The Journal of Neuroscience, March 1, 2000, *20*(5):1710–1721

Reconstitution of Muscarinic Modulation of the KCNQ2/KCNQ3 K^+ Channels That Underlie the Neuronal M Current

Mark S. Shapiro,[1] John P. Roche,[1] Edward J. Kaftan,[1] Humberto Cruzblanca,[1] Ken Mackie,[2] and Bertil Hille[1]

Departments of [1]Physiology and Biophysics and [2]Anesthesiology, University of Washington School of Medicine, Seattle, Washington 98195

Channels from KCNQ2 and KCNQ3 genes have been suggested to underlie the neuronal M-type K^+ current. The M current is modulated by muscarinic agonists via G-proteins and an unidentified diffusible cytoplasmic messenger. Using whole-cell clamp, we studied tsA-201 cells in which cloned KCNQ2/KCNQ3 channels were coexpressed with M_1 muscarinic receptors. Heteromeric KCNQ2/KCNQ3 currents were modulated by the muscarinic agonist oxotremorine-M (oxo-M) in a manner having all of the characteristics of modulation of native M current in sympathetic neurons. Oxo-M also produced obvious intracellular Ca^{2+} transients, observed by using indo-1 fluorescence. However, modulation of the current remained strong even when Ca^{2+} signals were abolished by the combined use of strong intracellular Ca^{2+} buffers, an inhibitor of IP_3 receptors, and thapsigargin to deplete Ca^{2+} stores. Muscarinic modulation was not blocked by staurosporine, a broad-spectrum protein kinase inhibitor, arguing against involvement of protein kinases. The modulation was not associated with a shift in the voltage dependence of channel activation. Homomeric KCNQ2 and KCNQ3 channels also expressed well and were modulated individually by oxo-M, suggesting that the motifs for modulation are present on both channel subtypes. Homomeric KCNQ2 and KCNQ3 currents were blocked, respectively, at very low and at high concentrations of tetraethylammonium ion. Finally, when KCNQ2 subunits were overexpressed by intranuclear DNA injection in sympathetic neurons, total M current was fully modulated by the endogenous neuronal muscarinic signaling mechanism. Our data further rule out Ca^{2+} as the diffusible messenger. The reconstitution of muscarinic modulation of the M current that uses cloned components should facilitate the elucidation of the muscarinic signaling mechanism.

Key words: K^+ channel; muscarinic receptor; G-protein; calcium; patch clamp; M current

A diverse family of neurotransmitters and hormones regulates Ca^{2+} and K^+ channels via G-protein-mediated signaling pathways (Wickman and Clapham, 1995; Brown et al., 1997; Dolphin, 1998). Nearly 20 years ago, several investigators gave the name M current to a noninactivating K^+ current with slow kinetics in sympathetic neurons that is strongly suppressed by muscarinic acetylcholine receptor (mAChR) agonists (Brown and Adams, 1980; Constanti and Brown, 1981). The M current is thought to play an important role in neuronal excitability, and its suppression increases responses to excitatory synaptic inputs (Jones et al., 1995; Wang and McKinnon, 1995). Modulation of the M current by muscarinic receptor agonists and by angiotensin (Constanti and Brown, 1981; Marrion, 1997; Shapiro et al., 1994a) is mediated by a G-protein of the $G_{q/11}$ class (Delmas et al., 1998; Haley et al., 1998) via a diffusible cytoplasmic second messenger (Selyanko et al., 1992) that is yet to be identified.

Although the buffering of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) to very low levels prevents muscarinic suppression of the M current in rat sympathetic neurons (Beech et al., 1991), no transients or rises of $[Ca^{2+}]_i$ have been detected on mAChR (Wanke et al., 1987; Beech et al., 1991) or angiotensin (Shapiro et al., 1994a) receptor stimulation. Such results have argued against a $[Ca^{2+}]_i$ signal as the unidentified cytoplasmic messenger. Another agonist, bradykinin, inhibits the M current (Jones et al., 1995) and does induce $[Ca^{2+}]_i$ rises in sympathetic neurons via the traditional phospholipase C/inositol trisphosphate (PLC/IP_3) pathway and the release of Ca^{2+} from intracellular stores (Cruzblanca et al., 1998). Whereas bradykinin inhibition is prevented when $[Ca^{2+}]_i$ is clamped at physiological levels or by blocking PLC or IP_3 receptors, the muscarinic action is unaffected (Cruzblanca et al., 1998). Thus, the muscarinic signaling pathway that inhibits the M current is distinct from that mediating bradykinin modulation.

The molecular identity of the M current also has been elusive. Recently, Wang et al. (1998) suggested that the M current is produced by heteromeric channels of the KCNQ (*KvLQT*) family. These channels have a predicted structure similar to the *Shaker* family of K^+ channels, but they possess a C terminus of unknown function that is longer than that of most other voltage-gated K^+ channels. When KCNQ2 and KCNQ3 channel subunits are coexpressed in *Xenopus* oocytes, a K^+ current is displayed that shares many characteristics with the M current of sympathetic neurons, including voltage dependence, kinetics, and pharmacology. These shared features, along with the neuronal localization of KCNQ2 and KCNQ3, are the basis for the proposal that KCNQ2/KCNQ3 channels are the molecular correlate of the M current (Wang et al., 1998). We sought to verify the correspondence to the M current by reconstituting appropriate muscarinic modulation. We demonstrate the modulation of cloned channels expressed in neurons and reconstitute the muscarinic suppression of KCNQ2 and KCNQ3 channels in a cell line via a

Received Oct. 13, 1999; revised Dec. 10, 1999; accepted Dec. 22, 1999.

This work was supported by National Institutes of Health Grants NS081734 and AR17803 (B.H.), DA00286 and DA11322 (K.M.), and DA07278 (J.R.); a grant from the Lalor Foundation (E.K); a Mexican government grant, CONACYT 4113P-N9607 (H.C.); and a Pew Latin American Fellowship in Biomedical Sciences (H.C.). We thank William N. Zagotta for comments on this manuscript.

M.S.S. and J.P.R. contributed equally to this work.

Correspondence should be addressed to Dr. Bertil Hille, Department of Physiology and Biophysics, University of Washington School of Medicine, G-424 Health Sciences Building, Box 357290, Seattle, WA 98195-7290. E-mail: mshapiro@u.washington.edu or hille@u.washington.edu.

Dr. Cruzblanca's present address: Centro Universitario de Investigaciones Biomedicas, Universidad de Colima, Av. 25 de Julio S/N, Col. Villa San Sebastian, Colima, Col. 28000 Mexico.

modulatory pathway that behaves remarkably similarly to that of native neurons. This work also reinforces the conclusion that the diffusible second messenger mediating muscarinic inhibition of the M current is not free Ca^{2+}. Our reconstituted system can form the basis for investigating the identity of the elusive cytoplasmic signal at a molecular level.

MATERIALS AND METHODS

Cells and expression of KCNQ2/KCNQ3 channels. Plasmids encoding human KCNQ2 (GenBank accession number AF110020) and rat KCNQ3 (GenBank accession number AF091247) were kindly given to us by David McKinnon (State University of New York, Stony Brook, NY), and the plasmid containing rat M_1 receptor was given by Neil Nathanson (University of Washington, Seattle, WA). Human tsA-201 (tsA) cells are a simian virus 40 (SV40) T-antigen-expressing derivative of the human embryonic kidney cell line 293 (HEK293). They were kindly given to us by Galen Flynn (University of Washington, Seattle, WA). To express KCNQ2 and KCNQ3 in tsA cells, we amplified the coding region for each channel by PCR, using primers incorporating *Hin*dIII and *Xba*I restriction sites. Then the amplified inserts were subcloned into the corresponding restriction sites of the pcDNA3 expression plasmid (Invitrogen, San Diego, CA). The fidelity of all amplified sequences was confirmed by dye terminator sequencing on both strands (Perkin-Elmer, Emeryville, CA). Cells were grown in 60 or 35 mm tissue culture dishes (Falcon, Oxnard, CA) in tsA growth medium (DMEM or DMEM/F-12 nutrient mixture plus 10% heat-inactivated fetal bovine serum plus 0.2% penicillin/streptomycin) in a humidified incubator at 37°C (5% CO_2) and passaged approximately every 5 d after exposure to Ca^{2+}-free saline for 2 min. Plasmids were transfected as follows: DNA (~2 μg total) was combined with 10 μl of Superfect transfection reagent (Qiagen, Chatsworth, CA) and 100 μl of serum/antibiotic-free DMEM medium. After a 10 min wait for complexes to form, this was mixed with 600 μl of tsA growth medium and incubated with tsA cells, grown to ~50% confluency, for 2 hr, and then returned to the incubator. The next day the cells were plated onto poly-L-lysine-coated coverslip chips and used within 3 d for electrophysiological experiments. As a marker for successfully transfected cells, 0.2 μg of DNA encoding green fluorescent protein was cotransfected with channel and receptor DNA. Using this protocol, we found that >95% of green-fluorescing cells express the M-like currents in control experiments.

Superior cervical ganglion (SCG) sympathetic neuron cultures. SCG neurons were taken from 5- to 6-week-old male rats (Sprague Dawley, Indianapolis, IN) and cultured for 1 d. Rats were anesthetized with CO_2 and decapitated. Neurons were dissociated by using the methods of Bernheim et al. (1991) and suspended twice in DMEM plus 10% heat-inactivated horse serum. Cells were plated on 4 × 4 mm glass coverslips (coated with poly-L-lysine) and incubated at 37°C (5% CO_2). Fresh culture medium containing nerve growth factor (50 ng/ml) was added to the cells 2 hr after plating.

