Single-walled Carbon Nanotubes Are a New Class of Ion Channel Blockers*

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Here we identify a novel class of biological membrane ion channel blockers called single-walled carbon nanotubes (SWNTs). SWNTs with diameter distributions peaked at ~0.9 and 1.3 nm, C60 fullerenes, multi wall nanotubes (MWNTs), and hyperfullerenes (nano-onions) were synthesized by several techniques and applied to diverse channel types heterologously expressed in mammalian cells. External as-fabricated and purified SWNTs blocked K+ channel subunits in a dose-dependent manner. Blockage was dependent on the shape and dimensions of the nanoparticles used and did not require any electrochemical interaction. SWNTs were more effective than the spherical fullerenes and, for certain diameters, SWNTs retained their blocking efficiency (data not shown). Annealed nanotubes retained their blocking efficiency (data not shown), indicating that blocking can be attributed to nanotubes alone and not to other chemical factors. The identification of new classes of molecules to target ion channels is of significant interest in biological research, and, therefore, we sought to explore the possibility of using novel materials such as selected single-walled carbon nanotubes (SWNTs),† as ion channel blockers. In recent years there have been several attempts to use nanotubes for biological purposes because of their unique mechanical, chemical, and electrical properties. For example, nanotubes have been successfully used for the helical crystallization of proteins and the growth of embryonic rat brain neurons (4) and as potential biosensors and bioreactors (5, 6). Here we show that SWNTs of certain diameters can efficiently block K+ channels.

MATERIALS AND METHODS

Nanotubes and Fullerene Synthesis—SWNTs, with a diameter distribution peaked in the 0.8–0.9 nm range, were grown by chemical vapor deposition (CVD) in a horizontal tube reactor using a three-stage process. In the first stage, the catalyst/support system was obtained via wet mixing followed by combustive calcination. Magnesium nitrate hexahydrate, cobalt nitrate hexahydrate, ammonium heptamolybdate tetrahydrate, and citric acid (the latter to induce combustion) were mixed with enough distilled water to give a clear solution, which was heated to 550 °C for 5–10 min in air. The resulting powder of general composition MgO1-x,Co1-xMox (where nanoscale Co-Mo is the catalyst in a typical molybdenuco/balt atomic ratio of 1:4, and MgO is the catalyst support) was taken out, ground to a fine consistency, and placed in a quartz boat in the CVD reactor. The deposition reaction zone was pumped down to 10−5 torr, back-filled with pure hydrogen to 1 atm, and the temperature was raised to 500 °C for 30 min. The temperature was then raised to 700 °C, and pure CO was introduced at 1 atm with a flow rate of 100 standard cubic centimeters per minute. The SWNT growth was carried out for 20 min. For purification, the as-prepared SWNTs, together with the catalyst and support, were immersed in 6 M hydrochloric acid, shaken for a few minutes, centrifuged, and decanted. The purified SWNTs were dispersed again in 6 M hydrochloric acid, sonicated for 5–10 min, centrifuged, and decanted. This process was repeated twice. The purified SWNTs were then washed 2–3 times with de-ionized water, filtered, and dried. X-ray diffraction and x-ray electron spectroscopy of the SWNT powder showed the absence of MgO and catalyst particles. The purified SWNTs (consisting only of SWNTs as determined by micro-Raman scattering spectroscopy and x-ray diffraction) were used after dispersion to a uniform and stable suspension by ultrasonic agitation for 1 min in a basic aqueous solution (pH near 10) containing 1 volume percentage of Triton X-100 surfactant. The purification of the Tubes@Rice SWNTs was carried out with 45 h of reflux in 2–3 M nitric acid to remove amorphous carbon and amorphous onions. Following reflux, the suspension was centrifuged, and the supernatant fluid was decanted. The SWNT sediment was washed, re-suspended, centrifuged, and again decanted. This process was repeated 3–4 times. The purified SWNTs were dispersed in a pH 10 aqueous solution containing 0.5 volume percentage of Triton X-100 by ultrasonic agitation for ~1 h. Stock solutions were prepared by rinsing the nanomaterials in water several times (until they formed clusters indicating removal of Triton) and resuspended in CHAPS. To confirm that this treatment was sufficient to remove Triton, nanotubes in which Triton was removed via annealing in a nitrogen atmosphere at 300 °C for 2 h were tested on HERG channels. The absence of the Triton subsequent to annealing was confirmed by Fourier transform infrared spectroscopy. Annealed nanotubes retained their blocking efficiency (data not shown), indicating that blocking can be attributed to nanotubes alone and not to the Triton.

