

Different Ability of Clenbuterol and Salbutamol to Block Sodium Channels Predicts Their Therapeutic Use in Muscle Excitability Disorders

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ABSTRACT

Activation of muscle β_2 -adrenergic receptors successfully counteracted sarcolemma inexcitability in patients suffering from hyperkalemic periodic paralysis (HPP), a hereditary disease caused by mutations in the gene encoding the skeletal muscle sodium channel. Looking for potential modulation of these channels by β_2 -adrenergic pathway using patch-clamp technique, we found that clenbuterol blocked sodium currents (I_{Na}) in rat skeletal muscle fibers and in tsA201 cells transfected with the human channel isoform, whereas salbutamol did not. The effects of clenbuterol were independent of β -adrenoceptor stimulation. Instead, clenbuterol structure and physicochemical characteristics as well as I_{Na} blocking properties resembled those of local anesthetics, suggesting direct binding to the

channels. Similar experiments with the chemically similar β -antagonists propranolol and nadolol, suggested the presence of two hydroxyl groups on the aromatic moiety of the drugs as a molecular requisite for impeding sodium channel block. Importantly, clenbuterol use-dependently inhibited action potential firing in rat skeletal muscle fibers, owing to β -adrenoceptor-independent I_{Na} block. From a clinical point of view, our study defines the rationale for the safe use of salbutamol in HPP patients, whereas clenbuterol may be more indicated in patients suffering from myotonic syndromes, a condition characterized by sarcolemmal overexcitability, because use-dependent I_{Na} block can inhibit abnormal runs of action potentials.

Modulation of skeletal muscle function by β -adrenoceptor agonists has long been described (Bowman and Nott, 1969); only recently, however, therapeutic use was proposed on the basis of experimental data, case reports, and rare clinical trials. In particular, the use of clenbuterol or salbutamol (albuterol) was proposed to counteract muscle atrophy that occurs in orthopedic patients, especially in elderly persons or patients in a malnutrition state, as well as in hereditary muscle dystrophies (Maltin et al., 1993; Hayes and Williams, 1998; Zeman et al., 2000; Herrera et al., 2001; Kissel et al., 2001). Potential benefits in these diseases are expected from the β_2 -adrenoceptor-mediated anabolic effects of these drugs (Choo et al., 1992; Hinkle et al., 2002). Interestingly, β_2 -agonists also successfully counteracted muscle paralysis in patients suffering from hyperkalemic periodic paralysis (HPP) (Wang and Clausen, 1976; Hanna et al., 1998). HPP is

a hereditary muscle disorder caused by mutations in the gene encoding the sodium channel expressed exclusively in the skeletal muscle (Cannon, 2001). The genetic defect produces a susceptibility to sarcolemmal depolarization, which renders the fiber inexcitable and leads to frequent attacks of muscle weakness lasting 1 to 3 h. The β_2 -agonists may be able to counteract the guilty membrane depolarization through activation of the electrogenic Na^+K^+ pump (Clausen et al., 1993). Use of salbutamol, metaproterenol, and terbutaline in patients with HPP have been reported, but their relative efficacy in this disorder remains to be established. Differing from periodic paralysis are the myotonic syndromes that are characterized by sarcolemmal overexcitability, which are common to a number of hereditary diseases resulting from various genetic defects, including sodium channelopathies (Moxley, 2000; Cannon, 2001; Meola, 2002). Local anesthetic-like drugs, such as mexiletine, prove useful in myotonic patients, owing to use-dependent block of sodium channels (Moxley, 2000). To our knowledge,

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ABBREVIATIONS: HPP, hyperkalemic periodic paralysis; PKA, cyclic AMP-dependent protein kinase; PKC, calcium- and phospholipid-dependent protein kinase; I_{Na} , sodium currents; hSkM1, human skeletal muscle sodium channels; K_R , drug affinity constant for closed sodium channels; K_I , drug affinity constant for inactivated sodium channels; hp, holding potential; CPT-cAMP, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate; H-89, *N*-[2-(*p*-bromocinnamylamino)-ethyl]-5-isoquinoline-sulfonamide; QX-314, *N*-(2,6-dimethylphenylcarbomylmethyl)-triethylammonium chloride.

there have been no reports about the use of adrenergic agents in myotonia, most probably because sodium channel block by these drugs was not expected in skeletal muscle.

The β_2 -subtype is the predominant β -adrenergic receptor expressed in skeletal muscle (Liggett et al., 1988). This G protein-coupled receptor exerts its physiological function through phosphorylation of specific effectors, such as ion channels, by cyclic AMP-dependent protein kinase (PKA) (Yang and McElligott, 1989). For instance, β_2 -agonists were shown to increase sodium channel activity in cardiac myocytes through the classic PKA-dependent pathway but also through a membrane-delimited pathway involving direct interaction between G α s protein and the channel (Matsuda et al., 1992; Lu et al., 1999). Nothing is known about possible similar mechanisms in the skeletal muscle.

In a previous study, we showed that two membrane-permeable analogs of cyclic AMP inhibited sodium currents (I_{Na}) in cell-attached patches of rat skeletal muscle fibers. The effect was not mimicked by externally-applied cAMP and persisted in the presence of the PKA inhibitor *N*-[2-(*p*-bromocinnamylamino)-ethyl]-5-isoquinoline-sulfonamide (H-89), indicating that cAMP acted within the cell to block skeletal muscle sodium channels independently of PKA activation (Desaphy et al., 1998). In the present study, we sought to determine whether β_2 -adrenoceptor agonists might increase cyclic AMP level sufficiently to block sodium channels. We found that clenbuterol but not salbutamol inhibited I_{Na} in rat skeletal muscle fibers or in tsA201 cells expressing the human skeletal muscle sodium (hSkM1) channels. We also showed that sodium channel block by clenbuterol can affect action potential property in skeletal muscle fibers. Those effects were independent of β_2 -adrenoceptor stimulation and did not involve PKA, calcium- and phospholipid-dependent protein kinase (PKC), or cyclic AMP. Thus, we propose that clenbuterol directly blocked sodium channels in a manner similar to local anesthetic drugs, and we defined some structural requirements in β -agonists and antagonists for obtaining such an effect. Because of the differences in blocking muscle sodium channels, salbutamol should be safely used in periodic paralysis patients, whereas clenbuterol may be more indicated in patients suffering from myotonic syndromes.

Materials and Methods

All experiments involving animals were conducted in accordance with the Italian Guidelines for the use of laboratory animals, which conform with the European Community Directive published in 1986 (86/609/EEC).

Sodium Current Measurement in Rat Skeletal Muscle Fibers. Fibers were enzymatically dissociated from flexor digitorum brevis muscles of adult rats, and sodium currents were recorded at room temperature in the cell-attached configuration of the patch-clamp method, as described previously (Desaphy et al., 1998a). Bath solution contained 145 mM CsCl, 5 mM EGTA, 1 mM MgCl₂, 10 mM HEPES, and 5 mM glucose. Pipette solution contained 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM HEPES. Both solutions were buffered at pH 7.3. In these conditions, muscle fibers were depolarized near 0 mV such that the cell-attached patch potential was close to that held by the AxoPatch 1D amplifier (Axon Instruments, Union City, CA). The patch generally contained tens of sodium channels, allowing recording of macroscopic-current-like sodium currents (I_{Na}) with rapid onset and total inactivation in less than 3 ms. Patches with I_{Na} exhibiting >15% run-down in 20 min or

anomalous activation and inactivation voltage-dependence were discarded from analysis (Desaphy et al., 1998a). Voltage-clamp protocols and data acquisition were performed using pCLAMP 6.0 software (Axon Instruments) through a digidata 1200 analog/digital interface. Current were low-pass filtered at 2 kHz (−3 dB) by the amplifier four-pole Bessel filter and digitized at 10 to 20 kHz.

Sodium Current Measurement in tsA201 Cells. The tsA201 cells were cotransfected with 10 μ g of plasmid DNA encoding the full-length hSkM1 cDNA and lower amount of a plasmid DNA encoding CD8 receptors using the calcium-phosphate precipitation method, as described previously (Desaphy et al., 2001). Successfully transfected cells were identified using Dynal microbeads coated with anti-CD8 antibody (Dynal A.S., Oslo, Norway). I_{Na} were recorded at room temperature using the whole-cell, patch-clamp method (Desaphy et al., 2001). Bath solution contained 150 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, and 5 mM glucose, and the pH was set to 7.4 with NaOH. The pipette solution contained 120 mM CsF, 10 mM CsCl, 10 mM NaCl, 5 mM EGTA, and 5 mM HEPES, and the pH was set to 7.2 with CsOH. Peak I_{Na} amplitudes ranging from 0.8 to 6 nA, stable series resistance errors less than 5 mV, and current run-down less than 5% within the experiment were our limiting criteria to consider the data for analysis.