Current recording and analysis. The whole-cell configuration of the patch-clamp technique was used to voltage-clamp and dialyze cells at room temperature (22–25°C). Electrodes were pulled from glass hematocrit tubes (VWR Scientific, Seattle, WA) and fire-polished. They had resistances of 1–3 MΩ when measured in Ringer's and filled with internal solution. Membrane current was measured under whole-cell clamp with pipette and membrane capacitance cancellation, sampled at 5 msec, and filtered at 200–500 Hz. The whole-cell access resistance was 3–10 MΩ. Junction potentials have been corrected by −2 or by −4 mV. For experiments without $[Ca^{2+}]_i$ measurement the cells were placed in a 100 μl chamber through which solution flowed at 1–2 ml/min. Inflow to the chamber was by gravity from several reservoirs, selectable by the activation of solenoid valves. Bath solution exchange was complete by <30 sec.

For all experiments except that shown in Figure 5, KCNQ2/KCNQ3 currents from tsA cells were studied by holding the cell at −20 or 0 mV and applying a 500–650 msec hyperpolarizing step to −60 mV, followed by a 650 msec pulse back to the holding potential, every 4–5 sec. The amplitude of the current usually was defined as the outward current at the holding potential sensitive to block by 50 μM XE991. TsA cells do have small endogenous voltage-gated K currents, like HEK293 cells (Yu and Kerchner, 1998). These currents have an onset rate >10-fold faster than KCNQ2/KCNQ3 channels, show little observable tail current (deactivation) at −60 mV, and are just starting to activate at −20 or 0 mV (data not shown). Thus, the KCNQ2/KCNQ3 current was distinguished easily from the endogenous current. In some experiments with a holding potential of 0 mV that did not use XE991 and had little leak or endogenous current, the KCNQ2/KCNQ3 current amplitude was the holding current. In the few cells with significant endogenous current or significant leak current, the amplitude of the KCNQ2/KCNQ3 current was taken as the difference between the holding current and a point 20–25 msec after the activating step back to the holding potential (which is enough time for activation of the endogenous current). Cells exhibited variable "run-down" in the amplitude of KCNQ currents and usually stabilized within several minutes of whole-cell dialysis. Cells in which the run-down was excessive were not studied.

In all experiments with pipette solutions containing 20 mM BAPTA, we waited >5 min before applying oxo-M to allow for the dialysis of BAPTA and other ingredients into the cell. M-type currents in SCG cells were studied by holding the membrane potential at −25 mV and applying a 500 msec hyperpolarizing pulse to −60 mV every 4 sec. The M-type current amplitude was measured at −60 mV from the decaying time course of deactivating current as the difference between the average of a 10 msec segment, taken 20–30 msec into the hyperpolarizing step, and the average during the last 50 msec of that step. All results are reported as mean ± SEM.

$[Ca^{2+}]_i$ measurement. $[Ca^{2+}]_i$ was measured by using fluorescence of the indicator indo-1. For experiments on intact cells bath-loaded with indo-1 dye, the cells were incubated at room temperature with indo-1 AM (2.4 μM; Molecular Probes, Eugene, OR) in Ringer's solution for 20 min. For simultaneous current recording and $[Ca^{2+}]_i$ measurement, indo-1 was dialyzed into the cell by adding 150 μM indo-1 pentapotassium salt to the pipette solution; we waited >3 min before recording to allow for indo-1 dialysis. For all $[Ca^{2+}]_i$ measurements the cells were transferred to a chamber mounted on the stage of an inverted microscope, using a 1.3 numerical aperture 40× oil objective, an attenuated (1.0 NDF) 75 W xenon source, and paired photon-counting detectors (Hamamatsu, Hamamatsu City, Japan). Indo-1 was excited at 365 nm, and emission was detected at 405 and 500 nm. A shutter limited exciting light to a 50 msec sampling period every second. For patched or AM-loaded cells, background measurements were taken of the cell to be studied before patching or in a cell-free area, respectively. $[Ca^{2+}]_i$ was calculated by using the equation: $[Ca^{2+}]_i = K^* (R - R_{min})/(R_{max} - R)$, where R is the ratio of emitted fluorescence (405/500 nm), and K^* was measured to be 1.0 μM, using the 20 mM BAPTA plus 10 mM Ca^{2+} solution for in-cell calibration (Beech et al., 1991). R_{min} (0.34) and R_{max} (3.4) were measured in tsA cells with a pipette solution containing 20 mM BAPTA (no added Ca^{2+}) or when a large bolus of ionomycin was added to the bath, respectively. A solenoid-controlled perfusion system allowed brisk solution changes and continuous superfusion of the cells.

Intranuclear microinjection. After overnight culture the SCG neurons were microinjected intranuclearly with an Eppendorf 5242 pressure microinjector and 5171 micromanipulator system (Eppendorf, Madison, WI), as previously described (Garcia et al., 1998). The injection solution contained DNA plasmids for KCNQ2 (0.12 mg/ml) and for green fluorescent protein (GFP) as an expression reporter (0.04 mg/ml) and 0.05% 10,000 kDa dextran-fluorescein (Molecular Probes) as an injection marker. Injection at pressures of 10–20 kPa for 0.5–0.8 sec resulted in no obvious increase in cell volume. After 12–16 hr successfully injected neurons were identified by their characteristic greenish-blue GFP fluorescence by using an inverted microscope equipped with epifluorescence and fluorescein optics.

Solutions and materials. The external Ringer's solution used to record KCNQ currents in tsA cells contained (in mM): 160 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 8 glucose, pH-adjusted to 7.4 with NaOH. For recordings from SCG neurons the $CaCl_2$ concentration was 5 mM. In one set of experiments on tsA cells the $CaCl_2$ was omitted from the medium. The 0.1 BAPTA pipette solution contained (in mM): 175 KCl, 5 $MgCl_2$, 5 HEPES, 0.1 1,2-bis(2-aminophenoxy)ethane *N,N,N′,N′*-tetra-acetic acid (BAPTA), 3 Na_2ATP, and 0.1 NaGTP, pH-titrated to 7.4 with KOH. In one set of experiments the BAPTA concentration was raised to 20 mM, and the KCl concentration was reduced to 120 mM. To make the BACaPPS cocktail pipette solution, we raised the BAPTA concentration to 20 mM, added 10 mM $CaCl_2$, reduced the KCl concentration to 110 mM, and then added pentosan polysulfate (PPS) to the pipette solution (100 μg/ml) from a stock solution at 100 mg/ml in water. The thapsigargin stock solution was 5 mM in DMSO. The staurosporine stock solution was 10 mM in DMSO.

Reagents were obtained as follows: oxotremorine methiodide (Research Biochemicals, Natick, MA); BAPTA (Molecular Probes); ATP and GTP (Pharmacia LKB Biotechnology); DMEM, DMEM/F-12 mix-

ture, fetal bovine serum, horse serum, nerve growth factor, and penicillin/streptomycin (Life Technologies, Gaithersburg, MD); *N*-ethylmaleimide (NEM) and PPS (Sigma, St. Louis, MO); thapsigargin (Calbiochem, La Jolla, CA); indo-1 and indo-1 AM (Molecular Probes). XE991 was a kind gift from Michael E. Schnee (DuPont Pharmaceuticals, Wilmington, DE).

RESULTS

KCNQ2/KCNQ3 channels are modulated by muscarinic agonists

We expressed KCNQ2 and KCNQ3 channels, individually and together, in tsA-201 (tsA) cells by transfecting their cDNA clones, and we recorded K^+ currents 1–5 d later by using whole-cell clamp. As a marker for successfully transfected cells, all transfections included cDNA coding for green fluorescent protein, and cells emitting green fluorescence were chosen for study. Cotransfection of the plasmids for the two channel subunits yielded slowly activating and deactivating currents with M-current-like voltage dependence, kinetics, and pharmacology similar to those reported previously in oocytes (Wang et al., 1998; Yang et al., 1998).

In sympathetic neurons from the SCG, muscarinic suppression of the M current uses the M_1 subtype of the muscarinic receptor (Bernheim et al., 1992; Hamilton et al., 1997). When tsA cells were cotransfected with the plasmids for the M_1 receptor and for KCNQ2 and KCNQ3 channels, bath application of the muscarinic agonist oxotremorine-M (oxo-M; 10 μM) strongly suppressed the expressed current (Fig. 1*A*), with a time course qualitatively similar to suppression of the M current in neurons (Beech et al., 1991). The mean muscarinic inhibition of the KCNQ2/KCNQ3 current in these experiments was 84 ± 8% (n = 5). As with muscarinic inhibition of the M current in sympathetic neurons (Beech et al., 1991; Cruzblanca et al., 1998), the inhibition of KCNQ2/KCNQ3 currents in tsA cells was partially reversible. The drug XE991 (50 μM), a selective blocker of M current (Wang et al., 1998), completely blocked the expressed KCNQ2/KCNQ3 current (Fig. 1*A*).

The initial experiments used a pipette (internal) solution like that used in previous work on sympathetic neurons that contained only a minimal amount of Ca^{2+} buffer (0.1 mM BAPTA), too little to prevent changes in $[Ca^{2+}]_i$ that might occur during stimulation. Although stimulation of M_1 receptors in many other cells causes release of Ca^{2+} from IP_3-sensitive stores via the actions of PLC (Felder, 1995), in rat SCG neurons muscarinic agonists do not induce an observable increase in $[Ca^{2+}]_i$ (Wanke et al., 1987; Beech et al., 1991). To see if stimulation of expressed M_1 receptors in tsA cells induces $[Ca^{2+}]_i$ rises, we exposed undialyzed cells to oxo-M and monitored $[Ca^{2+}]_i$ with indo-1 loaded as the cell-permeant indo-1 AM ester. In four cells tested this way, repeated exposures to oxo-M induced repeated $[Ca^{2+}]_i$ transients (Fig. 1*B*). Thus, similar to M current in sympathetic neurons, muscarinic agonists strongly suppress KCNQ2/KCNQ3 current in tsA cells. However, in contrast to the results obtained in SCG neurons, muscarinic agonists induce significant $[Ca^{2+}]_i$ signals in tsA cells.