Electrophysiology—CHO cells were transiently transfected with...
to form onion-like clusters (Fig. 1D) or multi-wall nanotubes (MWNT) (Fig. 1E). The range of diameters of the onions and MWNTs used in this study were 3–5 and 10–15 nm, respectively. To assay whether nanotubes, C_{60} fullerene, and fullerene-like onions can interact with ion-channels, we conducted electrophysiological experiments on different pore-forming channel subunits heterologously expressed in mammalian (CHO) cells. Surprisingly, as-fabricated and purified SWNTs had a significant effect on channels formed by C. elegans EXP-2, KVS-1, human KCNQ1 and Kv4.2 (not shown) as well as HERG potassium channels but not on endogenous CHO cell CIC-3 channels (Fig. 2, A–E) (5, 7–11). Qualitatively, these molecules acted to inhibit each current to a different extent, with KCNQ1 and HERG being the more susceptible proteins. Here, we use HERG as a representative example. Effects on EXP-2, Kv4.2, KVS-1, and KCNQ1 were significant but not conceptually different from those on HERG and are not covered in detail. Fig. 2E shows whole-cell HERG currents recorded in the absence and the presence of the SWNTs in the test solutions. SWNT inhibition was reversible, suggesting that the interaction takes place within extra-cellular domains of the channel. Calculated dose-response relationships for SWNTs with an average diameter of 1.3 nm fitted well to the Hill equation with K_i = 0.023 ± 0.003 mg/ml and a Hill coefficient n = 1.0 ± 0.1, suggesting that the blockade is not cooperative (Fig. 2F).

HERG channels open when depolarized to positive voltages; they are described as inwardly rectifying, however, because net ion movement through these channels is inward with symmetrical K^+. This conduction mechanism is the consequence of two singular gating characteristics, rapid inactivation and slow deactivation (12, 13), as shown by the sequence shown in Equation 1,

\[ C \circ O = I \]  

(1)