Action Potential Measurement in Rat Skeletal Muscle Fibers. Action potentials were recorded in vitro in rat skeletal muscle fibers as described previously (Desaphy et al., 1998b). Briefly, the extensor digitorum longus muscles were dissected from animals under urethane anesthesia and fixed through tendons in a recording chamber containing a 95% O₂/5% CO₂-gassed physiological solution. Action potentials were elicited in current-clamp mode using two intracellular microelectrodes. The membrane potential was held at −80 mV by injecting a steady current, and 100-ms depolarizing current pulses of increasing amplitude were applied up to elicit first a single action potential (threshold) and then a train of action potentials.

Drugs and Chemicals. Salbutamol, clenbuterol, DL-propranolol, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (CPT-cAMP), H-89, staurosporine, and okadaic acid were purchased from Sigma (Milan, Italy). QX-314 was a gift from Alomone Labs (Jerusalem, Israel). These compounds were directly dissolved in bath or pipette solution at the desired concentration, except for H-89 and staurosporine, which were first dissolved in dimethyl sulfoxide and then diluted in recording solutions. The final concentration of dimethyl sulfoxide did not exceed 0.1% and had no effect on sodium currents.

Average data are presented as mean \pm S.E.M. and statistical analysis was performed using Student's *t* tests for grouped data, considering *p* < 0.05 as significant.

Results

Effects of β_2 -Agonists on Sodium Currents in Rat Skeletal Muscle Fibers. Macroscopic-current-like sodium currents were elicited in cell-attached patches of rat skeletal muscle fibers by test pulses to the potential of −20 mV applied from a holding potential (hp) of −100 mV every 2 s (Desaphy et al., 1998a). Five minutes after seal formation, the peak I_{Na} amplitude was sufficiently stable to allow drug testing. As shown in Fig. 1A, bath application of 500 μ M salbutamol for about 5 min had no effect on I_{Na} . In five cells, the peak I_{Na} amplitude in the presence of salbutamol was 97.4 \pm 3.0% of control. Such an effect was not distinguishable from the spontaneous run-down observed in these experimental conditions (Desaphy et al., 1998a). In contrast to salbutamol, the other β_2 -adrenoceptor agonist, clenbuterol (500 μ M), reduced I_{Na} to 36.8 \pm 3.0% of control in four muscle fibers (Fig. 1B). The effect of clenbuterol was quite fully

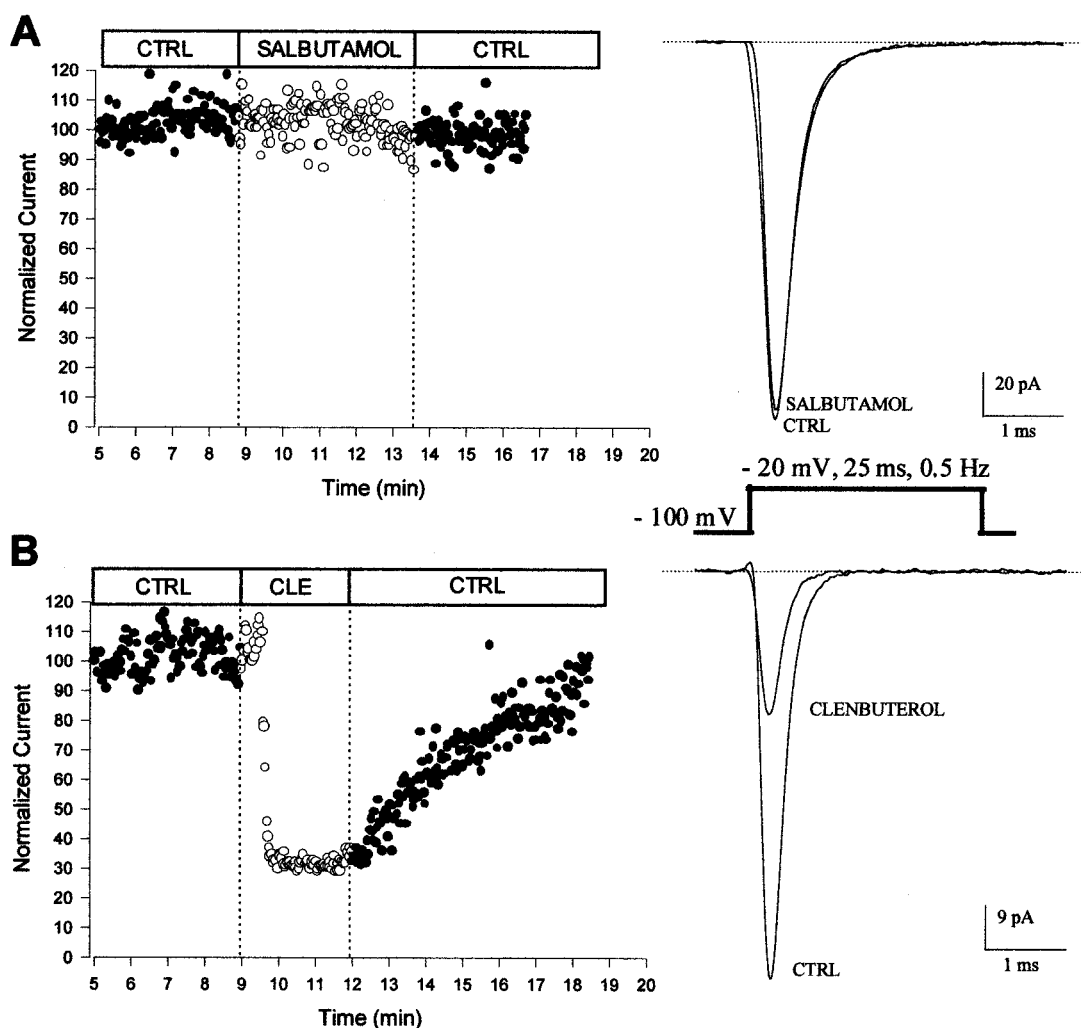


Fig. 1. Effects of β_2 -adrenoceptor agonists on sodium current amplitude in rat skeletal muscle fibers. Sodium currents were elicited in cell-attached patches by 25-ms depolarizing pulses to -20 mV applied every 2 s from a holding potential of -100 mV. Peak sodium current amplitude was reported as a function of time in control conditions (CTRL) and during external application of $500 \mu\text{M}$ salbutamol (A) or $500 \mu\text{M}$ clenbuterol (B). Ensemble average sodium currents were constructed from 10 consecutive traces recorded in absence (CTRL) and in presence of the drug.

reversible in 5 to 6 min, as shown in Fig. 1B. Normalized current-voltage relationships measured in three cells tested for clenbuterol effect are shown in Fig. 2A. Under control conditions, the current-voltage curve activated at -60 mV, peaked at -20 mV, and reached zero-current level at $+70$ mV. Clenbuterol reduced I_{Na} at all voltages and did not modify the voltage at which current amplitude was maximal. The voltage dependence of the activation curve was not modified by the drug (Fig. 2B), suggesting that no change in fiber V_m occurred in response to $500 \mu\text{M}$ clenbuterol. The midpoint potentials for activation were -40.9 mV in control and -42.8 mV in presence of clenbuterol. In contrast, clenbuterol shifted the voltage dependence of steady-state fast inactivation toward negative potentials, as assessed using a two-pulse protocol including a 200-ms conditioning pulse (Fig. 2C). The -8.5 mV shift of the half-maximum inactivation potential induced by the drug was larger than the spontaneous negative shift we generally observed in cell-attached patch recordings (i.e., usually -2 mV in 10 min) (Desaphy et al., 1998a). The effect of clenbuterol was dose-dependent because $1500 \mu\text{M}$ clenbuterol reduced peak I_{Na} to $10.7 \pm 6.0\%$ of control ($n = 4$, $p < 0.001$ versus $500 \mu\text{M}$). To further evaluate the role of PKA in current inhibition by clenbuterol, we applied the drug in presence of $10 \mu\text{M}$ H-89, a specific inhibitor of the kinase (Fig. 3). In 3 fibers, H-89 alone applied

externally for 10 min had no effect on I_{Na} , whereas further application of $1500 \mu\text{M}$ clenbuterol still reduced peak current with potency similar to that observed in the absence of H-89.