Modulation of KCNQ2/KCNQ3 channels does not require a Ca^{2+} signal

Although the G-protein-coupled suppression of the M current in sympathetic neurons does not use a $[Ca^{2+}]_i$ signal, the M current also can be suppressed by Ca^{2+} elevations (Marrion et al., 1991; Selyanko and Brown, 1996). Therefore, to reconstitute a muscarinic signaling pathway resembling that in neurons, we took several measures to eliminate the muscarinic $[Ca^{2+}]_i$ transient of tsA cells. First, we used pipettes containing 20 mM BAPTA plus 10 mM Ca^{2+}. This should "clamp" $[Ca^{2+}]_i$ at a physiological level (~150 nM), which permits muscarinic modulation of M current in sympathetic neurons (Beech et al., 1991; Cruzblanca et al., 1998). Because generation of $[Ca^{2+}]_i$ signals by M_1 muscarinic receptors usually is mediated by IP_3 receptors, we also inhibited the IP_3 receptors by adding pentosan polysulfate (PPS; 100 μg/ml) to the pipette solution. PPS is a polyanion that inhibits IP_3 binding in rat liver microsomes with an IC_{50} of 7 μg/ml (Tones et al., 1989) and is a competitive antagonist of IP_3 action (Ehrlich et al., 1994). PPS does not affect muscarinic modulation of M current in sympathetic neurons, but it strongly blocks inhibition of the M current by bradykinin by preventing IP_3-mediated $[Ca^{2+}]_i$ rises (Cruzblanca et al., 1998). To verify the effectiveness of these experimental manipulations, we simultaneously monitored KCNQ2/KCNQ3 currents and $[Ca^{2+}]_i$ by using indo-1 dye loaded into the cell from the whole-cell pipette. Figure 2 shows such an experiment. After several minutes to allow for cell dialysis from the pipette, repetitive voltage pulses from −20 to −60 mV were applied every 4 sec to monitor KCNQ2/KCNQ3 currents. Bath application of 10 μM oxo-M produced a robust suppression of the KCNQ2/KCNQ3 current with a time course similar to muscarinic suppression of the M current in SCG neurons (Beech et al., 1991), yet there was no change in $[Ca^{2+}]_i$, which appears "clamped" at a concentration of ~170 nM. Thus the combination of Ca^{2+} buffer and PPS effectively eliminated the $[Ca^{2+}]_i$ transient. In seven cells tested with this combination of 20 mM BAPTA, 10 mM Ca^{2+}, and 100 μg/ml PPS (BACaPPS cocktail) in the pipette, the mean inhibition of the KCNQ2/KCNQ3 current was 72 ± 6%, and in none of these cells was there a detectable $[Ca^{2+}]_i$ change caused by oxo-M. In conclusion, muscarinic agonists acting on M_1 receptors can inhibit KCNQ2/KCNQ3 channels expressed in tsA cells without an associated rise in $[Ca^{2+}]_i$, seemingly reconstituting the modulatory pathway of SCG neurons that acts on the M current without any detectable $[Ca^{2+}]_i$ signals. The extent of inhibition by oxo-M falls in the range seen with 20 mM BAPTA plus 10 mM Ca^{2+} pipette solutions in SCG neurons (60–80%) (Beech et al., 1991; Cruzblanca et al., 1998).

Even if the BACaPPS cocktail eliminates the spatially averaged $[Ca^{2+}]_i$ response, might there still be a "local" release of $[Ca^{2+}]_i$ from internal stores that we failed to detect? To guard further against this possibility, we tried another measure to prevent the generation of $[Ca^{2+}]_i$ signals from intracellular Ca^{2+} pools. We used thapsigargin to deplete intracellular Ca^{2+} stores by inhibiting endoplasmic reticulum Ca^{2+} pumps (Thastrup et al., 1990; Inesi and Sagara, 1992). Figure 3*A* shows a test with 0.1 mM BAPTA in the pipette on a cell transfected with KCNQ2/KCNQ3 channels and M_1 receptors. Bath application of 2 μM thapsigargin released Ca^{2+} from internal stores, producing an obvious $[Ca^{2+}]_i$ transient. Subsequent application of 10 μM oxo-M did not raise $[Ca^{2+}]_i$, presumably because all reticular stores had been depleted of Ca^{2+} by the thapsigargin treatment. However, oxo-M suppressed the KNCQ2/KCNQ3 current strongly. A similar experiment, but with the BACaPPS cocktail in the pipette, is shown in Figure 3*B*. Now, the exposure of the cell to thapsigargin did not produce an observable rise in $[Ca^{2+}]_I$, as expected, because there was a high concentration of BAPTA in the pipette. Nevertheless, subsequent application of oxo-M suppressed the KCNQ2/KCNQ3 current strongly. After thapsigargin treatment and with the BACaPPS cocktail in the pipette, the mean inhibition of the KCNQ2/KCNQ3 current was 60 ± 10% (n = 6), only slightly less (not significant at the $p < 0.1$ level) than

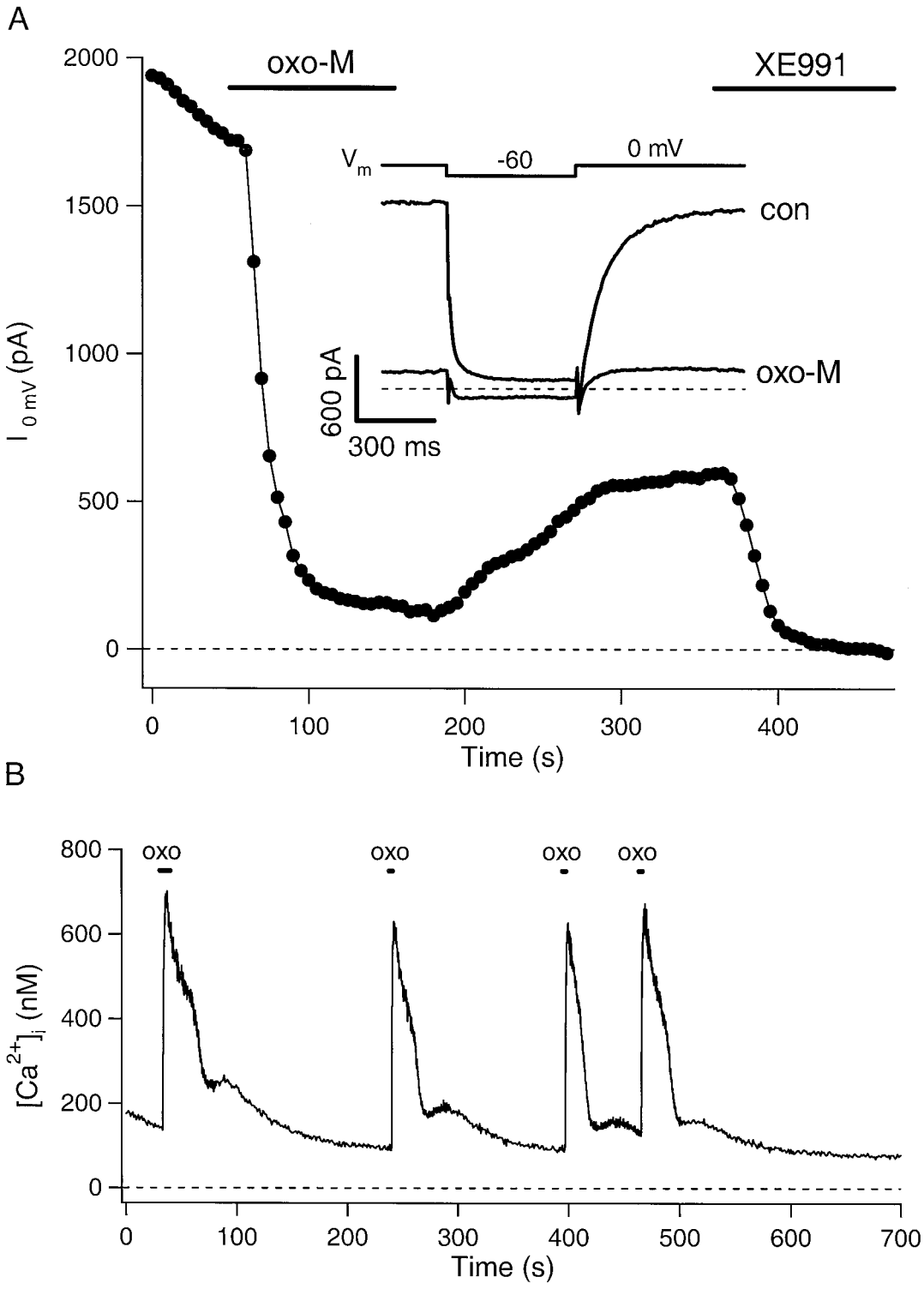

Figure 1. Inhibition of KCNQ2/KCNQ3 currents and generation of Ca^{2+} signals by a muscarinic agonist. *A*, Current amplitude at 0 mV in a tsA cell transfected with KCNQ2, KCNQ3, and M_1 muscarinic receptors. Oxo-M (10 μM) and XE991 (50 μM) were bath-applied during the periods that are marked. The pipette solution contained 0.1 mM BAPTA. The *inset* shows the pulse protocol that was used and the current wave forms before and after the application of oxo-M. The *dashed line* in the current traces is the zero current level. Pulses were given every 4 sec. *B*, Microfluorometric measurement of $[Ca^{2+}]_i$ in a tsA cell transfected with the M_1 muscarinic receptor and bath-loaded with indo-1 as the AM ester for 20 min before measurements were taken. The cell was not patch-clamped and is different from that in *A*. Oxo-M (10 μM) was applied during the four periods that are marked.

the inhibition without thapsigargin treatment. Thus, even with $[Ca^{2+}]_i$ highly buffered, IP_3 receptors blocked, and internal Ca^{2+} stores depleted, oxo-M can inhibit the KCNQ2/KCNQ3 current.

