Iberiotoxin-Induced Block of Ca\(^{2+}\)-Activated K\(^{+}\) Channels Induces Dihydropyridine Sensitivity of ACh Release from Mammalian Motor Nerve Terminals

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ABSTRACT
The role which Ca\(^{2+}\)-activated K\(^{+}\) (K\(_{Ca}\)) channels play in regulating acetylcholine (ACh) release was examined at mouse motor nerve terminals. In particular, the ability of the antagonist iberiotoxin to recruit normally silent L-type Ca\(^{2+}\) to participate in nerve-evoked release was examined using conventional intracellular electrophysiological techniques. Incubation of cut hemidiaphragm preparations with 10 μM nimodipine, a dihydropyridine L-type Ca\(^{2+}\) channel antagonist, had no significant effect on quantal content of nerve terminals. Nevertheless, 1 μM S-(−)-1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-trifluoromethyl)[phenyl]-3-pyridine carboxylic acid methyl ester (Bay K 8644) enhanced quantal content to 134.7 ± 3.5% of control. Iberiotoxin (150 nM) increased quantal content to 177.5 ± 9.9% of control, whereas iberiotoxin plus nimodipine increased quantal content to only 145.7 ± 10.4% of control. Coapplication of 1 μM Bay K 8644 with iberiotoxin did not significantly increase quantal content further than did treatment with iberiotoxin alone. The effects of iberiotoxin and nimodipine alone or in combination on the miniature end-plate potential (MEPP) frequency following KCl-induced depolarization were examined using uncut hemidiaphragm preparations. Nimodipine alone had no effect on MEPP frequency from preparations incubated in physiological saline containing 5 to 20 mM KCl. Moreover, iberiotoxin alone or combined with nimodipine also had no effect on MEPP frequency in physiological saline containing 5 to 15 mM KCl. At 20 mM KCl, however, iberiotoxin significantly increased MEPP frequency to 125.6% of iberiotoxin-free values; combined treatment with nimodipine and iberiotoxin prevented this increase in MEPP frequency. Thus, loss of functional K\(_{Ca}\) channels unmasks normally silent L-type Ca\(^{2+}\) channels to participate in ACh release from motor nerve terminals, particularly under conditions of intense nerve terminal depolarization.

Release of acetylcholine (ACh) from motor nerves is a highly controlled process that requires precisely controlled entry of Ca\(^{2+}\) through voltage-dependent Ca\(^{2+}\) channels into the nerve terminal (Augustine et al., 1987). Multiple Ca\(^{2+}\) channel subtypes have been described (for review, see Catterall, 1998). Often more than one subtype of Ca\(^{2+}\) channel coexists at the same nerve terminal to control transmitter release (Lemos and Nowycky, 1989; Turner et al., 1993; Elhamdani et al., 1998). Nevertheless, the specific Ca\(^{2+}\) channel phenotype primarily involved in ACh release is both species-dependent (Sano et al., 1987; De Luca et al., 1991; Protti et al., 1996) and age-dependent (Sugiura and Ko, 1997). Release of ACh from mature mammalian motor nerves relies primarily on entry of Ca\(^{2+}\) through P/Q-type (Uchitel et al., 1992; Protti et al., 1996) and not N-type Ca\(^{2+}\) channels, which control release of ACh from motor nerves of amphibians (Sano et al., 1987) and birds (De Luca et al., 1991). Developing mammalian motor nerves, on the other hand, possess multiple subtypes of Ca\(^{2+}\) channels that are involved in the release of ACh, some of which become less important during maturation (Sugiura and Ko, 1997).

L-Type Ca\(^{2+}\) channels, which colocalize with other Ca\(^{2+}\) channels, have been described (for review, see Catterall, 1998). Often more than one subtype of Ca\(^{2+}\) channel coexists at the same nerve terminal to control transmitter release (Lemos and Nowycky, 1989; Turner et al., 1993; Elhamdani et al., 1998). Nevertheless, the specific Ca\(^{2+}\) channel phenotype primarily involved in ACh release is both species-dependent (Sano et al., 1987; De Luca et al., 1991; Protti et al., 1996) and age-dependent (Sugiura and Ko, 1997). Release of ACh from mature mammalian motor nerves relies primarily on entry of Ca\(^{2+}\) through P/Q-type (Uchitel et al., 1992; Protti et al., 1996) and not N-type Ca\(^{2+}\) channels, which control release of ACh from motor nerves of amphibians (Sano et al., 1987) and birds (De Luca et al., 1991). Developing mammalian motor nerves, on the other hand, possess multiple subtypes of Ca\(^{2+}\) channels that are involved in the release of ACh, some of which become less important during maturation (Sugiura and Ko, 1997).