Effects of β_2 -Adrenoceptor Agonists and Antagonists on Human Skeletal Muscle Sodium Currents Expressed in tsA201 Cells. Wild-type hSkM1 channels were transiently expressed in tsA201 cells, and the resulting I_{Na} were recorded with patch-clamp technique in the whole-cell configuration (Desaphy et al., 2001). Externally applied clenbuterol produced both tonic and use-dependent block of I_{Na} elicited by depolarizing pulses to -30 mV from an hp of -120 mV (Fig. 4). Tonic block was assayed 3 min after drug application by measuring the reduction of I_{Na} elicited at 0.1 Hz, whereas use-dependent block was further obtained by increasing stimulation frequency to 10 Hz. In the presence of $100 \mu\text{M}$ clenbuterol, I_{Na} was reduced to 40% (tonic block) and 20% (10-Hz block) of control current (Fig. 4A). The inhibitory effect of clenbuterol was dose-dependent, with IC_{50} values of $76 \mu\text{M}$ for tonic block and $26 \mu\text{M}$ for 10 Hz-block (Fig. 4B). The Hill coefficients calculated from the fitting functions were close to unity, thereby indicating a 1:1 stoichiometry.

In tsA201 cells, the effect of clenbuterol was not mimicked by $500 \mu\text{M}$ CPT-cAMP, a membrane-permeable analog of cyclic AMP (Fig. 5A). This contrasts with the inhibitory effect of this compound on native I_{Na} recorded in skeletal muscle

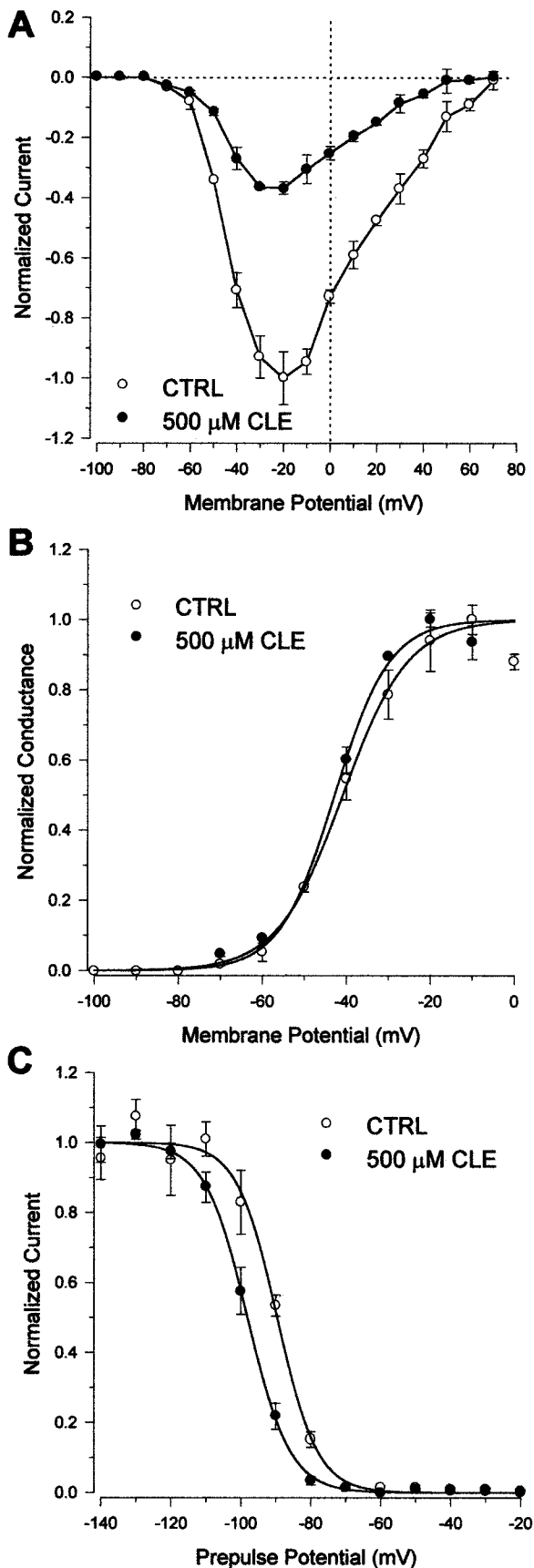


Fig. 2. Effects of clenbuterol on sodium current voltage-dependence in rat skeletal muscle fibers. **A**, current-voltage relationships constructed before (CTRL) and after application of 500 μ M clenbuterol (CLE). The

fibers (Desaphy et al., 1998a). The lack of CPT-cAMP effect on heterologously expressed sodium channels was not caused by an endogenous activation of phosphatases in the system of expression, because it persisted in presence of 300 nM okadaic acid. Also, application of 10 μ M H-89 had no effect on I_{Na} in tsA201 cells, suggesting that basal phosphorylation of the channels had not masked effect of CPT-cAMP. The effects of 100 μ M clenbuterol assayed in the presence of either 10 μ M H-89 or 1 μ M staurosporine in the pipette solution were not significantly different from that observed with standard pipette solution, indicating that neither PKA nor PKC were involved in the inhibitory effect of the drug (Fig. 5B). To verify whether clenbuterol effect depends on β_2 -adrenoceptor stimulation, the drug was applied in presence of 10-fold more concentrated nadolol, a specific β -antagonist (Fig. 5B). Nadolol by itself had no effect of hSkM1 channels, and the effect of 100 μ M clenbuterol measured in presence of the β -antagonist was very similar to that recorded with clenbuterol alone.

All together, the results described so far suggested a direct interaction of clenbuterol with sodium channels that resembles that of local anesthetics. Use-dependent block of I_{Na} and negative shift of channel availability are generally observed for drugs having greater affinity for the inactivated channel compared with the closed channel. At the hp of -120 mV, a small proportion of the channels are inactivated (Fig. 6A) and the tonic block measured at this hp most probably reflects the combination of binding to resting (closed) and inactivated channels, as described previously for the local anesthetic drug, mexiletine (Desaphy et al., 2001). Determination of affinity constant for inactivated channels (K_I) is complicated by the existence of several kinetically-different inactivated states and superimposing of kinetics for drug binding/unbinding and inactivation development/recovery (for recent review, see Takahashi and Cannon, 2001). In an attempt to determine K_I for clenbuterol, we measured the shift of sodium channel steady-state availability as a function of clenbuterol concentration (Bean et al., 1983), and calculated a K_I value of 19 μ M (Fig. 6B). Another way to estimate K_I is to measure block of depolarized channels (Nau et al., 1999; Takahashi and Cannon, 2001). To dissociate channel inactivation from drug block, both occurring during a depolarized prepulse, it is necessary to include a recovery period before to

patches were held at -110 mV and depolarized every 10 s to potentials ranging from -100 to $+70$ mV, applied in 10-mV increments. Each data point is the mean \pm S.E.M. from three patches. **B**, activation curves were constructed from current-voltage relationships by converting current (I_{Na}) to conductance (g_{Na}) using the equation $g_{Na} = I_{Na}/(V_m - E_{Na})$, where V_m is the membrane potential and E_{Na} is the equilibrium electrochemical potential for sodium ions, estimated to be $+70$ mV. Activation curves were fitted with the Boltzmann equation, $g_{Na}/g_{Na,max} = 1/(1 + \exp[(V_m - V_{1/2})/K])$, to determine the half-maximum activation potential ($V_{1/2}$) and the slope factor (K). In control, the values of $V_{1/2}$ and K along with the S.E. of the fit were -40.9 ± 0.9 mV and 7.7 ± 0.8 mV, respectively. With 500 μ M clenbuterol, $V_{1/2}$ was -42.8 ± 0.9 mV and K was 6.5 ± 0.8 mV. **C**, availability curves for sodium current were constructed using a standard double-pulse protocol. The patches were held at -110 mV and received a 200-ms conditioning prepulse ranging in amplitude from -140 to -20 mV followed by a test pulse to -20 mV. Data points were calculated as the mean \pm S.E.M. of three patches and were reported as a function of the prepulse potential. The inactivation relationships were fitted with the Boltzmann equation, $I_{Na}/I_{Na,max} = 1/(1 + \exp[(V_m - V_h)/K])$ to determine the half-maximum inactivation potential (V_h) and the slope factor (K). The values of V_h and K along with the S.E. of the fit were -89.6 ± 0.6 mV and 5.8 ± 0.6 mV in control conditions. With 500 μ M clenbuterol, V_h was -98.1 ± 0.2 mV and K was 6.1 ± 0.2 mV.

apply a test pulse. The recovery period should be long enough for channels to recover from inactivation but insufficient for recovery from drug block. We first measured recovery time of hSkM1 channels at -120 mV in the absence and in the presence of $100 \mu\text{M}$ clenbuterol (Fig. 6C). In the absence of clenbuterol, most of the channels ($>97\%$) recovered from fast inactivation with a single exponential time constant ($\tau_1 = 2.02 \pm 0.06$ ms). Clenbuterol introduced a second, longer exponential time constant ($\tau_2 = 11.2 \pm 3.3$ s). It is clear from Fig. 6C that a recovery period of 35 ms allowed recovery from inactivation without affecting the proportion of drug-bound channels. We thus measured block of channels depolarized for 1.5 s at -70 mV, a conditioning pulse at which about 75% of the channels are inactivated, using a recovery period at -120 mV for 35 ms (Fig. 6D, inset). This protocol applied in absence of drug produced less than 5% channel block, whereas a dose-dependent block was observed in the presence of clenbuterol with an IC_{50} value of $\sim 30 \mu\text{M}$ (Fig. 6D). A