Our standard external solution contains 2 mM Ca^{2+}. Might receptor-stimulated entry of external Ca^{2+} somehow play a role in the muscarinic modulation of KNCQ2/KCNQ3 channels? To

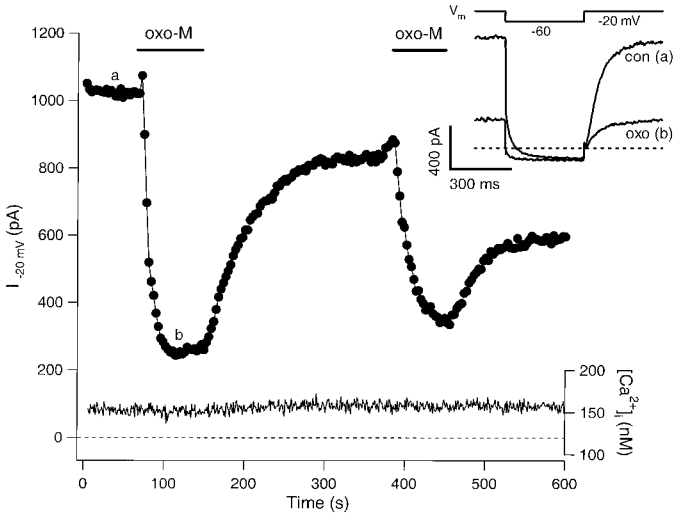

Figure 2. Inhibition of KCNQ2/KCNQ3 currents by muscarinic agonists without Ca^{2+} signals. The holding current amplitude (*filled circles*) is plotted for a tsA cell transfected with KCNQ2, KCNQ3, and M_1 muscarinic receptors. The $[Ca^{2+}]_i$ trace (*bottom*) was measured simultaneously from the fluorescence of indo-1 dialyzed into the cell from the patch pipette. Oxo-M (10 μM) was bath-applied during the periods that are shown. To prevent $[Ca^{2+}]_i$ changes, the pipette solution contained the BACaPPS cocktail, which includes 20 mM BAPTA, 10 mM Ca^{2+}, and 100 μg/ml PPS. The *inset* shows the pulse protocol and current traces before and after the first application of oxo-M. The *dashed line* in the current traces is the zero current level. Pulses were given every 4 sec.

test this, we used an external solution with no added Ca^{2+} (estimated Ca^{2+}, <50 μM) and tested oxo-M action with the BACaPPS cocktail in the pipette. The ability of oxo-M to suppress the KCNQ2/KCNQ3 current was little affected (inhibition = 70 ± 20%; n = 3) by the removal of external Ca^{2+}. The ability of high concentrations of intracellular Ca^{2+} chelators without added Ca^{2+} to reduce muscarinic inhibition of the M current, coupled with its restoration with added Ca^{2+} (Beech et al., 1991), has been interpreted to reflect a minimum permissive level of Ca^{2+} required for the muscarinic signal to operate in SCG neurons. To see if modulation of KCNQ2/KCNQ3 channels is similarly sensitive to intracellular Ca^{2+} chelators in tsA cells, we did experiments with 20 mM BAPTA in the pipette, without any added Ca^{2+}. Under these conditions $[Ca^{2+}]_i$ should be reduced to <10 nM (Beech et al., 1991). As for the M current in SCG neurons, the suppression of KCNQ2/KCNQ3 currents by oxo-M in tsA cells was reduced significantly with 20 mM BAPTA in the pipette, to a value of 27 ± 7% (n = 5).

The muscarinic inhibition of KCNQ2/KCNQ3 channels under the experimental conditions presented so far is summarized in Figure 3*C*. We expect that, with low BAPTA concentrations in the pipette, muscarinic agonists can suppress KCNQ2/KCNQ3 currents both via IP_3-mediated $[Ca^{2+}]_i$ rises and via the unidentified muscarinic cytoplasmic messenger. Prevention of $[Ca^{2+}]_i$ transients with the BACaPPS cocktail in the pipette and depletion of internal stores by thapsigargin modestly reduced the total inhibition, because presumably the former pathway became inoperative under these conditions. Removal of external Ca^{2+} did not reduce the muscarinic modulation significantly. However, if $[Ca^{2+}]_i$ was strongly buffered to very low, unphysiological levels, the muscarinic inhibition was attenuated. These experiments reinforce the conclusion that muscarinic modulation of the KCNQ2/KCNQ3 channels is not mediated by a $[Ca^{2+}]_i$ *signal*, but a minimum permissive *level* of $[Ca^{2+}]_i$ is required for the muscarinic pathway to operate. They also present direct evidence that the muscarinic modulation of KCNQ2/KCNQ3 channels expressed in tsA cells and recorded with the BACaPPS pipette solution is indistinguishable from the muscarinic modulation of the M current in SCG neurons.

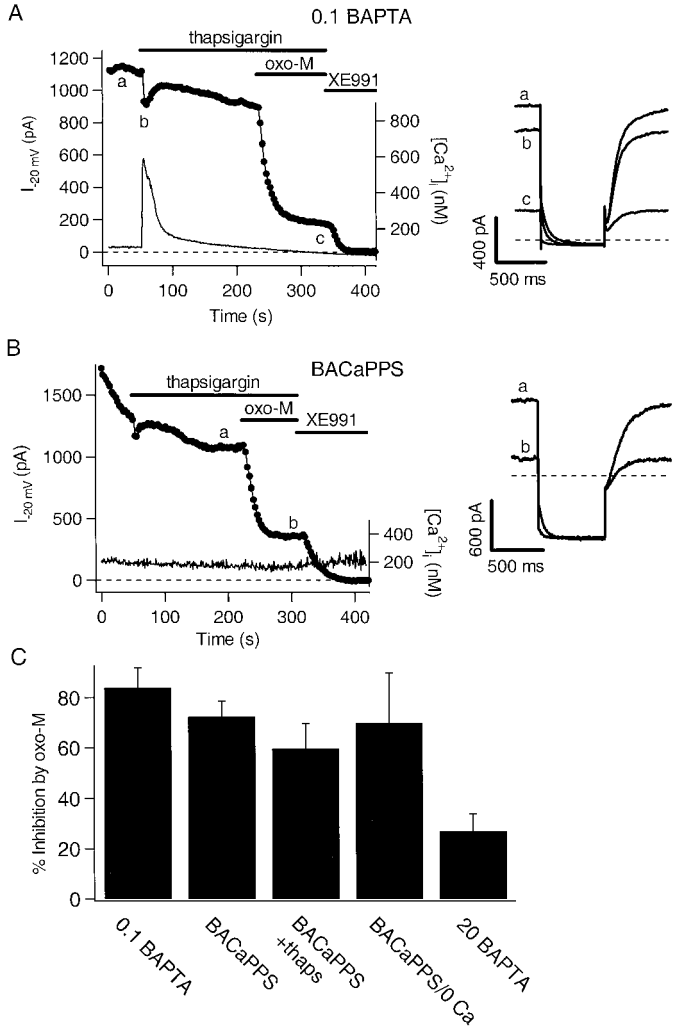

Figure 3. Depletion of internal stores by thapsigargin does not prevent the muscarinic inhibition of KCNQ2/KCNQ3 currents. Thapsigargin (2 μM) was bath-applied to cells transfected with KCNQ2, KCNQ3, and M_1 muscarinic receptors to deplete internal Ca^{2+} stores, followed by the application of oxo-M (10 μM) to test for muscarinic inhibition. *A*, The pipette solution contained 0.1 mM BAPTA without PPS. *Filled circles* are current amplitudes, and the *line* is the $[Ca^{2+}]_i$ trace from indo-1 fluorescence. Thapsigargin, oxo-M, and XE991 were applied as shown by the *horizontal bars*. Selected current traces during the experiments are shown on the *right*. The *dashed line* in the current traces is the zero current level. *B*, The pipette contained the BACaPPS cocktail. Traces are as in *A*. *C*, Summary of KCNQ2/KCNQ3 channel inhibition under the various conditions as described in Results.

