was that \(\beta\)-EMTBL only reduced low-voltage-activated (T-type) currents, but had a biphasic effect on high-voltage-activated currents, producing an increase at low concentrations (\(\leq 300 \mu\)M) and a decrease at higher concentrations. By contrast, \(\alpha\)-EMTBL only reduced low-voltage- and high-voltage-activated currents and \(\alpha\)-EMGBL had no effect on Ca\(^{2+}\) currents. The predominant effects of the thiobutyrolactones were on the T-, N- and L-type current components, with little effect on the P- and Q-type current components. Furthermore, thiobutyrolactone-induced reductions in the N-type current component were voltage dependent.

**Butyrolactone structure and action on Ca\(^{2+}\) channels.** Thiobutyrolactone actions were concentration dependent and exhibited some selectivity for Ca\(^{2+}\) channel subtypes. These findings stand in contrast to the lack of effect of the \(\gamma\)-butyrolactones and suggest that Ca\(^{2+}\) channel subtypes represent specific targets for the thiobutyrolactones. The inability of \(\alpha\)-EMGBL to affect low-voltage- or high-voltage-activated currents, or to block the actions of \(\alpha\)-EMTBL, for example, illustrates this difference in binding sites. (This last result highlights a clear difference in the effects of these compounds, at similar concentrations, on the GABA\(_{\alpha}\) receptor, at which \(\alpha\)-EMGBL acts as an “antagonist” to \(\alpha\)-EMTBL [Holland et al., 1990].) It is possible, however, that \(\alpha\)-EMGBL may bind at the thiobutyrolactone site on Ca\(^{2+}\) channels, but with a markedly lower affinity or with less potency. Additional experiments will be required to distinguish between these possibilities, but the present results make clear that the identity of the ring heteroatom is of primary importance in determining actions on Ca\(^{2+}\) channels. Studies with additional analogs may allow firmer conclusions regarding the importance of the ring structure in targeting Ca\(^{2+}\) channels of different subtypes. Ethosuximide, for example, which differs from \(\alpha\)-EMTBL in its ring heteroatom and in the number of ring carbonyl groups, targets only T-type channels.

The present results allow some conclusions regarding the importance of the alkyl substitutions in targeting channel subtypes. Low-voltage-activated (T-type) currents were only reduced by thiobutyrolactones. The action of \(\beta\)-EMTBL on T-type currents was slightly more efficacious but less potent than for \(\alpha\)-EMTBL. Thus, for low-voltage-activated currents, the position of the alkyl substitution confers differing properties and imparts to...
β-substituted compounds an unusual characteristic, Ca\(^{++}\) current enhancement.

The structure of a given compound may determine its action on particular Ca\(^{++}\) channel subtypes. Unlike many Ca\(^{++}\) channel blockers, which show preferences for either single-channel subtypes, such as α-conotoxin GVIA for the N-type channel (for example, Regan et al., 1991), or a relative preference for either low-voltage- or high-voltage-activated channels, such as Ni\(^{++}\) or Cd\(^{++}\), respectively (Fox et al., 1987), the thiobutyrolactones affect both low-voltage-activated (T-type) and high-voltage-activated Ca\(^{++}\) current components. Of the latter, both N- and L-type current components were affected, with little apparent effect on the P- and Q-type current components. This last finding must remain tentative, however, because DRG neurons express relatively little P- and Q-type current. In experiments with 500 nM α-agatoxin IVA, for example, the high-voltage-activated currents were reduced by only 10 to 15% by this toxin, similar to the findings of others who used this cell type (Mintz et al., 1992; Regan et al., 1991). Thus, it may have been difficult to determine with certainty whether and to what extent these current components are affected by thiobutyrolactones. Additional experiments will be required, with either expressed channels or cells, such as cerebellar Purkinje or granule cells, which express a greater proportion of P- and Q-type channels.

**Voltage dependence of thiobutyrolactone action.** The thiobutyrolactone effects on T- and L-type currents did not appear to be voltage dependent as did the α-conotoxin GVIA-sensitive effects on the N-type current component. The mechanism of this voltage-dependent effect on N-type channels is uncertain, although the present results suggest possible alternative explanations. The finding that thiobutyrolactones, applied at \(V_h = -80\) mV, had little effect on peak currents, but increased the rate of apparent current inactivation at \(+10\) mV, suggests that the major effect may have been on open channels. This hypothesis predicts that Ca\(^{++}\) channels needed to be in the open state for the thiobutyrolactones to effect a reduction in current, either by an inhibitory effect on channel activity or by blocking open channels. The finding that peak currents were substantially reduced by thiobutyrolactones applied at \(V_h = -40\) mV, a potential at which high-voltage-activated channels were not open, counters this hypothesis and suggests, rather, that closed channels can be affected by these compounds. Further, the fact that inactivation rates of currents evoked from \(-40\) mV were not substantially affected by thiobutyrolactones suggests that the open state of high-voltage-activated channels may not be the only channel conformation to be affected by the thiobutyrolactones. Indeed, if “open channel block” were the only or predominant mechanism of action, a shift in the apparent \(E_{Ca}\) would be expected, which is not evident in the present results.