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channel phenotypes, participate in release of noradrenaline from chromaffin cells of the adrenal medulla (Owen et al., 1989) and in the release of oxytocin and vasopressin (Lemos and Nowacky, 1989). Pharmacological evidence has implicated a potential role of normally silent L-type Ca$^{2+}$ channels in release of ACh from mature mammalian motor nerves (Atchison and O’Leary, 1987; Atchison, 1989). Moreover, during certain pathological conditions, L-type Ca$^{2+}$ channels can participate in release of ACh as well (Katz et al., 1996; Santafe et al., 2000; Flink and Atchison, 2002; Giovannini et al., 2002).

The control of ACh release from motor nerves requires not only precise and rapid opening of Ca$^{2+}$ channels at the nerve terminal but also mechanisms to close these channels as well. One such mechanism involves the opening of K$^+$ channels, which return the depolarized membrane to resting potential and thus alter the open state of the voltage-dependent Ca$^{2+}$ channels (Llinás et al., 1981; Augustine, 1990). Three types of K$^+$ currents have been identified at mammalian motor nerve terminals: a slow and fast voltage-dependent K$^+$ current and a Ca$^{2+}$-dependent K$^+$ current (Mallart, 1985; Tabti et al., 1989). Evidence suggests that K$_{Ca}$ channels are not only colocalized with voltage-dependent Ca$^{2+}$ channels at the motor nerve terminal but also probably participate in attenuating transmitter release by contributing to membrane repolarization, thus altering the open state of voltage-dependent Ca$^{2+}$ channels (Mallart, 1985; Robitaille and Charlton, 1992; Robitaille et al., 1993; Xu and Atchison, 1996).

We recently reported that passive transfer of Lambert-Eaton myasthenic syndrome (LEMS), a neuromuscular disorder that causes functional loss of P/Q-type Ca$^{2+}$ channels and thus a decrease in the depolarization-induced entry of Ca$^{2+}$ into the motor nerve terminal (Lambert and Elmqvist, 1971; Fukunaga et al., 1983; Hewett and Atchison, 1991), appears to “unmask” the contribution of L-type voltage-gated Ca$^{2+}$ channels to ACh release (Flink and Atchison, 2002; Giovannini et al., 2002). This is similar to the changes that occur in the phenotype of Ca$^{2+}$ channel involved in transmitter release at reinnervating (Katz et al., 1996) or botulinum toxin-poisoned motor nerves (Santafe et al., 2000). LEMS, however, has neither been shown to damage the nerve terminal nor cause sprouting of newly formed terminals (Fukunaga et al., 1983; Tsujihata et al., 1987). In LEMS, reduced entry of Ca$^{2+}$ into the nerve terminal following membrane depolarization could attenuate activation of K$_{Ca}$ channels. This could, in turn, slow repolarization of the motor nerve terminal and thus increase the probability that colocalized or spatially removed Ca$^{2+}$ channels become involved in ACh release. For example, a component of transmitter release from sympathetic and motor nerves has been shown to depend upon Ca$^{2+}$ entry through L-type channels under conditions of intense stimulation or following inhibition of voltage-dependent K$^+$ currents (Hong and Chang, 1990; Somogyi et al., 1997; Correia-de-Sá et al., 2000a,b). Therefore, the present study was designed to determine whether loss of functional K$_{Ca}$ channels unMASKS normally silent L-type Ca$^{2+}$ channels involved in release of ACh from mammalian motor nerves. To accomplish this, iberiotoxin, a specific antagonist for K$_{Ca}$ channels (Galvez et al., 1990), was used to block K$_{Ca}$ channels, and the resulting sensitivity of release of ACh to DHP-type antagonists such as nimodipine was tested at murine motor nerve terminals.