where C, O and I represent the closed, open, and inactivated state respectively. Rapid inactivation means that, when the channel is opened by depolarization, it enters a non-conducting (inactivated) state passing very little current. In contrast, when the membrane potential is returned to its resting value (repolarization), the channel probably retraces its conformational steps and passes through the open state on the way back to the closed configuration. The return to the closed state, or deactivation, is slow and, therefore, the channel spends a significant time in the conducting open state. SWNTs exerted several effects on HERG gating (Fig. 3, A–F). They lowered the half-maximal threshold for activation (Fig. 3A) and speeded deactivation at any voltage (−2-fold; Fig. 3, B and C). These gating modifications, however, can account only marginally for the instantaneous decrease of the tail current (Fig. 2E) that appears to be the principal cause of blocking. We noticed that depolarization increased current inhibition (Fig. 3D), which was also dependent on the duration of the depolarizing pulse (Fig. 3E). Although sustained depolarization produced larger tail currents (by moving an increasingly large number of channels into the inactivated state), in the presence of SWNTs this effect was significantly suppressed (Fig. 3E). These observations can be interpreted by assuming that either the SWNTs impair the forward transition from the open to the inactivated state (O → I) or that, alternatively, they impair the reverse transition (I → O) by stabilizing the inactive state. In the presence of SWNTs, the number of inactivated channels was significantly increased compared with control conditions (Fig. 3F), and, thus, we conclude that the blockade results from the combined effect of stabilizing the channel in the inactivated state and accelerating deactivation kinetics. Other mecha-
nisms seem less likely; for example, a process in which the SWNTs prevent the channel to open (\(C_{3O}\)) could be possible in principle but would challenge the observation that depolarization facilitates block (Fig. 3D). The fact that as-fabricated and purified SWNTs are chemically inert suggests that the geometry and not the chemical nature of the molecule must be the primary factor governing blockade. Fig. 3G shows the dependence of the inhibition constant \(K_i\), assumed to be an indicator of blocking efficiency, as a function of nanotube/nanoparticle diameter for HERG, KCNQ1, and KVS-1. The extent of the block varies among channel types, but in all cases blocking is stronger with small diameters and is suppressed with large diameter nanostructures (MWNTs did not exert any block; in Fig. 3 the \(K_i\) is fixed to the astronomical 1 kg/ml value for fitting purposes). Thus, SWNTs having an average diameter of 0.9 nm blocked better than those with a diameter of 1.3 nm with \(K_i(0.9)/K_i(1.3)\) ratios of 1.9, 1.5, and 1.4 for HERG, KCNQ1, and KVS-1, respectively. Block was affected not only by the dimensions but also by the shape of the molecule. In Fig. 3H we compare the effects of fullerenes and nanotubes at a fixed concentration (0.1 mg/ml) in the test solution. Both small diameter nanotubes (i.e. SWNTs) and the \(C_{60}\) fullerene blocked HERG channels and, significantly, the former were roughly 3- to 2-fold more efficient. We conclude that geometrical factors play a key role in determining a blockade whose relative intensity is probably affected by structural differences among the various channel types.

Docking simulations with the crystal structure of KcsA (Fig. 4A) provide insights into the geometrical basis of these interactions (14). In KcsA, a fullerene with a diameter of 0.72 nm can fit into the mouth of the selectivity filter and, like a cork in a bottle, obstruct the flow of ions (Figs. 4B-C). Similarly, larger onions or MWNTs (that have diameters \(\geq 3\) nm) would be too big to fit into this or other channel crevices. The case of SWNTs is more intriguing, considering that these structures block more efficiently than fullerenes (Fig. 3G). SWNT synthesis and purification can result in two physically distinct and co-existing species, namely SWNTs having spherically shaped closed ends (Fig. 4D) and those in which one or both ends are open (Figs. 4E-F). Thus, the same considerations used for fullerenes can be evoked to explain the interaction of closed end SWNTs with the selectivity filter (Fig. 4D). However, open-
ended SWNTs might be the most efficient type because the rim can be chemically reactive. Fig. 4, F–H shows open-ended SWNTs with different diameters sitting and stably anchoring on the top of the selectivity filter after having established weak electrical and/or chemical bonds. It is a common view that the three-dimensional structure of the pore and the selectivity filter is conserved among K⁺ channels because of their universal TVGYG signature (15), a notion corroborated by the recent crystallization of a bacterial, voltage-gated K⁺ channel (16). It is therefore not surprising that phylogenetically distant K⁺ channels all appear susceptible to nanotubes, whereas chloride channels are not. Local structural differences must also exist, however, to account for the diverse extent of blocks exhibited by different channel types.

SWNTs constitute a new class of universal K⁺ channel inhibitors exploiting rather elementary principles of blocking. The mechanism appears to be governed by geometrical factors and lacks any other physical/chemical component that is usually required by conventional agents. Tetraethylammonium (TEA) ions, for instance, are driven into and clog K⁺ pores by their free positive charge. Short chain scorpion toxins accomplish docking by establishing electrochemical bonds with specific residues of the target protein (1). In contrast, SWNTs do not resort to any of those mechanisms but instead hamper channel function probably by fitting into the pore and thus either hindering ion movement or alternatively preventing further conformational steps. In virtue of their electrochemical neutrality, nanotubes can provide precise information about ion channel structures. For instance, our data suggest that the pore vestibules of different K⁺ channels are not. Local structural differences must also exist, however, to account for the diverse extent of blocks exhibited by different channel types.