NEM-insensitive G-proteins and muscarinic receptors mediate the inhibition

Sensitivity to pertussis toxin and to *N*-ethylmaleimide (NEM), a sulfhydryl-alkylating agent, can be used to distinguish the involvement of G-proteins of the $G_{o/i}$ class from the others (Jakobs et al., 1982; Milligan, 1988; Shapiro et al., 1994a; Choi and Lovinger, 1996; Viana and Hille, 1996). In SCG neurons the

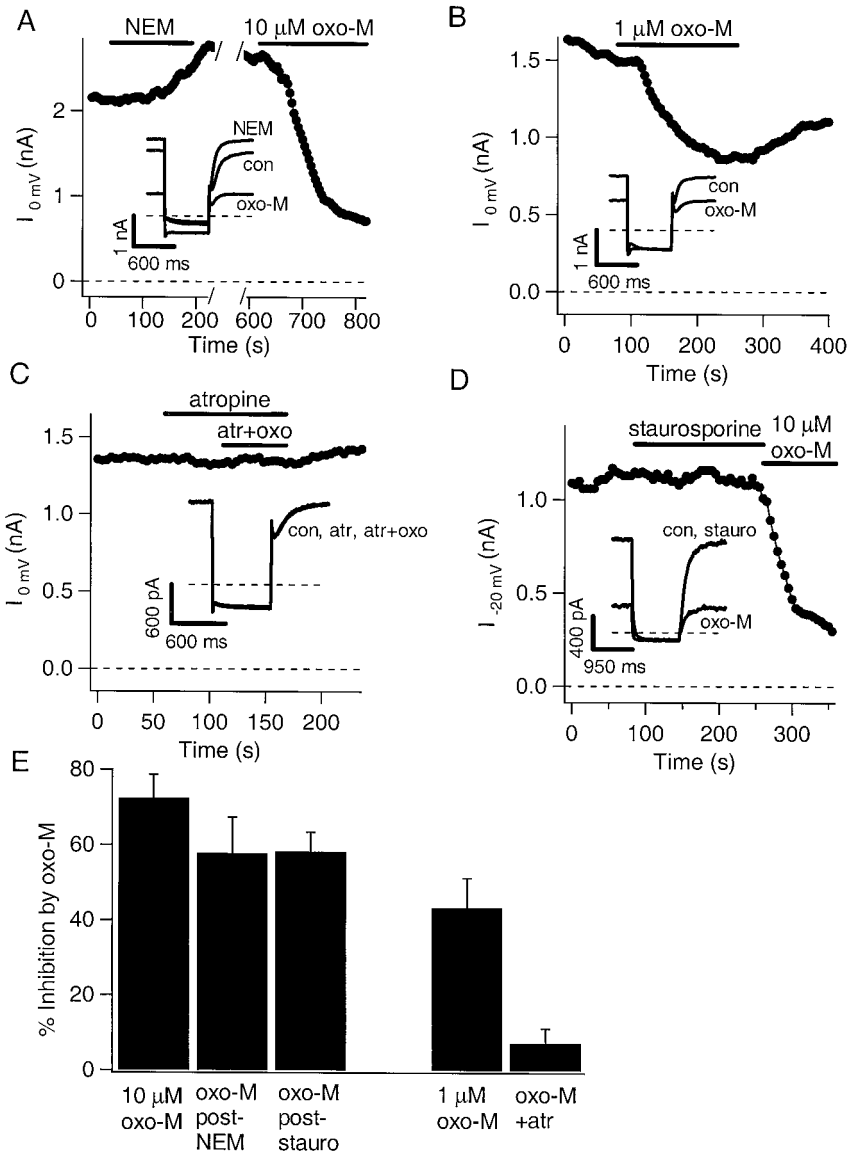

Figure 4. Muscarinic inhibition of KCNQ2/KCNQ3 currents is NEM-insensitive, uses M_1 receptors, and is not mediated by protein kinases. Plotted in *A–D* are current amplitudes at the indicated holding potential in tsA cells transfected with KCNQ2, KCNQ3, and M_1 receptors. The pipette solution contained the BACaPPS cocktail. *A*, NEM (50 μM) and oxo-M (10 μM) were bath-applied as indicated. The record contains a gap of 5 min. Current traces before NEM, after NEM, and after oxo-M applications are shown in the *inset*. The *dashed line* in the current traces is the zero current level. *B*, Oxo-M (1 μM) was bath-applied as indicated. *C*, Atropine (100 μM) and oxo-M (1 μM) were bath-applied as indicated. *D*, Staurosporine (1 μM) and oxo-M (10 μM) were bath-applied as indicated. The pipette also contained 1 μM staurosporine. *E*, Summary of current inhibition under these conditions.

muscarinic suppression of the M current is mediated by the $G_{q/11}$ class of G-proteins, which are not sensitive to pertussis toxin (PTX) (Haley et al., 1998) and should not be sensitive to NEM. To confirm that inhibition of the KCNQ2/KCNQ3 current by oxo-M in tsA cells is not NEM-sensitive, we treated cells with 50 μM NEM for 2 min and then tested the inhibition of the KCNQ2/KCNQ3 current by oxo-M with the BACaPPS cocktail in the pipette (Fig. 4*A*). Unexpectedly, application of NEM by itself produced a modest *increase* in the current. Subsequent application of oxo-M still strongly suppressed the current. In seven cells NEM increased the KCNQ2/KCNQ3 current by 33 ± 4%, and subsequent application of oxo-M inhibited the current by 58 ± 10% ($n = 7$) (Fig. 4*C*). In similar tests on SCG neurons, we also found that NEM (20 μM) increased the amplitude of the M current at −25 mV by 60 ± 9% and did not affect subsequent inhibition by oxo-M substantially (55 ± 7%, $n = 4$). Thus, as in SCG neurons, NEM increases current in expressed KCNQ2/KCNQ3 channels in tsA cells, and the muscarinic modulation of the current is not mediated by G-proteins of the $G_{o/i}$ class.

We wished to confirm that oxo-M modulates the channels in tsA cells by acting on the expressed M_1 receptors and not by some direct action on the channels. This was motivated by a report that muscarinic agonists can inhibit the I_{Ks} current in heart in a manner that is not blocked by muscarinic antagonists (Freeman and Kass, 1995) and the realization that cardiac I_{Ks} current is produced by channels containing the KCNQ1 subunit, a close relative of KCNQ2 and KCNQ3. In cells transfected with KCNQ2 and KCNQ3, but not the M_1 receptor, the application of oxo-M did not suppress the current ($n = 2$). We then tested the ability of the muscarinic antagonist atropine to block modulation by 1 μM oxo-M when M_1 receptors are expressed. By itself, 1 μM oxo-M suppressed the KCNQ2/KCNQ3 current (Fig. 4*B*), and in the presence of 100 μM atropine it failed to act on the current at all (Fig. 4*C*). Atropine by itself did not affect the current. On average, 1 μM oxo-M inhibited the expressed current by 43 ± 8% ($n = 7$), slightly less than the inhibition by 10 μM oxo-M; in the presence of atropine 1 μM oxo-M produced an inhibition of only 7 ± 4% ($n = 6$) (Fig. 4*E*). These experiments show that muscarinic agonists suppress KCNQ2/KCNQ3 currents via the M_1 receptors.

In sympathetic neurons the muscarinic modulation of the M current is not prevented by blockers of protein kinases and is not

occluded by the activation of protein kinase C (PKC) (Grove et al., 1990; Shapiro et al., 1996). It has been reported that cloned KCNQ2/KCNQ3 channels are modulated by protein kinase A (PKA), with PKA phosphorylation on the N terminus of KCNQ2 causing an upregulation of the current (Schroeder et al., 1998). To verify that muscarinic modulation of the expressed KCNQ2/KCNQ3 channels in tsA cells is not mediated by a protein kinase, we pretreated cells with the broad-spectrum protein kinase inhibitor, staurosporine, and tested whether staurosporine would block oxo-M action. Staurosporine blocks the activity of PKC and PKA, and many other kinases, at nanomolar concentrations (Tamaoki et al., 1986; Ruegg and Burgess, 1989; Meggio et al., 1995). Figure 4*D* shows an experiment in which a tsA cell expressing KCNQ2/KCNQ3 channels and M_1 receptors was treated with staurosporine (1 μM in pipette and bath, 2 min bath application) in a manner that has been shown to block the PKC-mediated actions of the phorbol ester PMA on Ca^{2+} channels (Shapiro et al., 1996). Staurosporine treatment did not prevent the suppression of the KCNQ2/KCNQ3 current by 10 μM oxo-M (58%). These data are summarized in Figure 4*E*. In cells treated with staurosporine the subsequent suppression of the KCNQ2/KCNQ3 current was still robust (58 ± 5%, $n = 10$) and only slightly less than in nonstaurosporine- and non-NEM-treated cells. Thus, as in the muscarinic modulation of the M current in sympathetic neurons, the modulation of KCNQ2/KCNQ3 current in tsA cells is not mediated by PKA or PKC.

Muscarinic modulation of KCNQ2/KCNQ3 currents does not change channel voltage dependence

One well studied G-protein pathway that inhibits several different types of neuronal Ca^{2+} channels involves a direct action of G-protein $\beta\gamma$ subunits on the channels that shifts the voltage dependence of channel activation to more depolarized potentials (Bean, 1989; Herlitze et al., 1996; Ikeda, 1996). To test whether the muscarinic action on the KCNQ2/KCNQ3 channels involves a shift in the voltage dependence of channel activation, we elicited a family of currents over a range of test potentials before and after oxo-M application (Fig. 5*A*). Cells were held at the potential of −70 mV, and voltage steps were given from −80 to 40 mV. To eliminate the possibility of voltage shifts from some other kinase-mediated pathway activated by muscarinic stimulation, we performed these experiments by using the same staurosporine protocol as in Figure 4*D*. To quantify the action on KCNQ2/KCNQ3 currents, we focused on the slow deactivation transients ("tail currents") at −70 mV, which are specific for KCNQ2/KCNQ3 channels. The amplitude of the tail currents will reflect the activation of the channels by the preceding voltage step. Plotted in Figure 5*B* are the amplitudes of the tail currents versus test potential, averaged for three cells. The inhibition of the current by oxo-M was not voltage-dependent and results in a similar reduction of the current at all test voltages. We quantified the voltage dependence of channel activation by fitting the data to Boltzmann relations. Before the application of oxo-M the voltage that produces half-maximal activation of the conductance ($V_{1/2}$) was −21 mV; after oxo-M application $V_{1/2}$ was −19 mV. Thus, muscarinic modulation of KCNQ2/KCNQ3 channels is not associated with shifts in channel voltage dependence.