Materials and Methods

Electrophysiology. Experiments were performed using male ICR mice (20–22 g; Harlan Sprague-Dawley Laboratories, Madison, WI) in accordance with local university (Michigan State University Laboratory Animal Resources) and national guidelines. Animals were sacrificed by decapitation following anesthesia with 80% CO$_2$ and 20% O$_2$. The diaphragm muscle was then removed (Barstad and Liljeheil, 1968) and pinned out at resting tension in a Sylgard-coated chamber. The tissue was perfused continuously at a rate of approximately 1 to 5 ml/min with buffered saline solution containing 137.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 11 mM Na-glucose, 4 mM HEPES, with a pH adjusted to 7.4 at room temperature (23–25°C) using NaOH and kept under continual oxygenation (100% O$_2$). The diaphragm muscle was transected into the two hemidiaphragms, and one hemidiaphragm was then cut approximately 4 mm on either side of the main intramuscular nerve branch to prevent muscle contraction following stimulation of the phrenic nerve (Glavinovic, 1979; Atchison, 1989). This technique does not produce significant changes in the muscle cable properties or neurotransmitter release (Glavinovic, 1979). Depolarization-induced nerve conduction block that occurs when K$^+$ is released from the cut-muscle fibers was prevented by the use of a buffered saline solution, containing 2.5 mM KCl, throughout the experiments (Glavinovic, 1979; Atchison, 1989). Only one hemidiaphragm preparation per mouse was used for any given experiment.

Intact uncut muscle preparations were used in experiments examining the effect of iberiotoxin on asynchronous release of ACh (measured as changes in MEPP frequency) in the presence of varying concentrations of KCl (5–20 mM). Osmolarity was adjusted for changes in concentrations of KCl with an equiosmolar change in the NaCl. All experiments were performed at room temperature of 23–25°C using conventional intracellular recording techniques. EPPs (end-plate potentials) and MEPPs (miniature end-plate potentials) were recorded using borosilicate glass microelectrodes (1.0-mm o.d.; WP Instruments, Sarasota, FL) and having resistance of 5 to 15 MΩ when filled with 3M KCl. The phrenic nerve was stimulated supramaximally at a frequency of 0.5 Hz using a suction electrode attached to a stimulus isolation unit (Grass SIU; Grass Instruments, Quincy, MA) and stimulator (Grass S88). Signals were amplified using a WPI 721 amplifier and digitized into a computer for inspection using Axoscope 8.0 (Axon Instruments, Foster City, CA) software and analyzed using MiniAnalysis 5.0 software (Synaptosoft, Decatur, GA).

Data Analysis and Statistics. Control recordings were first made from untreated muscle preparations, and subsequent recordings were made from the same preparation following incubation with the relevant drug treatment for the time indicated in the figure legends. Recordings from at least five different end-plates from the same neuromuscular junction preparation were used to determine the mean amplitude of the EPPs (average of 10 recordings per endplate) and MEPPs for each drug treatment, yielding an n value of 1. Details of electrophysiological data manipulation and analyses have been published previously (Atchison, 1989). Averaged EPP and MEPP amplitudes were first standardized to a membrane potential of −50 mV to correct for changes in membrane potential driving force. EPPs were then corrected for nonlinear summation using the formula $V_{corr} = V/(1 - 0.8 \cdot V/E)$, where $V$ is the uncorrected EPP amplitude, $E$ is the resting membrane potential, and $V_{corr}$ is the corrected EPP amplitude. Quantal content was calculated using the ratio of the mean amplitude of the corrected EPPs to the mean amplitude of the corrected MEPPs. Statistical significance between the various treatment groups was analyzed using a one-way analysis of variance followed by Tukey’s test. A two-way analysis of variance was used to compare the effect of drug treatments on MEPP ampli-
tudes in the presence of varying concentrations of KCl. P values were set to <0.05 for all statistical tests.