In these experiments, filled symbols indicate the absence and open symbols the presence of 0.1 mg/ml SWNTs in the test solutions. A, steady-state activation is altered by SWNTs. Voltage protocol is in the inset. Lines according to the Boltzmann equation \((1 + \exp(V - V_\text{half})/k)\), where \(V_\text{half}\) is half-maximal voltage and \(k\) the slope factor. \(V_\text{half}\) was 6.9 ± 0.5 mV and −8.3 ± 2.2 mV and \(V_\text{half}\) was 9.4 ± 0.4 mV and 15.4 ± 2.0 mV in the absence and presence of SWNTs, respectively. Data are from four cells. B and C, voltage dependence of deactivation kinetics. Deactivating tail currents were evoked by the voltage protocol shown in the inset (0.5-s depolarizing steps to +20 mV, 1-s test pulses from −120 to 40 mV in 20-mV increments) and fitted to a double exponential function \(I_e = I_e \exp(-t/\tau_e) + I_i \exp(-t/\tau_i)\). Data are from groups of five cells. D and E, dependence of block on the magnitude and duration of depolarization. In panel D, steady-state fractional tail current at −40 mV was evaluated as a function of the indicated depolarizing pulses. Depolarization increases inhibition, suggesting that opening of the channel is a prerequisite for block. Under the same conditions as in panel D, tail currents were evaluated in panel E as a function of the duration of the depolarizing step (in the inset in panel D the bar indicates a 20-mV variable prepulse in 16-ms increments followed by a 1-s repolarizing step to −40 mV). Data were normalized to the amplitude of the control current at time = 0. Data are from four cells. F, steady-state inactivation was evaluated by using a voltage protocol in which channel inactivation comes to equilibrium at various voltages during a prepulse so brief that little deactivation can occur; then, the fraction of channels in the inactive state is assessed by stepping the voltage to a test potential (inset depicts a 1-s depolarizing step to +20 mV, 30-ms repolarizing steps from −120 to 0 mV in 10-mV increments followed by a 1-s depolarizing step to +20 mV). For comparison, data were normalized. Data are from groups of four cells. G, SWNTs block various K⁺ selective pores differently. Inhibition constants were obtained by fitting dose-response curves to the Hill function (see Fig. 2F) and fitted to the function \(a_t = \alpha + \beta t\), with \(c\) varying from 7 to 3 in the cases considered. Nanotubes having the smallest diameter (0.9 nm) exerted maximum block on all of the three different channel types. Data are from groups of 3–5 cells. H, dependence of block on the shape and diameter of nanostructures. HERG channels were exposed to test solutions containing nanotubes (N) or fullerenes having the indicated average diameters. With both basic shapes, large diameter nanoparticles did not block the channels. In all cases, \(p < 0.05\). Data are from groups of 3–5 cells.
singular materials with unique characteristics. Here we unveil another previously unknown novel property of these materials that represents a further step forward in the effort to utilize them for biological purposes.

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Fig. 4. Nanotubes block K\textsuperscript{+} channels through a pore occlusion mechanism. A, the crystal structure of the Ksc\textalpha{} K\textsuperscript{+} channel. This and other images were constructed by RasMol and the file 1BL8 from the Protein Data Bank. B and C, docking simulation of a fullerene. A fullerene with an average diameter of 0.7 nm can fit into the entrance of the selectivity filter and act as a cork in a bottle to stop ion permeation. D, docking simulation of a capped SWNT showing that, because of its spherical end, it can fit into the selectivity filter like a fullerene. The figure shows a SWNT with an average diameter of 0.9 nm, similar to those used in this report. E and F, docking simulation of an open-ended SWNT. An open-ended nanotube can sit on top of the selectivity filter establishing weak bonds. Simulations of SWNTs with average diameters of 0.9 and 1.3 nm are shown in panels E and F, respectively.