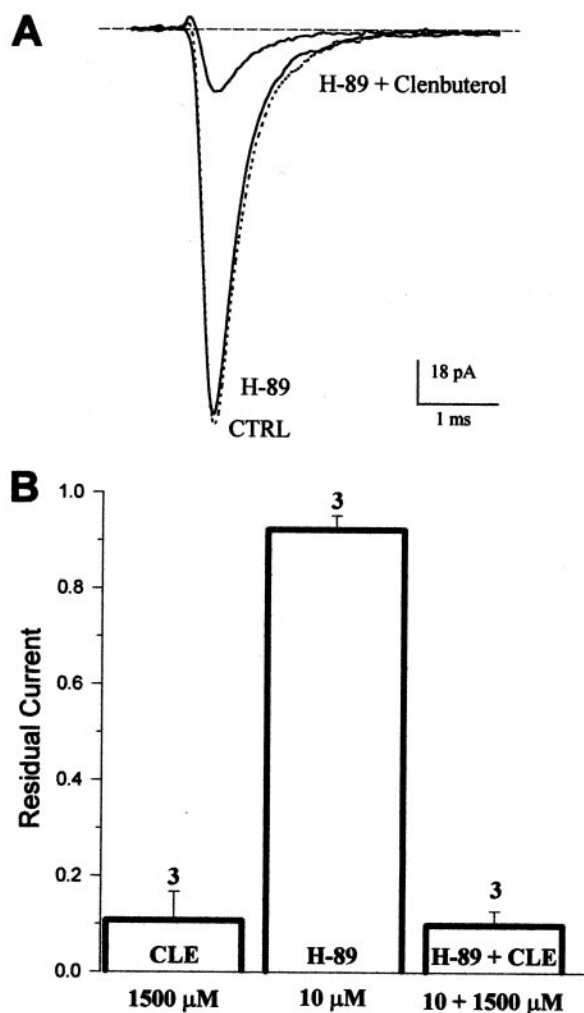


Fig. 3. Effects of clenbuterol on sodium currents in rat skeletal muscle fibers in presence of the cyclic AMP-dependent protein kinase inhibitor, H-89. A, ensemble average sodium currents were constructed from 10 consecutive traces elicited from -100 to -20 mV in a cell-attached patch exposed to control bath solution (CTRL, dashed line), then to $10 \mu\text{M}$ H-89, and then to $10 \mu\text{M}$ H-89 + $1500 \mu\text{M}$ clenbuterol. B, the protocol described in A was repeated in three patches and data, normalized with respect to control current, were averaged as mean \pm S.E.M., and reported together with average data obtained from three patches exposed to $1500 \mu\text{M}$ clenbuterol alone.

quite similar block was obtained using a shorter conditioning pulse duration of 1 s, indicating that steady-state block of fast inactivated channels was reached (not shown). On the other hand, prolonging the conditioning pulse to 2 s produced a greater reduction of sodium current, most probably because of development of slow inactivation (not shown). To evaluate clenbuterol affinity for closed sodium channels (K_R), we constructed concentration-response curves for tonic block from an hp of -180 mV. At this potential, the entire population of hSkM1 channels is in the closed state, ready to open in

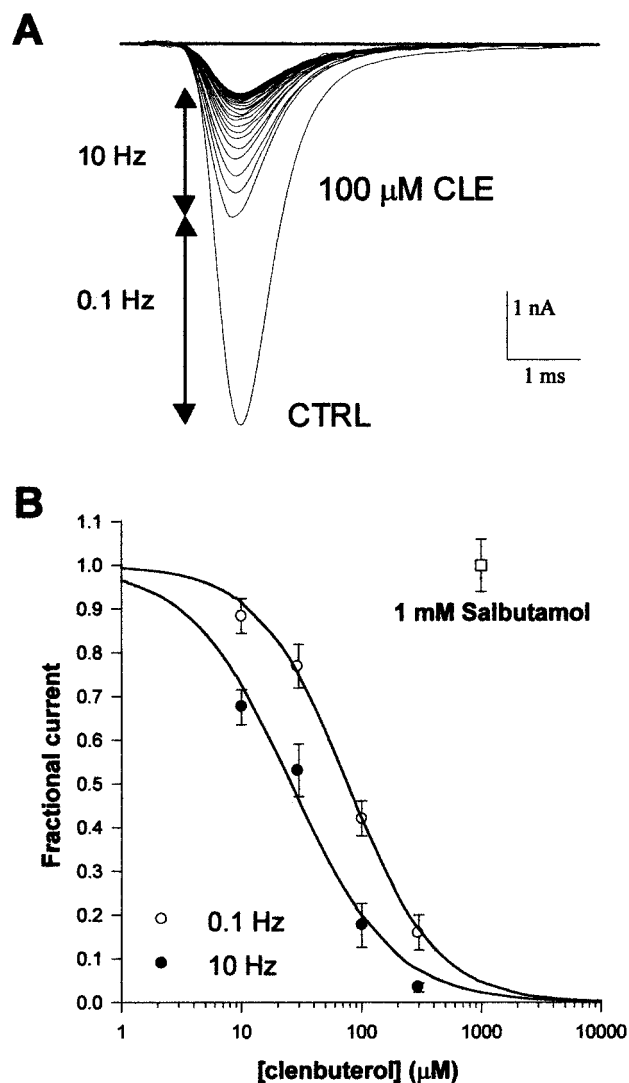


Fig. 4. Dose-dependent effects of clenbuterol on human skeletal muscle sodium currents in tsA201 cells. A, whole-cell I_{Na} were recorded in tsA201 cells transiently transfected with the hSkM1 channel. The cells were held at -120 mV and 25-ms test pulses were applied to -30 mV. Currents were recorded under control conditions, 3 min after application of $100 \mu\text{M}$ clenbuterol at a low frequency stimulation (0.1 Hz), and during high-frequency stimulation (10 Hz). B, dose-response relationships were constructed using the protocol described in A at both 0.1 Hz (tonic block) and 10 Hz (use-dependent block). Each data point was calculated as the mean \pm S.E.M. from 4 to 13 cells. The relationships were fitted with the Hill binding function, $I_{\text{drug}}/I_{\text{control}} = 1/[1 + ([\text{drug}]/\text{IC}_{50})^{n_H}]$, to calculate the half-maximum inhibitory concentration (IC_{50}), and the logistic slope factor (n_H). For tonic block, the values of IC_{50} and n_H together with the S.E. of the fit were $76.4 \pm 5.0 \mu\text{M}$ and 1.17 ± 0.09 , respectively. For use-dependent block (10 Hz), IC_{50} was $25.9 \pm 4.9 \mu\text{M}$ and n_H was 1.03 ± 0.21 . Effect of 1 mM salbutamol (mean \pm S.E.M., $n = 3$) obtained in the same experimental conditions is also reported for comparison.

response to depolarization. The K_R of clenbuterol calculated from the first-order binding function was 242 μM (Fig. 6D). Using the K_R value and the IC_{50} value calculated for depolarized channels, a value of K_I was estimated from the modulated receptor model equation: $1/\text{IC}_{50} = h/K_R + (1 - h)/K_I$, where the terms h and $(1 - h)$ are the proportions of closed and inactivated channels at the potential considered (Bean et al., 1983). The value for h in the cells used for IC_{50} determination at -70 mV was 0.25, which gives a K_I value of ~ 23 μM .

Direct interaction of β -adrenoceptor antagonists, including propranolol, with cardiac sodium channels was proposed on the basis of their effect on the maximum upstroke velocity of action potential (Ban et al., 1985; Courtney, 1990). Although we failed to find an effect of nadolol on hSkM1 channels, the previous studies suggested that other β -antagonists may block I_{Na} . We choose to test propranolol because chemical differences with nadolol were comparable with those between salbutamol and clenbuterol (Table 1). The external application of 1 mM propranolol greatly inhibited I_{Na} elicited to -30 mV at 0.1 Hz from an hp of -120 mV, the effect being rapid and fully reversible (Fig. 7A). Both tonic (0.1 Hz) and use-dependent (10 Hz) blocks were observed in a dose-dependent manner, with IC_{50} values of 69 and 8 μM , respectively (Fig. 7B).