**Drugs and Chemicals.** Nimodipine, S(−)-Bay K 8644, and HEPES were purchased from Sigma-Aldrich (St. Louis, MO). Iberiotoxin was obtained from Alomone Labs (Jerusalem, Israel). All other reagents were of analytical grade or better. Nimodipine and S(−)-Bay K 8644 were prepared as a 20 and 10 mM stock solution, respectively. The final working solution in physiological saline with nimodipine and S(−)-Bay K 8644 contained only 0.05 and 0.01% ethanol (v/v), respectively. Control experiments contained an equivalent concentration of the respective vehicle. Experiments performed in the presence of nimodipine or S(−)-Bay K 8644 were done in the dark to prevent photo-oxidation of these compounds. Iberiotoxin was prepared as a stock solution in distilled water containing 0.01% bovine serum albumin (w/v) and was used within a 2-week period. Before incubation with either toxin, 0.01% bovine serum albumin was added to the buffered saline solution to prevent nonspecific binding of toxin to the chamber, tubing, and glassware.

**Results**

**Effects of K_{Ca} and L-Type Ca^{2+} Channels on Neuromuscular Transmission.** Incubation of cut neuromuscular preparations with iberiotoxin increased quantal content of nerve-evoked release of ACh as shown in sample records (Fig. 1A) and composite data (Fig. 1B) to 177.5 ± 9.9% of control values. Pretreatment of neuromuscular preparations with nimodipine (10 μM), a dihydropyridine type L-type Ca^{2+} channel antagonist, in the presence of iberiotoxin significantly reduced the increase of quantal content observed with iberiotoxin alone to 145.7 ± 10.4% of control (Fig. 1, A and B). The final ethanol concentration (0.05%) used in physiological saline containing nimodipine and iberiotoxin did not significantly affect EPP amplitude, MEPP amplitudes or frequency, or muscle resting membrane potentials in comparison with iberiotoxin alone (data not shown). Similarly, iberiotoxin and nimodipine had no effect on resting membrane potentials, MEPP frequency, or MEPP amplitudes recorded from the cut preparations (Figs. 2, A–C). Finally, incubation of preparations with iberiotoxin vehicle (0.01% bovine serum albumin) alone or with nimodipine also had no effect on quantal content in comparison to untreated controls (Fig. 1C).

Although L-type Ca^{2+} channels do not normally participate in nerve-stimulated release of ACh from adult mammalian motor nerve terminals, the dihydropyridine L-type Ca^{2+} channel agonist, Bay K 8644 has been shown to enhance quantal content at rat motor nerve terminals (Atchison and O’Leary, 1987; Atchison, 1989). Addition of Bay K 8644 (1 μM) to cut neuromuscular preparations enhanced quantal content to 134.7 ± 3.5% of control (Fig. 3B). Treatment of cut preparations with Bay K 8644 plus iberiotoxin did not cause a significantly greater increase in quantal content from the effect of iberiotoxin alone (Fig. 3A).

**Effects of K_{Ca} and L-Type Ca^{2+} Channels on Asynchronous Release of ACh.** The effect of iberiotoxin and nimodipine on asynchronous release of ACh (measured as changes in MEPP frequency) from motor nerve terminals was examined in the presence of varying concentrations of KCl. Resting membrane potentials recorded from uncut muscle preparations in the absence of any drug treatments (control), following incubation with iberiotoxin alone or with nimodipine, were not significantly different from each other (Table 1). Although MEPP amplitudes (Fig. 5B) appeared to increase slightly in the presence of nimodipine as KCl concentration was increased, comparisons of results for all treatment groups within and between different KCl concentrations (5–20 mM) revealed no significant differences among these values (P > 0.05). MEPP frequency, on the other hand, increased in conjunction with the increase in KCl concentrations (Figs. 4, A and B, and 5A). Treatment of preparations with iberiotoxin alone or plus nimodipine had no significant effect on MEPP frequency in comparison to one another or to control treatment in physiological saline containing either 5, 10, or 15 mM KCl (Fig. 4, A and B). When the physiological