Expression and modulation of homomeric KCNQ2 and KCNQ3 channels

Will homomeric KCNQ2 or KCNQ3 channels also express well, and can they also be inhibited by muscarinic agonists? Transfection with KCNQ2 or KCNQ3 alone resulted in currents with very similar voltage dependence and kinetics to those produced by the cotransfection of KCNQ2 and KCNQ3. The amplitude of the currents was somewhat smaller for KCNQ3 and several-fold smaller for KCNQ2, compared with the cotransfection of KCNQ2 and KCNQ3. KCNQ3 channels also expressed well in Chinese hamster ovary cells (data not shown). The similarity to the heteromeric currents made it seem prudent to check that the currents were indeed attributable to homomeric expression. One pharmacological characteristic that should distinguish KCNQ2 from KCNQ3 is sensitivity to external tetraethylammonium ion (TEA) (Wang et al., 1998; Yang et al., 1998). The KCNQ2 channel has a tyrosine at position 284, analogous to position 449 in *Shaker* K^+ channels, which has been shown to confer high sensitivity to external TEA (MacKinnon and Yellen, 1990; Heginbotham and MacKinnon, 1992), and KCNQ2 homomultimers are very TEA-sensitive (Wang et al., 1998; Yang et al., 1998). The KCNQ3 channel has a threonine at this position and is much less sensitive to TEA (Yang et al., 1998). We compared the TEA sensitivity of putative homo- and heteromeric channels. They

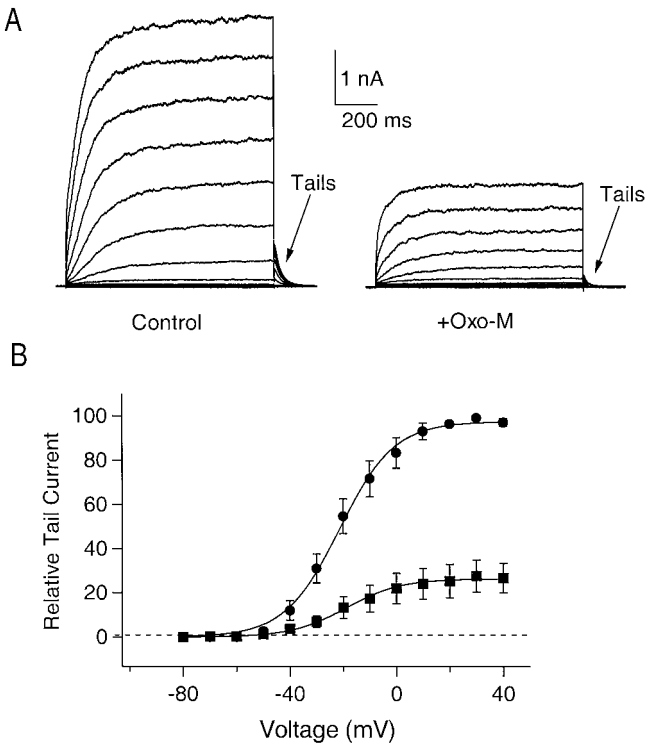

Figure 5. Voltage independence of muscarinic modulation. *A*, Families of current elicited by voltage steps from −70 to 40 mV, in 10 mV intervals, before and after the addition of 10 μM oxo-M to the bath. Cells were pretreated with staurosporine (1 μM) for 2 min before the addition of oxo-M. The holding potential and the tail current potential were −70 mV. The pipette solution contained the BACaPPS cocktail plus 1 μM staurosporine. The inhibition by oxo-M that is shown may be overestimated slightly because of run-down, although it was not excessive in this cell. Tail currents are marked by *arrows*. *B*, Amplitudes of the tail currents versus test potential for all experiments like those in *A*. Tail currents were quantified by measuring the average amplitude for 20 msec at 30 msec after repolarization to −70 mV. The data were fit with Boltzmann relations of the form: $\% I/I_{max} = 100 \cdot I_{max}/\{1 + \exp[(V_{1/2} - V)/k]\}$, where $V_{1/2}$ is the voltage that produces half-maximal activation of the conductance and k is the slope factor. Before oxo-M application, $V_{1/2}$ was −21 mV and k was 10.3 mV; I_{max} was set to unity. After oxo-M application, I_{max} was 0.26, $V_{1/2}$ was −18 mV, and k was 10.3 mV.

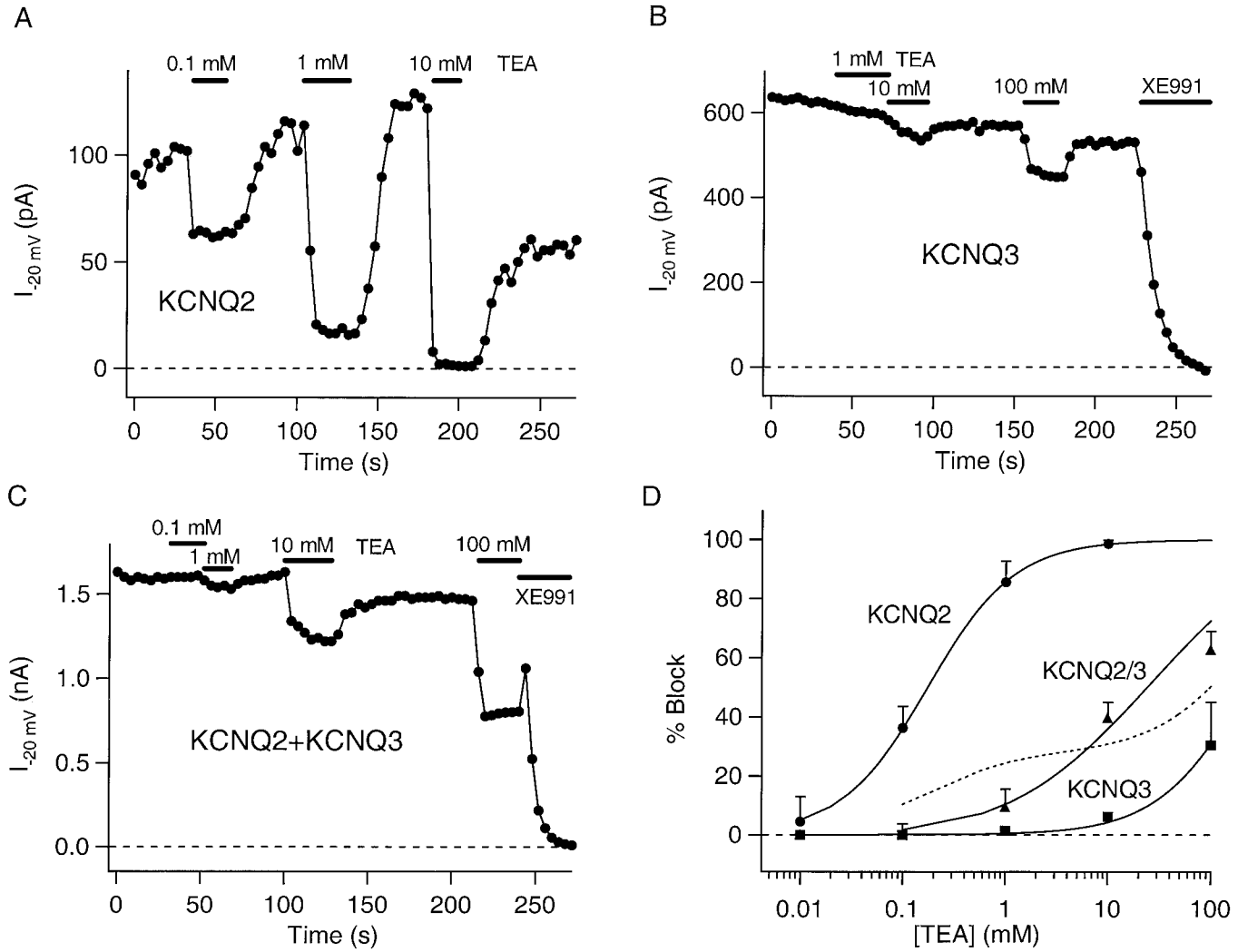

Figure 6. TEA sensitivity of KCNQ2 and KCNQ3 homo- and heteromultimers. Shown are current amplitudes at −20 mV in cells transfected with KCNQ2 (*A*), KCNQ3 (*B*), or KCNQ2 and KCNQ3 (*C*). The pipette solution contained the BACaPPS cocktail. TEA was applied at the indicated concentrations. The dose–response data are summarized in *D*. The observations (*symbols*) were fit by a binding equation (*curves*) of the form: % Block = $100([TEA]/(K_{1/2} + [TEA]))$, where $K_{1/2}$ is the concentration for half-block. The fitted $K_{1/2}$ was 174 μM for KCNQ2 and 224 mM for KCNQ3, but the data for KCNQ3 do not extend high enough for a good determination. For KCNQ2 plus KCNQ3, we first tried to fit the data as the sum of two Hill equations, with $K_{1/2}$ values taken from the homomeric data (174 μM and 224 mM) (*dotted line*). We then fit the data by the sum of five binding equations for channels, with zero to four of each type of subunit (*solid line*). The distribution of channel arrangements was governed by the binomial distribution with a fit ratio of KCNQ2/KCNQ3 subunits of 0.35:0.65. The $K_{1/2}$ values for KCNQ2 and KCNQ3 were taken from the homomeric data, and the affinities for channels with the various subunit arrangements were calculated assuming energy additivity (see Results). The predicted $K_{1/2}$ values for channels with one KCNQ2 and three KCNQ3 subunits, two KCNQ2 and two KCNQ3 subunits, and three KCNQ2 and one KCNQ3 subunits were 37.4, 6.24, and 1.04 mM, respectively. For all of the fits the maximal block was constrained to be 100%. The binomial modeling of the TEA data is qualitative and meant to demonstrate the expression of heteromeric versus homomeric channels. The data are not sufficient to determine the ratio of expressed KCNQ2 and KCNQ3 subunits with precision. Because the $K_{1/2}$ value for TEA block of the KCNQ3 currents is not well determined, the heteromeric data also were fit by allowing that value to be a free parameter. This did not change the fit significantly.

were obviously different (Fig. 6). Currents expressed by transfection of KCNQ2 alone showed substantial block at 0.1 mM TEA and almost full block at 1 mM (Fig. 6*A*), whereas currents expressed by transfection of KCNQ3 alone showed negligible block at 1 mM and less than half-block at 100 mM TEA (Fig. 6*B*). The dose–response relation for KCNQ2 was well fit by a Hill equation, with the half-blocking concentration ($K_{1/2}$) of 174 μM. That for KCNQ3 was fit by a $K_{1/2}$ value of 224 mM, but because the inhibition was so weak, the $K_{1/2}$ was not well determined (Fig. 6*D*). For both channels the Hill coefficient was very near unity.