Thus, in contrast to nadolol, propranolol did block I_{Na} and was even more potent than clenbuterol in producing use-dependent block. As already mentioned, block of sodium channels by clenbuterol and propranolol was very similar to that produced by the local anesthetic mexiletine. It is generally admitted that binding of local anesthetic drugs to their putative molecular receptors within the ion-conducting pore of skeletal muscle sodium channels requires the drugs to cross the cell membrane and to reach their binding sites from the intracellular mouth of the pore (Hille, 2001). As shown in Table 1, the presence of two hydroxyl groups on the aromatic

moiety of salbutamol and nadolol greatly reduces the lipophilicity (Log P) of these drugs compared with the sodium channel blockers, suggesting that the externally applied compounds may be retained outside the cell by the plasma membrane before to reach their binding site. To verify this hypothesis, we compared the effects of salbutamol and nadolol with those of the membrane-impermeant quaternary derivative of lidocaine, QX-314 (Frazier et al., 1970). The drugs were diluted in the pipette solution, which allowed direct access to the intracellular side of the channels. Potential effect of the drugs was assayed by measuring use-dependent block of I_{Na} (Fig. 8). In the presence of 300 μM QX-314, use-dependent block of I_{Na} developed to $\sim 50\%$ of control. In contrast, neither 1 mM salbutamol nor 1 mM nadolol modified I_{Na} in response to 10-Hz stimulation.

Effects of β_2 -Agonists and Antagonists on Action Potentials of Rat Skeletal Muscle Fibers. We looked at the effect of clenbuterol on action potential behavior in rat skeletal muscle fibers by means of two intracellular microelectrodes (Desaphy et al., 1998b). The membrane potential was clamped to -80 mV before to apply depolarizing currents of increasing amplitude up to elicit a single action potential and then a train with the maximal number of action potentials. After collection of data in control conditions, clenbuterol was applied to the muscle, and action potentials were recorded after a short delay of ~ 5 min. At the concentration of 3 μM , clenbuterol had no significant effect on the single action potential but reduced by $\sim 50\%$ the maximum number of spikes elicited (Fig. 9B). At 30 μM , clenbuterol reduced the amplitude of the single action potential to $\sim 80\%$ of control and completely inhibited action potential firing (Fig. 9A). In the presence of 300 μM clenbuterol, only 3 fibers of 7 were able to elicit a single action potential, which was $\sim 65\%$ of control amplitude (Fig. 9C). Thus the inhibitory effect of clenbuterol on action potential was dose-dependent and use-dependent, because the drug affected the number of spikes at

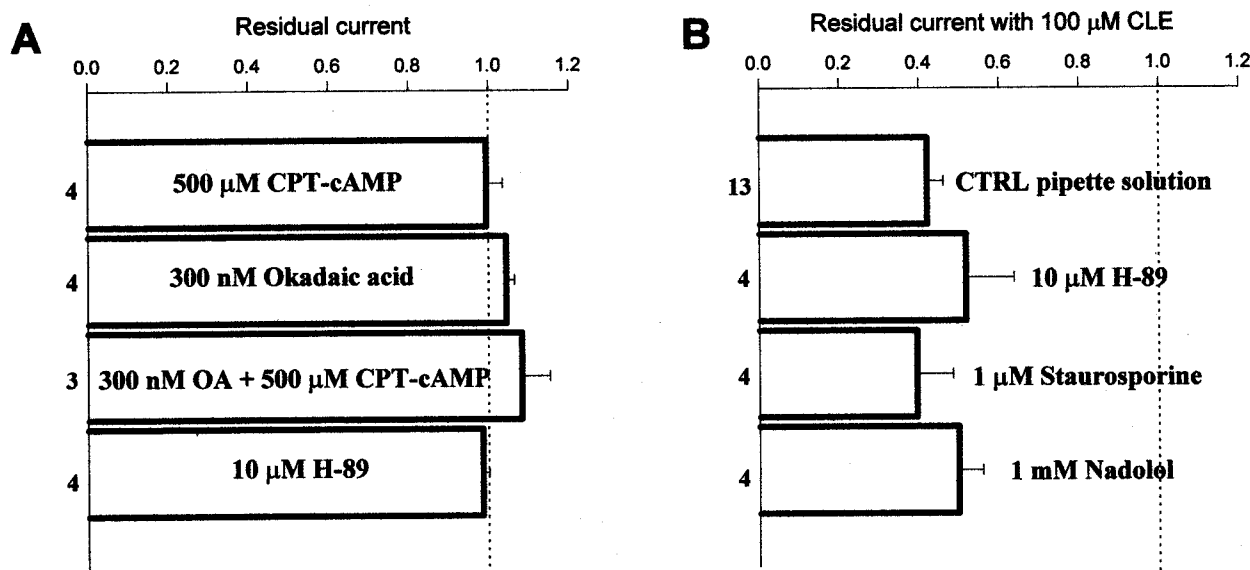


Fig. 5. No role for cyclic AMP, PKA, PKC, and β -adrenergic receptor in the inhibitory effect of clenbuterol on human skeletal muscle sodium currents. A, whole-cell I_{Na} were recorded in tsA201 cells transiently transfected with hSkM1 channel. The cells were held at -120 mV and received a 25-ms depolarizing pulse to -30 mV every 10 s. Each bar represents the mean \pm S.E.M. from the number of cells indicated on the left of the bar of the residual current ($I_{\text{drug}}/I_{\text{control}}$) measured 4 to 5 min. after external application of 500 μM CPT-cyclic AMP, 300 nM okadaic acid, 300 nM okadaic acid + 500 μM CPT-cyclic AMP, and 10 μM H-89. B, effects of 100 μM clenbuterol was measured as in A in patches containing control pipette solution alone, or supplemented with 10 μM H-89 or 1 μM staurosporine, or in the presence of 1 mM external nadolol.