### Fig. 1

Iberiotoxin-induced increase in quantal content at mouse neuromuscular junction is due to part of activation of L-type voltage-gated Ca^{2+} channels. A, effects of iberiotoxin on EPP amplitudes at single neuromuscular junctions. Each representative tracing is the average of 10 EPPs at a stimulation frequency of 0.5 Hz, recorded from a single end-plate before (control) after the addition of 150 nM iberiotoxin and following incubation with iberiotoxin (150 nM) and nimodipine (10 μM). Each representative tracing was recorded from the same diaphragm preparation. B, effects of iberiotoxin (IBTx) and nimodipine (nimod); or C, nimodipine in the absence of iberiotoxin on quantal content of mouse hemidiaphragm end-plates. Recordings were made in the absence of any drug treatments (control) and, then subsequently, after 1 h incubation with either 150 nM iberiotoxin or iberiotoxin vehicle (0.01% BSA) alone and then following further incubation with 10 μM nimodipine for 25 min in the presence of iberiotoxin or iberiotoxin vehicle. Paired comparisons are made for each preparation between the drug-free treatment (control), iberiotoxin, or vehicle and then following application of nimodipine in the presence of vehicle or iberiotoxin. Values are expressed as the percentage of quantal content from drug-treated preparations to that of the drug-free control value preparations. Each value represents the mean ± S.E.M. of at least four different preparations. The asterisk (*) and double dagger (‡) indicates a value significantly different from either control or control and iberiotoxin alone, respectively (P < 0.05).
saline contained 20 mM KCl, however, iberiotoxin significantly increased MEPP frequency to approximately 125.6% of control treatment (Fig. 4, A and B). Further addition of nimodipine to preparations in the presence of iberiotoxin significantly reduced the enhancement of MEPP frequency caused initially by iberiotoxin alone. Therefore, normally silent L-type Ca$^{2+}$ channels can participate in ACh release (Xu and Atchison, 1996). Thus, antagonism of K$_{ca}$ channels influences Ca$^{2+}$ current flow and, in turn, enhances the release of ACh.

Further treatment of muscle preparations with nimodipine, a DHP-sensitive L-type Ca$^{2+}$ channel antagonist in the presence of iberiotoxin, reduced the enhanced ACh release caused initially by iberiotoxin alone. Therefore, normally silent L-type Ca$^{2+}$ channels can participate in ACh release from motor nerve terminals lacking functional K$_{ca}$ channels. The observation that nimodipine did not completely abolish the effects of iberiotoxin suggests that the activity or number of any drug treatments (control), then subsequently after 1 h of incubation with either 150 nM iberiotoxin or iberiotoxin vehicle (0.01% BSA) alone, and then following further incubation with 1 μM Bay K 8644 for 25 min in the presence of iberiotoxin or iberiotoxin vehicle. Paired comparisons are made for each preparation between the drug-free treatment (control), iberiotoxin, or vehicle and then following application of Bay K 8644 in the presence of vehicle or iberiotoxin. Values are expressed as the percentage of quantal content from drug-treated preparations to that of control preparations. Each value represents the mean ± S.E.M. of at least four different preparations.

### Table 1

Muscle resting membrane potentials (RMP) from control, iberiotoxin, and iberiotoxin plus nimodipine-treated mouse neuromuscular junction preparations at various levels of KCl-induced depolarization.

<table>
<thead>
<tr>
<th>KCl (mM)</th>
<th>Control</th>
<th>Iberiotoxin$^a$</th>
<th>Iberiotoxin + Nimodipine$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>$-70.1 \pm 2.4^c$</td>
<td>$-71.3 \pm 2.0$</td>
<td>$-70.5 \pm 2.0$</td>
</tr>
<tr>
<td>10</td>
<td>$-60.2 \pm 2.0$</td>
<td>$-60.9 \pm 3.1$</td>
<td>$-60.0 \pm 2.6$</td>
</tr>
<tr>
<td>15</td>
<td>$-53.3 \pm 2.1$</td>
<td>$-51.9 \pm 1.8$</td>
<td>$-51.7 \pm 1.0$</td>
</tr>
<tr>
<td>20</td>
<td>$-45.8 \pm 1.3$</td>
<td>$-42.0 \pm 1.7$</td>
<td>$-43.1 \pm 1.9$</td>
</tr>
</tbody>
</table>

$^a$ Iberiotoxin = 150 nM.

$^b$ Nimodipine = 10 μM.

$^c$ Each value represents the mean RMP ± S.E.M. from at least four preparations for each KCl concentration.

Fig. 2. Effects of iberiotoxin (IBTx) and nimodipine (nimod) on resting membrane potential (A), MEPP frequency (B), and MEPP amplitude (C) at mouse neuromuscular junctions. Recordings were made in the absence of any drug treatments (control), following 1 h of incubation with 150 nM iberiotoxin alone, and following further incubation with 10 μM nimodipine and 150 nM iberiotoxin for an additional 25 min. Each value represents the mean ± S.E.M. of at least four different preparations.