As expected for KCNQ2/KCNQ3 heteromultimers, cotransfection of KCNQ2 and KCNQ3 produced currents with TEA sensitivity intermediate between the two homomultimers (Fig. 6*C*). In addition, a fit of the TEA dose–response relation to a single Hill equation had a Hill coefficient of 0.56, suggesting that cotransfection results in a mixed population of channel types with different TEA affinities. The data also were not well fit by the sum of two Hill equations with the $K_{1/2}$ values for the two types of homomultimers (Fig. 6*D, dotted line*), suggesting that coexpression of KCNQ2 and KCNQ3 does not result only in two homomeric populations. If association of expressed subunits into channels is random, then coexpression of KCNQ2 and KCNQ3 should result in five classes of tetramers containing from zero to four

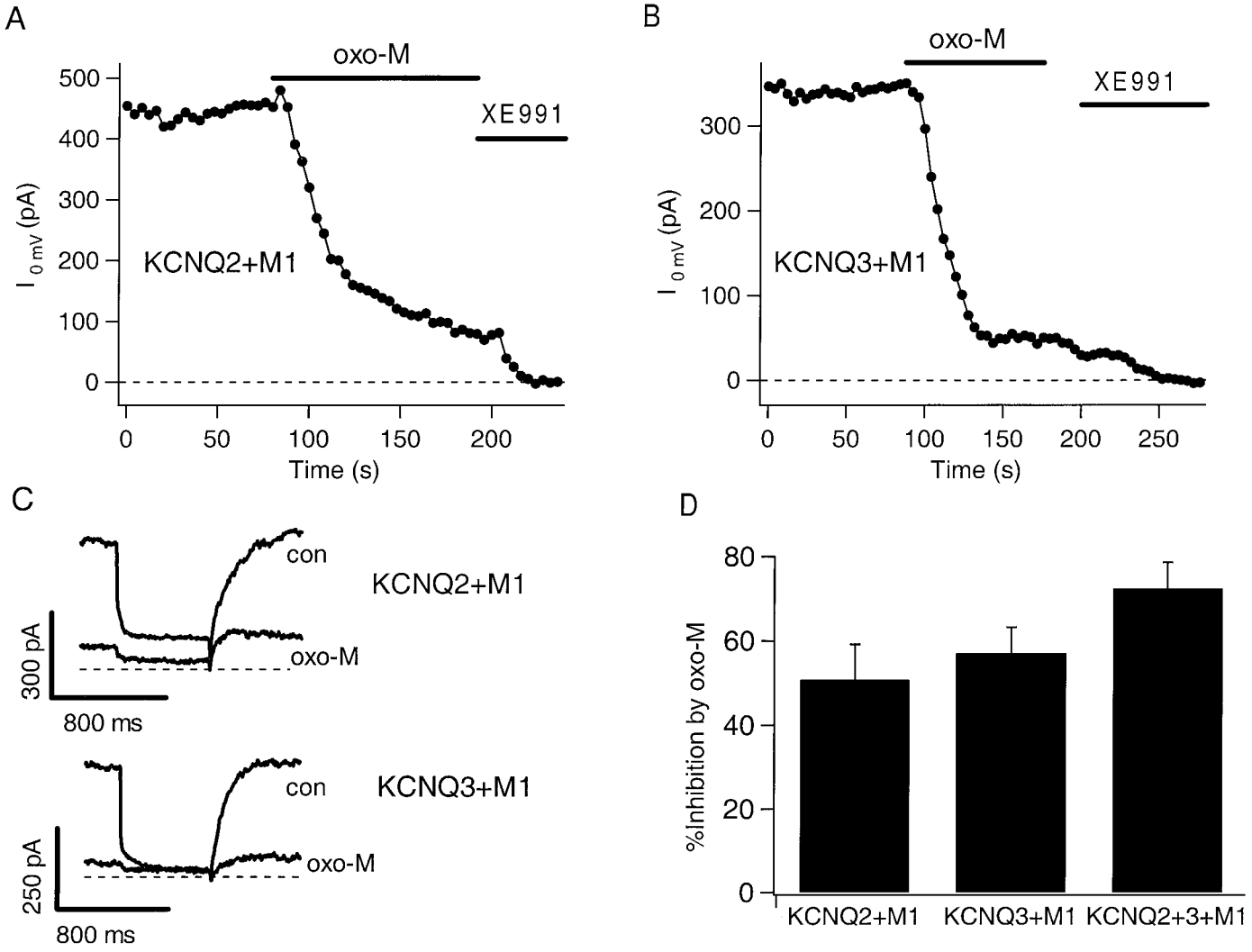

Figure 7. KCNQ2 and KCNQ3 are modulated individually by oxo-M. *A*, Current amplitudes recorded with the BACaPPS cocktail at 0 mV in cells transfected with KCNQ2 and M_1 receptors. Oxo-M (10 μM) and XE991 (50 μM) were applied as indicated. *B*, A similar experiment but with cells transfected with KCNQ3 and M_1 receptors. *C*, Current traces before and after the application of oxo-M. The *dashed line* shows the zero current level. *D*, Summary of inhibition with KCNQ2 or KCNQ3 as compared with results from KCNQ2/KCNQ3 channels taken from Figure 3*C*. They are not significantly different at the $p < 0.05$ level.

subunits of each type, with a distribution governed by the binomial distribution. In other K^+ channels, external TEA ions block in the pore at a site coordinated by the four subunits, with a blocking affinity in heteromeric channels that can be predicted by adding up the energy of interaction with each subunit (Heginbotham and MacKinnon, 1992; Kavanaugh et al., 1992; Liman et al., 1992). Thus, the dose–response relation for KCNQ2/KCNQ3 was nicely fit by the sum of five Hill equations, assuming the binomial distribution and energy additivity (Fig. 6*D, solid line*). The only free parameter was the relative abundance of the two subunit types, and the best fit was obtained with a ratio of KCNQ2/KCNQ3 of 0.35:0.65. Because we performed the cotransfections by using a 1:1 ratio of DNA, the greater abundance of KCNQ3 may reflect greater transcriptional or translational efficiency of KCNQ3 versus KCNQ2 subunits, in agreement with the larger currents obtained with transfection of individual KCNQ3 versus KCNQ2 subunits. In sum, the TEA experiments confirm that transfection with individual subunits yields properties expected of homomultimers, and cotransfection with KCNQ2 and KCNQ3 produces a mixed population of heteromultimers.

To our surprise, both homomeric channels were strongly modulated by muscarinic agonists. Again to avoid $[Ca^{2+}]_i$ elevations, these experiments were done with the BACaPPS cocktail in the pipette. Figure 7*A* shows robust suppression of KCNQ2 on the application of oxo-M (10 μM) and complete block with the M current-selective blocker XE991. Figure 7*B* shows a similar experiment and similar results with the KCNQ3 current. Sample current traces from these experiments are shown in Figure 7*C*. Such data are summarized in Figure 7*D*. Suppression by 10 μM oxo-M was 51 ± 8% ($n = 11$) for KCNQ2 currents and 57 ± 6% ($n = 9$) for KCNQ3 currents. For comparison, the suppression of KCNQ2/KCNQ3 current under the same conditions was 72 ± 6% ($n = 7$). Thus, homomeric KCNQ2 and KCNQ3 channels also form M-like currents that are suppressed by muscarinic agonists only slightly less strongly ($p < 0.11$) than are KCNQ2/KCNQ3 heteromultimers. Both subunits must contain the fea-

tures needed for modulation by the unidentified muscarinic second messenger and needed for block by XE991.

KCNQ2 channels are modulated when expressed in SCG neurons also

The results thus far suggest that muscarinic modulation of heterologously expressed KCNQ2/KCNQ3 channels in tsA cells is very similar to muscarinic modulation of the M current in SCG neurons. The clearest proof of similarity would be to express the cloned channels in differentiated sympathetic neurons and ask whether the endogenous signaling machinery can suppress the current. To answer that question, we overexpressed KCNQ2 channels in cultured SCG neurons by injecting DNA directly into the nucleus (Ikeda, 1996). We choose to inject KCNQ2 because its high sensitivity to TEA would be useful to distinguish the expressed KCNQ2 current from endogenous M current, which has only a moderate TEA sensitivity (Brown, 1988). We first measured the effect of TEA on the M current in uninjected SCG neurons. As shown in Figure 8*A*, TEA (1 mM) only slightly reduced the M current amplitude, much as for heteromeric KCNQ2/KCNQ3 currents (see Fig. 6*C*). In 10 such uninjected SCG cells, TEA reduced the M current by 14.5 ± 3%, and oxo-M (10 μM) produced an inhibition of 80 ± 4% (Fig. 8*C*).