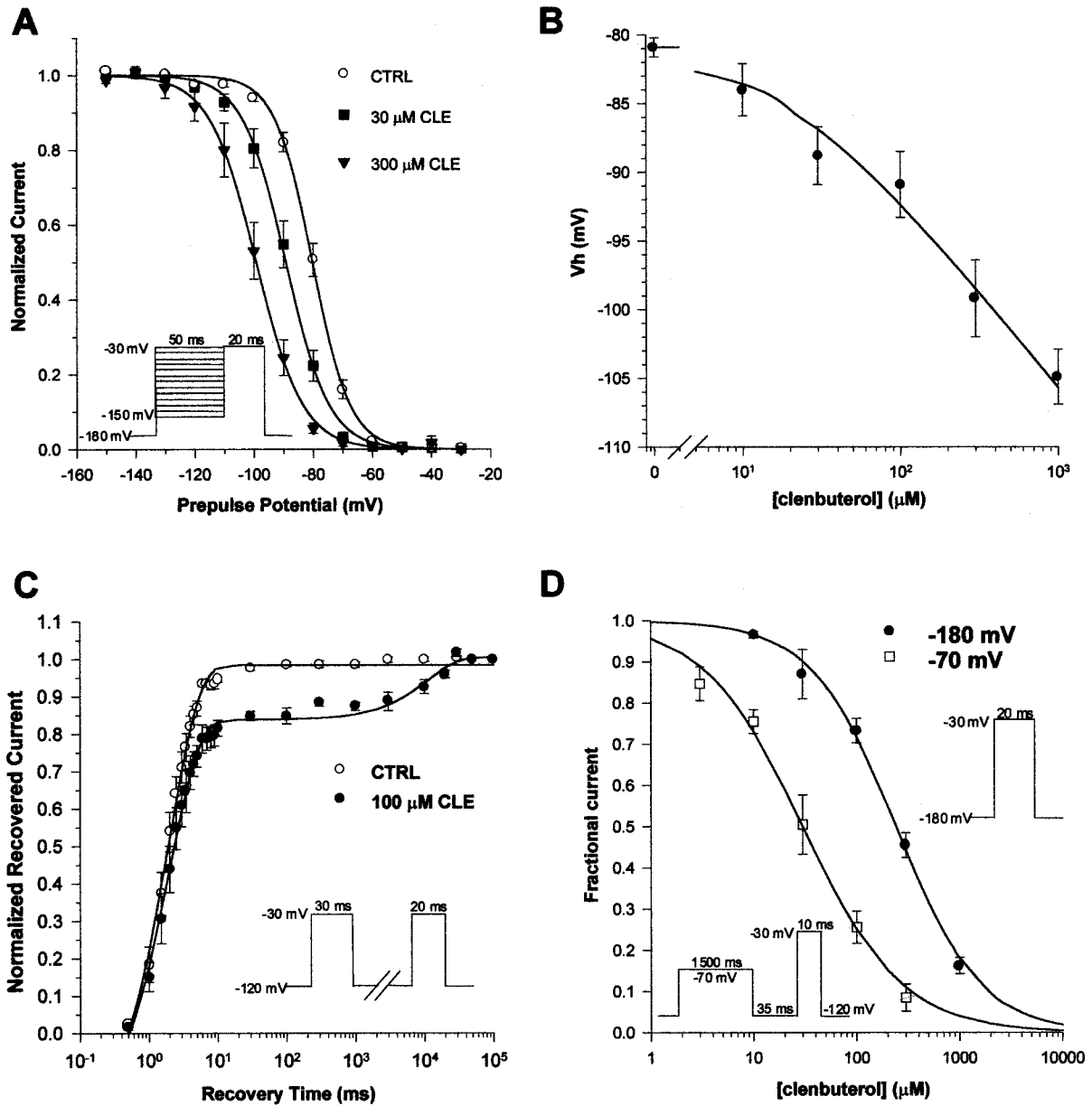


Fig. 6. State-dependent affinities of human skeletal muscle sodium channels for clenbuterol. **A**, voltage-dependence of I_{Na} availability in tsA201 cells transfected with hSkM1 channel. I_{Na} were evoked by a 20-ms test pulse to -30 mV after 50-ms conditioning prepulses to potentials ranging from -150 to -30 mV in 10-mV increments. Pulses were delivered at 10-s intervals and hp was -180 mV. The peak I_{Na} recorded during the test pulse was normalized with respect to the maximal I_{Na} , and means \pm S.E.M. were calculated from n cells to be plotted against the prepulse potential. The relationship was determined in control conditions (CTRL) and in the presence of various concentrations of clenbuterol. Only the effects of 30 and 300 μM clenbuterol are shown. The relationships were fitted with the Boltzmann equation as in Fig. 2. The values of V_h and K along with the S.E. of the fit were -80.2 ± 0.3 mV and 6.3 ± 0.2 mV in CTRL ($n = 13$). In the presence of 30 μM clenbuterol, V_h was -89.2 ± 0.2 mV and K was 7.3 ± 0.3 mV ($n = 10$). In presence of 300 μM clenbuterol, V_h was -99.2 ± 0.3 mV and K was 7.9 ± 0.2 mV ($n = 4$). **B**, the affinity of clenbuterol for inactivated channels (K_I) was estimated by plotting the half-maximum inactivation potential (V_h), determined as in **A**, as a function of clenbuterol concentration. Each data point was the mean \pm S.E.M. from 4 to 33 cells. The relationship was fitted with the equation, $V_h = K_{\text{CTRL}} \times \ln(1/(1 + ([\text{drug}]/K_I))) + V_{h,\text{CTRL}}$, where K_{CTRL} and $V_{h,\text{CTRL}}$ were the values of K and V_h measured in control conditions. The values of K_I determined along with the S.E. of the fit was 18.8 ± 2.1 μM . **C**, recovery from inactivation and clenbuterol block of hSkM1 channels. The cells were held at -120 mV. A recovery pulse at the hp of increasing duration was included between two test pulses at -30 mV. The peak I_{Na} recorded during the second test pulse was normalized with respect to the peak I_{Na} recorded during the first test pulse and means \pm S.E.M. were calculated from n cells to be plotted against the recovery time. The relationship determined in control conditions (CTRL) was fitted with a monoexponential function, $I(t) = A_0 + A_1 \times [1 - \exp(-t/\tau_1)]$, whereas the relationship determined in presence of 100 μM clenbuterol was fitted with a two-exponential function, $I(t) = A_0 + A_1 \times [1 - \exp(-t/\tau_1)] + A_2 \times [1 - \exp(-t/\tau_2)]$, using the value of τ_1 determined in CTRL. Fit parameters with the S.E. of the fit were $A_0 = -0.27 \pm 0.03$, $A_1 = 1.25 \pm 0.03$, $\tau_1 = 2.02 \pm 0.06$ ms, $A_2 = -0.24 \pm 0.02$, $A_1' = 1.08 \pm 0.02$, $A_2' = 0.17 \pm 0.01$, and $\tau_2 = 11.2 \pm 3.3$ ms. **D**, dose-response curves for depolarized and closed channels in tsA201 cells. The affinity of clenbuterol for depolarized channels was determined by eliciting I_{Na} during a test pulse at -30 mV after a 1.5-s depolarization at -70 mV followed by a 35-ms recovery period at -120 mV. Peak I_{Na} measured in presence of clenbuterol was normalized with respect to control peak I_{Na} and each data point is the mean \pm S.E.M. from at least four cells. The dose-response relationship was fitted using the Hill binding function, with $\text{IC}_{50} = 30.1 \pm 2.7$ μM and $n_H = 0.91 \pm 0.07$. The affinity of clenbuterol for closed channels (K_R) was determined by holding the cells at -180 mV and measuring the dose-response relationship at 0.1-Hz stimulation frequency. Each data point was calculated as the mean \pm S.E.M. from 3 or 4 cells. The relationship was fitted using the Hill binding function, $I_{\text{drug}}/I_{\text{control}} = 1/(1 + ([\text{drug}]/K_R)^{n_H})$. The values of K_R and n_H together with the S.E. of the fit were 242.3 ± 15.0 μM and 1.06 ± 0.07 , respectively.

TABLE 1

Chemical structures and physicochemical properties

p*K*_a and ionization refer to the amine group of the drugs. Log P and p*K*_a were calculated using Advanced Chemistry Development Software Solaris v4.67; ionization was calculated from Henderson-Hasselbalch equation.

Compound	Structure	M.W. (free base)	Log P	p <i>K</i> _a	Ionization (mol%, pH 7.4)
Clenbuterol		277.19	2.612 ± 0.382	13.29 ± 0.20	>99.9
Salbutamol		239.31	0.015 ± 0.301	9.83 ± 0.20	99.6
Propranolol		259.34	3.097 ± 0.193	13.84 ± 0.20	>99.9
Nadolol		309.40	1.288 ± 0.344	13.91 ± 0.2	>99.9
Mexiletine		179.26	2.160 ± 0.228	8.58 ± 0.20	93.4

lower concentrations than those required to affect the single action potential. As expected from patch-clamp data, the effect of clenbuterol on action potentials was also independent of β_2 -adrenoceptor stimulation, because it persisted in presence of nadolol (Fig. 9D).

Discussion

Looking for potential modulation of skeletal muscle sodium channels by the β -adrenergic signaling pathway using patch-clamp technique, we observed that the β -adrenergic receptor