Discussion

Incubation of muscle preparations with iberiotoxin, a K$_{ca}$ channel antagonist, significantly increased quantal content in comparison to untreated terminals. This finding is consistent with those of earlier reports (Robitaille and Charlton, 1992; Robitaille et al., 1993; Vatanpour and Harvey, 1995) and those in which K$_{ca}$ channel block affects perineurial Ca$^{2+}$ currents recorded at motor nerve terminals (Xu and Atchison, 1996). Thus, antagonism of K$_{ca}$ channels influences Ca$^{2+}$ current flow and, in turn, enhances the release of ACh.

Further treatment of muscle preparations with nimodipine, a DHP-sensitive L-type Ca$^{2+}$ channel antagonist in the presence of iberiotoxin, reduced the enhanced ACh release caused initially by iberiotoxin alone. Therefore, normally silent L-type Ca$^{2+}$ channels can participate in ACh release from motor nerve terminals lacking functional K$_{ca}$ channels. The observation that nimodipine did not completely abolish the effects of iberiotoxin suggests that the activity or number
of other Ca\(^2+\) channel subtypes, most likely representing the P/Q-type (Ca\(_{2.1}\)) normally involved in release of ACh, is also increased in the presence of iberiotoxin. Therefore, nimodipine only partially blocks the enhanced effect of iberiotoxin on release of ACh. These findings are consistent with those reported by Hong and Chang (1990) in which L-type Ca\(^2+\) channels (Ca\(_{1.3}\)) are involved in ACh release from mammalian motor nerve terminals pretreated with 3,4-diaminopyridine (DAP), which blocks voltage-dependent \(K^+\) channels. Interestingly, block of L-type Ca\(^2+\) channels reduces the increased duration, but not the enhanced amplitude of EPPs caused by DAP. Unlike DAP, iberiotoxin had no effect on the duration of EPPs but only affects their amplitude. The reason for this difference is unclear but may reflect functional and local differences between voltage-dependent-\(K^+\) channels and K\(_{Ca}\) channels. Block of K\(_{Ca}\) channels, which are colocalized with voltage-gated Ca\(^2+\) channels, enhance transmitter release faster than did block of voltage-gated \(K^+\) channels (Vatanpour and Harvey, 1995). Also, voltage-dependent \(K^+\), but not K\(_{Ca}\) channels, cause repetitive firing of the action potential following a single stimulation (Hong and Chang, 1990; Vatanpour and Harvey, 1995), which may influence the gating of L-type channels. The intimate colocalization of K\(_{Ca}\) with Ca\(^2+\) channels involved normally in release may allow K\(_{Ca}\) channels to have a faster and more direct effect on these Ca\(^2+\) channels than that observed with loss of voltage-gated K\(^+\) channels alone. Our findings and those reported by Hong and Chang (1990) are, however, in contrast with those reported by Giovannini et al. (2002) in which enhanced ACh release from mouse motor nerve terminals in the presence of the voltage-dependent K\(^+\) channel antagonist 4-aminopyridine was unaffected by L-type Ca\(^2+\) channel antagonists. This discrepancy most likely reflects differences in experimental protocols.

The effect of iberiotoxin on asynchronous release of ACh (MEPP frequency) was also examined. Addition of 5, 10, 15, or 20 mM KCl to the physiological saline caused a corresponding depolarization of the endplate resting membrane potential and presumably of the nerve terminal as well. MEPP frequency increased significantly as the resting membrane potential became more depolarized. This was unaffected by iberiotoxin and/or nimodipine in physiological saline containing 5, 10, or 15 mM KCl. In the presence of 20 mM KCl, however, addition of iberiotoxin caused a further significant increase in MEPP frequency, whereas addition of nimodipine in conjunction with iberiotoxin attenuated this effect. The amplitude of MEPPs was unaltered in the presence of iberiotoxin at all concentrations of KCl tested. This indicates that iberiotoxin had no direct or indirect effects on postsynaptic function. Thus, the effect of iberiotoxin on ACh release appears to occur only when the membrane is initially depolarized at KCl concentrations greater than 15 mM. This is consistent with its effect on nerve-stimulated release in which the membrane potential is depolarized markedly from rest. It is also consistent with the biophysical properties of L-type Ca\(^2+\) channels, which require strong depolarization from rest to induce opening.