The present data could be explained if thiobutyrolactones have voltage-dependent binding: little effect would be seen at very negative \(V_h\), with increasing current reduction at \(V_h\) of \(+10\) mV (and thus more rapid current “inactivation”); at less negative \(V_h\), greater effects would be seen on peak current, with little greater effect produced by increased depolarization. This hypothesis predicts that drug binding is relatively fast and that maximal binding is achieved at potentials near \(-40\) mV. Our findings that thiobutyrolactone effects on high-voltage-activated currents were relatively low in affinity and that onset and reversal of effects was rapid support this view. Alternatively, thiobutyrolactones may bind in a voltage-independent fashion, but may change the voltage-dependent properties of the target channel, resulting in an increase in steady-state inactivation and/or inactivation at depolarized potentials. Clearly, additional experiments are needed, perhaps with expressed N-type channels, for a fuller explanation of the mechanism of action of these compounds and their voltage dependence on this channel subtype.

**Anticonvulsant action and the block of Ca\(^{++}\) channels.** Butyrolactone analogs afford an opportunity to place in perspective the role of Ca\(^{++}\) channel blockade as an anticonvulsant mechanism of action. Some antiepileptic drugs currently in use have the ability to block Ca\(^{++}\) currents, although in most cases it is not clear whether this action is either necessary or sufficient or even contributes to clinical efficacy. Phenytoin, for example, reduces synaptosomal Ca\(^{++}\) uptake, but does so at concentrations above the therapeutic range (Sohn and Ferrendelli, 1976). Similarly, the barbiturates markedly reduce high-voltage-activated Ca\(^{++}\) currents, but at concentrations that are relevant only in the treatment of status epilepticus, when anesthetic concentrations are achieved (Gross and Macdonald, 1988a,b). The Ca\(^{++}\) channel block is substantial at these concentrations, however, and may account for the cardiovascular suppression commonly seen when patients are under barbiturate anesthesia. The reduction of T-type currents by ethosuximide is the best example of an antiepileptic agent that acts on Ca\(^{++}\) channels at therapeutic concentrations; to date, T-type current reduction is probably the most likely mechanism for ethosuximide’s efficacy in treating primary generalized (absence) seizures (Coulter et al., 1989). Valproic acid may also reduce T-type currents, although whether this contributes to its broad antiseizure efficacy is not certain (Kelly et al., 1990).

In this context, the thiobutyrolactones have significant effects on Ca\(^{++}\) currents, at concentrations within the presumed active range. In particular, the reduction of T-type currents by thiobutyrolactones occurs at lower concentrations than an equivalent reduction of T-type currents by ethosuximide or the thiobutyrolactone-induced reduction of high-voltage-activated currents. These concentrations are similar to the concentrations effective in regulating GABA\(_A\) receptor activity (Holland et al., 1990). The effects on high-voltage-activated Ca\(^{++}\) currents are more complicated. It is worth noting that β-EMTBL, a convulsant compound, not only blocks T-type Ca\(^{++}\) currents but also increases high-voltage-activated Ca\(^{++}\) currents, at concentrations that reduce GABA\(_A\)-mediated inhibition. At high concentrations, β-EMTBL blocks both low-voltage and high-voltage-activated Ca\(^{++}\) currents, but also increases GABA\(_A\)-mediated inhibition (Holland et al., 1995). It would seem likely that, at low concentrations, the block of GABA\(_A\) inhibition by β-EMTBL is by far the more significant clinical (convulsant) effect than its potentially anticonvulsant reduction of T-type Ca\(^{++}\) currents. It is also possible that enhancement of high-voltage-activated currents may be contributing to the convulsant actions of this compound. As for α-EMTBL, its anticonvulsant action is likely caused by enhancement of GABA\(_A\) receptor activity, but may be caused in part by Ca\(^{++}\) current reduction, particularly of T-type currents. It will be interest-
ing to determine whether α-EMTBL, like ethosuximide, is effective for control of absence seizures.

Acknowledgments

The authors thank Robert W. Subiaga, Jr. and Mark J. Gallagher for expert technical assistance.

References


Send reprint requests to: Robert A. Gross, M.D., Ph.D., Departments of Neurology and Pharmacology and Physiology, University of Rochester, Box 711, 601 Elmwood Avenue, Rochester, NY 14642.