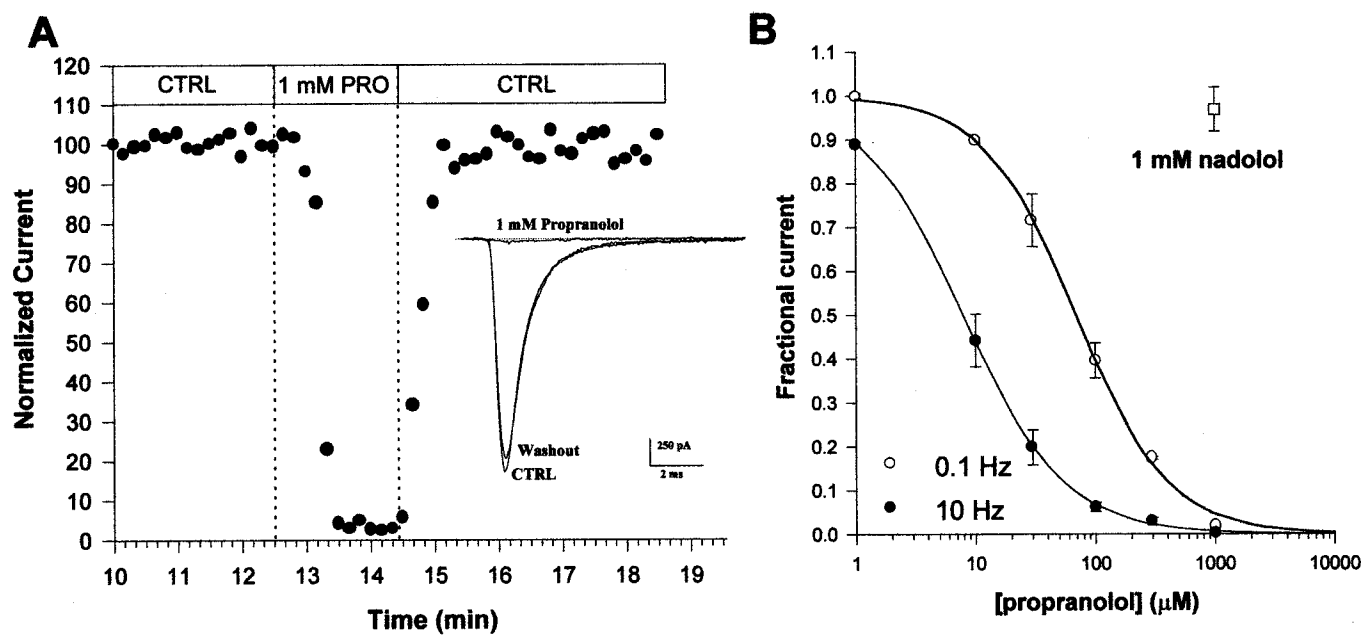


Fig. 7. Effects of the β -adrenoceptor antagonist, propranolol, on human skeletal muscle sodium channels in tsA201 cells. Whole-cell I_{Na} were elicited in tsA201 cells transiently transfected with hSkM1 channels. The cells were held at -120 mV and received a 25 ms-long depolarizing pulse to -30 mV every 10 s. **A**, peak sodium current amplitude was reported as a function of time in control conditions (CTRL) and during external application of 1 mM propranolol. Inset are shown I_{Na} traces recorded before, during, and after (washout) application of propranolol. **B**, dose-response relationships were constructed using the protocol described in **A** at both 0.1 Hz (tonic block) and 10 Hz (use-dependent block). Each data point was calculated as the mean \pm S.E.M. from three to five cells. The relationships were fitted with the Hill binding function, $I_{drug}/I_{control} = 1/(1 + ([drug]/IC_{50})^{n_H})$, to calculate the half-maximum inhibitory concentration (IC_{50}), and the logistic slope factor (n_H). For tonic block, the values of IC_{50} and n_H together with the S.E. of the fit were 68.9 ± 2.8 μ M and 1.13 ± 0.05 , respectively. For use-dependent block (10 Hz), IC_{50} was 7.9 ± 0.2 μ M and n_H was 1.03 ± 0.02 . Effect of 1 mM nadolol (mean \pm S.E.M., $n = 4$) obtained in the same experimental conditions is also reported for comparison.

agonist clenbuterol blocked I_{Na} in native rat skeletal muscle fibers or in tsA201 cells expressing the human skeletal muscle sodium channel. This effect was independent of β -adrenoceptor modulation and rather resembled the sodium channel block by local anesthetic-like drugs, thereby suggesting direct binding of the drug to the channels. In contrast, the β -agonist salbutamol had no effect on I_{Na} . Such observation may have important implications for the therapeutic use of these drugs. For example, this difference between the two drugs defines the rationale for the use of salbutamol in patients suffering from periodic paralysis (sarcolemmal inexcitability), whereas clenbuterol may be more indicated in patients presenting myotonic syndromes (sarcolemmal overexcitability).

Although membrane-permeable analogs of cyclic AMP inhibited sodium currents in rat muscle fibers (Desaphy et al., 1998a), the nucleotide CPT-cAMP had no effect on skeletal muscle sodium channels expressed in tsA201 cells, as reported by others (Bendahhou et al., 1995). As discussed elsewhere (Desaphy et al., 1998a), such a difference suggests that the heterologous system of expression lacks a component present in skeletal muscle fibers and responsible for the effect of cAMP on sodium channels. β -Adrenergic stimulation with salbutamol was not able to increase cyclic AMP sufficiently to block sodium channels in our experimental conditions, thereby raising concerns about the physiological significance of sodium channel direct modulation by the nucleotide.

Nevertheless, the β_2 -agonist clenbuterol produced a rapid and reversible block of rat and human skeletal muscle sodium channels. This effect persisted in presence of PKA or PKC inhibitors, was not mimicked by salbutamol, and was

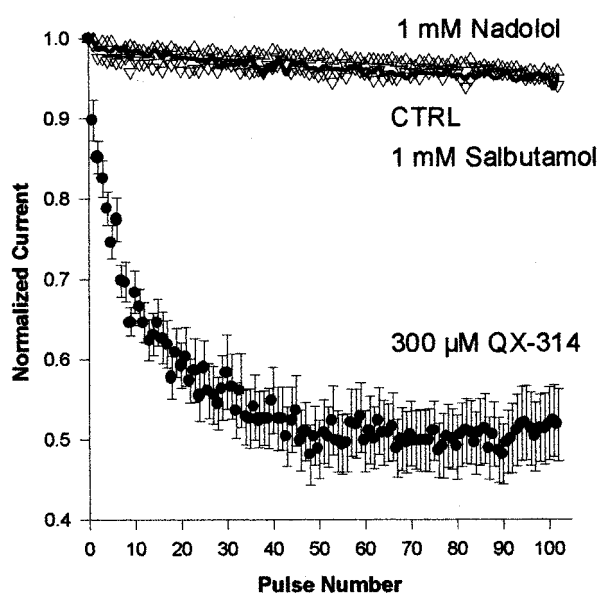


Fig. 8. Development of use-dependent block after internal diffusion of control pipette solution (CTRL, plain line), or pipette solution supplemented with 1 mM nadolol (Δ), 1 mM salbutamol (∇), or 300 μ M QX-314 (\bullet). The hSkM1-transfected tsA201 cells were held at -120 mV and received a 25-ms depolarizing pulse to -30 mV every 10 s to elicit I_{Na} . This protocol was applied about 5 min after achieving whole-cell configuration to allow pipette solution to diffuse well within the cell. Peak I_{Na} measured at each test pulse was normalized with respect to the first pulse I_{Na} . Each data point is the mean \pm S.E.M. from five cells in each condition. The S.E.M. bars are omitted for CTRL, nadolol, and salbutamol to improve clarity.

not antagonized by nadolol, a β -adrenoceptor antagonist. Together, these data indicate that the inhibitory effect of clenbuterol on I_{Na} was independent of β -adrenoceptor stimulation. On the other hand, the effect of clenbuterol on I_{Na} was very similar to that of local anesthetic drugs that bind and block sodium channels, including a 1:1 stoichiometry, voltage- and use-dependent properties, and negative shift of voltage-dependence of sodium channel availability (Ragsdale et al., 1996). Interestingly, use-dependent block of sodium channels was also observed in cardiomyocytes (Fischer et al., 2001). Such properties have been explained by the modulated receptor hypothesis that forecasts the drug binding dependence on channel state as a result of change in receptor affinity (Hille, 2001). Using specific voltage clamp protocols, we estimated the affinities for closed and inactivated channels to be ~ 240 and ~ 20 μ M, respectively. For comparison, using the same expression system, a typical inactivated-channel blocker such as mexiletine showed closed-channel affinity of ~ 800 μ M and inactivated-channel affinity of ~ 7 μ M (Desaphy et al., 2001). It should be noted that higher clenbuterol concentrations were required to block I_{Na} in skeletal muscle fibers to the same extent as in tsA201 cells, although voltage-clamp protocols should be more favorable to block in the native system, where less negative hp and higher frequency of stimulation were used. Hypothetic causes for such include differences in receptor affinity between the rat and the human sodium channels or differences in intracellular medium (for muscle fiber) and experimental solutions between the two systems. Importantly, inhibition of muscle action potential firing was obtained in more physiologic conditions (e.g., hp = -80 mV) with clenbuterol concentrations lower than those required to block I_{Na} in cell-attached patches of muscle fibers, as described below.

The putative molecular receptor for local anesthetic-like drugs includes amino acids of the S6 segments of domains I, III, and IV that face the ion-conducting pore of voltage-gated sodium channel α -subunits (Ragsdale et al., 1994; Nau et al., 1999; Wang et al., 2000; Yarov-Yarovoy et al., 2001; 2002). It was proposed that the two pharmacophore moieties of many local anesthetics, constituted of an uncharged aromatic ring and a charged tertiary amine, may bind to amino acid side chains through hydrophobic and cation- π interactions, respectively (Ragsdale et al., 1994). Interestingly, clenbuterol also presents a hydrophobic ring at one extreme and an amine group at the other end (Table 1). The ring confers to clenbuterol a lipophilicity comparable with that of mexiletine, as evidenced by the Log P value. Moreover, the pK_a of clenbuterol is very similar to that of mexiletine, and drug molecules are mostly protonated at physiological pH. Thus, molecular structure, physicochemical properties, and sodium channel block feature of clenbuterol strongly suggest that the drug binds to the sodium channel at the local anesthetic receptor.

It has long been hypothesized that β -adrenoceptor antagonists may exert part of their antiarrhythmic action by blocking directly cardiac sodium channels. Indeed direct interaction of β -adrenoceptor antagonists, including propranolol, with sodium channels was proposed on the basis of $^{22}Na^+$ uptake measure in rat brain membrane (Matthews and Baker, 1982), cardiac action potential modulation (Ban et al., 1985; Courtney, 1990), and 3H -batrachotoxin-A 20- α -benzoate binding studies to rat cerebrocortical synaptosomes

(Chidlow et al., 2000). The present study confirms inhibition of I_{Na} by propranolol using patch clamp technique. As clenbuterol, propranolol blocked human sodium channels in a use-dependent manner and shifted negatively the voltage dependence of channel availability (not shown). The IC_{50} value for tonic block at a hp of -120 mV was similar to that

of clenbuterol, whereas use-dependent block was three-fold more pronounced with the β -antagonist. The structure of propranolol that includes a strongly lipophilic naphthalene moiety and a protonated amine fulfills the general structural requirements for sodium channel binding and block by local anesthetic-like drugs, as described above for clenbuterol.