The exact mechanism involved in unmasking silent L-type
Ca$^{2+}$ channels during block of KCa channels is unclear and may be multifaceted. KCa channels may exert a direct effect by altering localized membrane potentials and, thus, prevent opening of L-type Ca$^{2+}$ channels, which require strong depolarization for activation (Miller, 1987). For instance, recruitment of silent L-type Ca$^{2+}$ channels involved in ACh release is evident during prolonged or high frequency stimulation of motor (Correia-de-Sá et al., 2000a,b) and sympathetic nerves (Somogyi et al., 1997) or during block of voltage-dependent K$^+$ channels (Hong and Chang, 1990). Also, L-type Ca$^{2+}$ channels, which may be located at a site distinct from active zone regions (for example, see Polo-Pareda et al., 2001), may require prolonged periods of openings to allow diffusion of Ca$^{2+}$ through these channels to reach the release machinery (Miller, 1987; Elhamdani et al., 1998).

Alternatively, activation of L-type Ca$^{2+}$ channels in the presence of iberiotoxin may involve more indirect mechanisms. Enhanced release of ACh from mammalian motor nerves by α1 adrenergic-receptor activation is abolished following antagonism of L-type Ca$^{2+}$ channels (Wessler et al., 1990). Intense and prolonged depolarization has also been implicated to enhance Ca$^{2+}$ currents by increasing phosphorylation of L-type Ca$^{2+}$ channels (Sculteouren et al., 1995). In the presence of muscarinic receptor-dependent activation of protein kinase C, L-type Ca$^{2+}$ channel activity at adult rat major pelvic ganglia is increased. Furthermore, it has been postulated that at mammalian motor nerve terminals, L-type Ca$^{2+}$ channels are in close proximity to A2A adenosine receptors and activation of A2A receptors during prolonged depolarization most likely unmask L-type Ca$^{2+}$ channels via activation of protein kinases (Correia-de-Sá et al., 2000a). More direct evidence also supports the role of protein kinases in activating L-type Ca$^{2+}$ channels involved in ACh release from mature mammalian motor nerves (Urbano et al., 2001).

The ability of Bay K 8644, a DHP-sensitive L-type channel agonist to enhance ACh release in comparison to control treatments, further supports the notion that silent L-type channels exist at mammalian motor nerve terminals and corroborates previous reports (Atchison and O’Leary, 1987; Atchison, 1989). Incubation of motor nerve terminals with Bay K 8644 in the presence of iberiotoxin did not enhance further the release of ACh over that seen with iberiotoxin alone. Thus, if iberiotoxin simply unmasked silent L-type channels, then addition of Bay K 8644, which increases the duration of L-type Ca$^{2+}$ channel openings (Nowycky et al., 1985), should have increased further ACh release. It is possible that mechanisms involved in L-type Ca$^{2+}$ channel activation or L-type channel involvement with ACh release become saturated in the presence of iberiotoxin, and thus, Bay K 8644 has no additional effect. Alternatively, iberiotoxin and Bay K 8644 may affect normally silent L-type channels by similar mechanisms, which reach a maximum state of activation in the presence of either drug alone. Although, iberiotoxin does not bind to L-type Ca$^{2+}$ channels, loss of KCa channels in the presence of iberiotoxin may activate secondary pathways that act in a manner similar to that of Bay K 8644 directly. The precise reason for the inability of Bay K 8644 to enhance the iberiotoxin-induced release of ACh is still unclear, however.

In conclusion, loss of functional KCa channels, which presumably delays nerve terminal membrane repolarization, activates normally silent L-type Ca$^{2+}$ channels involved in ACh release at adult mammalian motor nerves. The role that these silent L-type Ca$^{2+}$ channels play at the adult mammalian motor nerve terminal remains unclear but may offer a means to maintain a certain level of ACh release during periods of intense nerve stimulation or in certain pathologi cal conditions in which involvement of Ca$^{2+}$ channels normally responsible for ACh release is impaired.

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