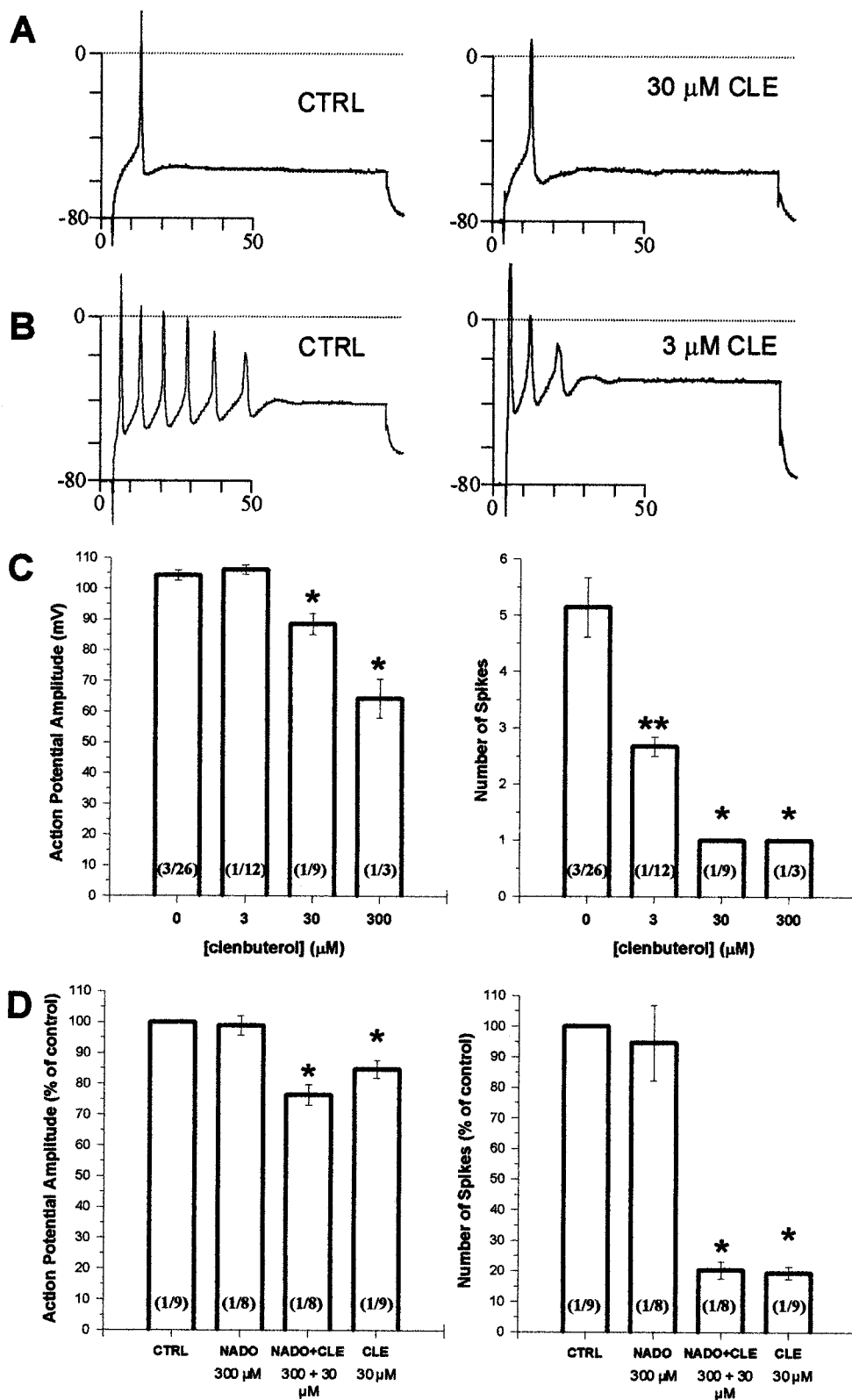


Fig. 9. Effects of clenbuterol on action potentials in rat skeletal muscle fibers. Action potentials were recorded in rat muscle fibers using two-micro-electrode current clamp method. A, representative single action potentials elicited by threshold current in absence (CTRL) and presence of 30 μ M clenbuterol (CLE). B, representative train of action potentials elicited by subthreshold current in absence (CTRL) and presence of 3 μ M clenbuterol (CLE). C, amplitude of single action potential elicited as in A (left) and maximum number of spikes obtained as in B (right), in the absence or presence of 3, 30, and 300 μ M clenbuterol, are reported as means \pm S.E.M. from n fibers of N rats, indicated in parenthesis as (N/n). D, amplitude of single action potential elicited as in A (left) and maximum number of spikes obtained as in B (right), were measured in control conditions (CTRL), in presence of 300 μ M nadolol (NADO), and then in presence of 300 μ M nadolol and 30 μ M clenbuterol (NADO+CLE) and reported as percentage of control. For comparison, effect of 30 μ M clenbuterol alone (CLE) is also reported. Each bar is the mean \pm S.E.M. from n fibers of N rats, indicated as (N/n). Statistical differences were assessed with unpaired Student's t test (*, $p < 0.001$; **, $p < 0.005$).

In contrast to clenbuterol and propranolol, salbutamol and nadolol had no effect on I_{Na} . From Table 1, it seems that the two inactive compounds are characterized by the presence of two hydroxyl groups on the aromatic moiety that render them far less lipophilic compared with clenbuterol and propranolol. It can be hypothesized that the hydroxyl groups may impede the hydrophobic interaction between the aromatic moiety and the local anesthetic receptor. Interestingly, it should be noted that most of the β_2 -agonists present two hydroxyl groups on their aromatic moieties, and that the atypical clenbuterol may be the unique β_2 -agonist able to block sodium channels. On the other hand, nadolol is the unique β -adrenoceptor antagonists with two hydroxyl groups on the aromatic moiety, suggesting that sodium channel blocking activity may be shared by many β -antagonists, as suggested by previous studies (Matthews and Baker, 1982; Ban et al., 1985; Courtney, 1990; Chidlow et al., 2000).

Consistent with its blocking action on sodium channels, clenbuterol inhibited action potentials in skeletal muscle fibers. This effect was use-dependent because 3 μ M clenbuterol drastically reduced the number of spikes without affecting the amplitude of a single action potential. Clenbuterol effect persisted in presence of the β -adrenoceptor antagonist nadolol; thus, inhibition of action potential firing most probably resulted from direct block of sodium channels by the drug. Clenbuterol concentration can reach 1 to 2 ng/g in skeletal muscles of rats treated with 1 mg/kg body weight/day, which is the safe therapeutic clenbuterol dose in humans (Zeman et al., 2000; Von Deutsch et al., 2002). Such a concentration is quite lower than that we used to block depolarized channels, but caution should be used in comparing the results obtained in the heterologous system of expression with clinical data. Interestingly, the K_1 value for clenbuterol is near that measured under the same experimental conditions for mexiletine ($\sim 7 \mu$ M; Desaphy et al., 2001) and flecainide ($\sim 15 \mu$ M; J.-F. Desaphy and D. Conte Camerino, unpublished observations), two drugs used with success in myotonic patients. Thus, higher doses of clenbuterol may affect tissue excitability; such an effect may occur especially in conditions of hyperexcitability owing to use-dependent block of sodium channels. Interestingly, such a mechanism of action was recently proposed for the beneficial effect of clenbuterol in various seizure models of experimental epilepsy (Fischer et al., 2001). At the skeletal muscle level, sarcolemmal hyperexcitability leads to myotonia, a condition of muscle stiffness shared by various genetic muscle diseases (Cannon, 2001; Moxley, 2000; Meola, 2002). On the basis of our results, it would be important to verify the therapeutic potential of clenbuterol in the myotonic syndromes. In particular, because of the possibility of combining antimyotonic activity with its well known anabolic action, clenbuterol might be remarkably indicated in the treatment of myotonic dystrophy, the most common hereditary disease of skeletal muscle, characterized by muscle wasting together with permanent or fluctuans myotonia (Meola, 2002). On the other hand, because sodium channel block may accentuate paralysis, clenbuterol should not be administered to patients with HPP, whereas other β_2 -agonists, such as salbutamol, have proven to be beneficial in those patients, most probably because β_2 -adrenoceptor stimulation activates the Na,K-AT-Pase and consequently hyperpolarizes the muscle fiber

(Wang and Clausen, 1976; Clausen et al., 1993; Hanna et al., 1998).

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