Figure 8*B* shows the functional expression of KCNQ2 subunits in SCG neurons, as revealed by a large increase in the fraction of current blocked by 1 mM TEA. In addition to the higher TEA sensitivity found 24 hr after intranuclear injection of KCNQ2, there was also an increase in total M-like current density (8.7 ± 1 pA/pF; $n = 11$; $p < 0.03$) as compared with control cells (5.3 ± 1 pA/pF; $n = 10$), showing that additional functional channels were expressed as a consequence of KCNQ2 injection. In 11 KCNQ2-injected neurons, TEA reversibly reduced the amplitude of the M current by 46 ± 3%. As in the uninjected neuron the application of oxo-M produced a strong suppression of the K^+ current (77 ± 4%, $n = 10$). The large increase in TEA sensitivity in KCNQ2-injected neurons shows that channels containing additional KCNQ2 subunits are now being expressed in the SCG neurons in addition to the endogenous M current, resulting in an increased TEA sensitivity of the M-like current (Fig. 8*B*). Without a more detailed TEA dose–response study we cannot determine whether the extra channels are homomultimers of KCNQ2 or heteromultimers of, say, three KCNQ2 subunits and one endogenous KCNQ3 subunit, resulting in a population different from typical M channels with intermediate TEA sensitivity. Nevertheless, the equivalent suppression of the M-like current in the KCNQ2-injected cells shows that the expressed channels, containing mostly expressed KCNQ2 subunits, also are modulated by oxo-M, like the endogenous M current. Thus, we conclude that KCNQ2 subunits are strongly inhibited by the endogenous second messenger pathway that modulates the native M current in SCG neurons.

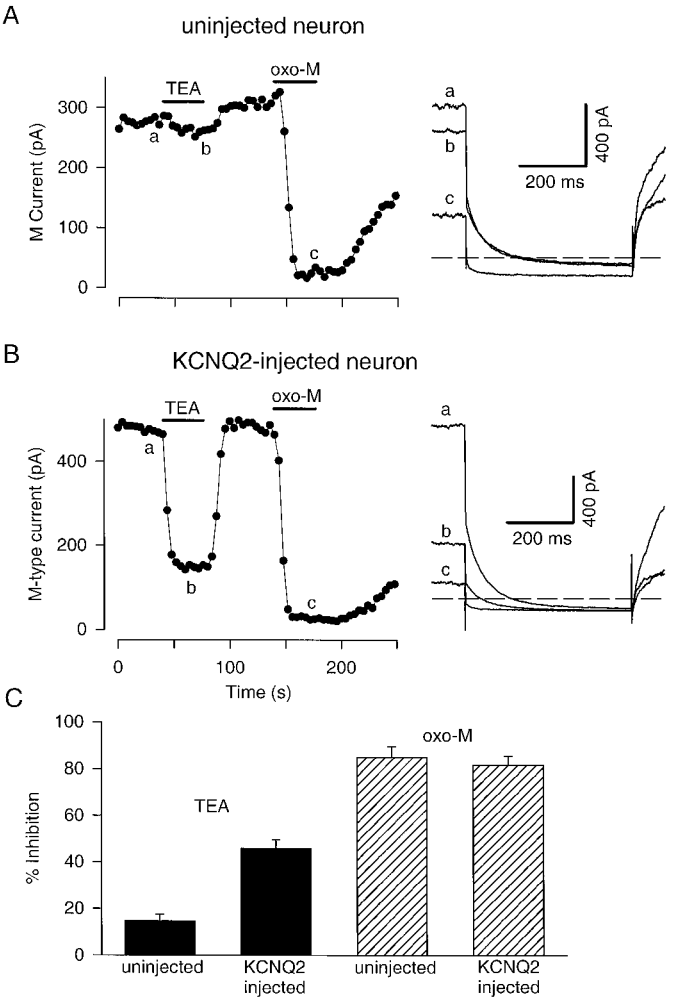

Figure 8. KCNQ2 channels can be expressed in SCG neurons and modulated by the endogenous muscarinic pathway. Shown are amplitudes of the time-dependent current at −60 mV from voltage pulses given every 4 sec in SCG neurons. *A*, An uninjected neuron. TEA (1 mM) and oxo-M (10 μM) were bath-applied as indicated. Current traces are shown on the *right* in the control (*a*) and in the presence of TEA (*b*) or oxo-M (*c*). The *dashed line* in the current traces is the zero current level. *B*, Similar experiment with a SCG neuron injected intranuclearly the previous day with plasmid for KCNQ2 (see Materials and Methods for a description of injections and a definition of current amplitude). *C*, Mean suppression of M current by TEA (*black bars*) and oxo-M (*dashed bars*). For uninjected and KCNQ2-injected cells the numbers of cells tested were 10 and 11, respectively. Two of the cells labeled *uninjected* were injected only with the GFP marker.

DISCUSSION

The KCNQ (formerly *KvLQT*) family of K^+ channels has come into prominence only recently. The importance of each member is reflected in human genetic disorders attributed to them. The first identified, KCNQ1, localizes to the heart, where it associates with the minK (IsK) subunit to produce the cardiac current called I_{Ks} (Barhanin et al., 1996; Sanguinetti et al., 1996). Mutations in KCNQ1 cause a form of long-QT syndrome of cardiac arrythmias and deafness (Barhanin et al., 1996; Sanguinetti et al., 1996; Neyroud et al., 1997). KCNQ2 and KCNQ3 are not expressed in the heart but rather in numerous brain regions and sympathetic ganglia (Biervert et al., 1998; Wang et al., 1998; Yang et al., 1998). Several mutations in either of these neuronal channel genes cause a form of neonatal epilepsy in humans (Biervert et al., 1998; Charlier et al., 1998; Singh et al., 1998). If neuronal M current indeed does result from KCNQ2 and KCNQ3 channel expression, then the dysfunction seen in individuals with non- or weakly functional mutant forms of these channels demonstrates that the M current plays an important stabilizing role in the nervous system. Finally, mutations in the newest member of this gene family to be identified, KCNQ4, result in a distinct syndrome of human deafness (Coucke et al., 1999; Kubisch et al., 1999).

In this work, we develop significant additional evidence for the identification of KCNQ2 and KCNQ3 channels with the M current of sympathetic ganglia, and we introduce a reconstituted

system capable of generating the elusive cytoplasmic muscarinic messenger of sympathetic neurons. The new evidence is primarily modulation. Like M currents in SCG, the KCNQ2/KCNQ3 current expressed in our tsA cell system, with the BACaPPS cocktail in the whole-cell pipette, is strongly suppressed by muscarinic agonists acting via M_1 muscarinic receptors. As for the M current, this modulation does not require a transient elevation of $[Ca^{2+}]_i$, and it is blocked if $[Ca^{2+}]_i$ is clamped to well below physiological levels. Treatments with NEM increase the amplitude of both currents but do not disturb the G-protein-coupled signaling from M_1 receptors to the channels. The modulation is not mediated by protein kinases and has no apparent voltage dependence. Finally, KCNQ2 current can be expressed exogenously in SCG cells and is modulated by the endogenous muscarinic second messenger system of these native neurons. Hence we are in full accord with the original suggestion of Wang and colleagues (1998) that the M current of SCG neurons is composed of KCNQ2/KCNQ3 subunits.

Although we do not yet know the identity of the diffusible cytoplasmic signaling molecule, the ability of channels formed by KCNQ2 and KCNQ3 homomultimers to be suppressed by muscarinic agonists suggests that the modulatory site is common to both subunits. There may well be constraints on the subunit assembly of KCNQ channels that dictate the allowed stoichiometry and arrangement of subunits, and there may be structural requirements that determine which combinations yield functional channels. However, the ability to express these channels separately and in combination raises the possibility that native M current channels, even in one neuron, may be a heterogeneous population of proteins with different subunit combinations. Single-channel kinetic analysis of the M current in sympathetic neurons has indicated remarkably complex gating behavior (Selyanko and Brown, 1999). If the observed M current arises from a mixture of channels, then the analysis of macroscopic properties will be made even more subtle by the heterogeneity of structure as well as from the complexity of gating mechanism. For example, this heterogeneity may explain the observation of M channels in the same cell with different unitary conductances and sensitivities to intracellular Ca^{2+} (Stansfeld et al., 1993; Selyanko and Brown, 1996). Furthermore, in different cell types the subunit arrangements may differ, and perhaps KCNQ4 subunits are included as well (Coucke et al., 1999; Kubisch et al., 1999). Recently, it has been suggested that some of the channels underlying M-like currents in a neuroblastoma cell line, but not in rat or mouse sympathetic neurons, are formed from Erg1 channel subunits, as well as others formed by KCNQ2 and KCNQ3 (Selyanko et al., 1999). Thus, M-type currents may have a range of potential subunit forms with subtly different physiological and pharmacological properties. As with the subunit assembly of many other channels, cells probably exercise control over which, and how much, of each type of subunit to express, providing a more refined means of regulating neuronal function.

For many years several laboratories have tried unsuccessfully to identify the second messenger that mediates the modulation of M currents by muscarinic receptors and by several other receptors coupled to $G_{q/11}$ G-proteins (for summary, see Hille, 1994; Marrion, 1997). In this paper we show that muscarinic M current modulation persists even when three measures are used *simultaneously* to block $[Ca^{2+}]_i$ transients (BAPTA, PPS, and thapsigargin). In SCG neurons any one of these three treatments *individually* suffices to block the Ca^{2+}-mediated modulation of the M current by bradykinin (Cruzblanca et al., 1998). Thus, as in previous papers, we reject the hypothesis that a $[Ca^{2+}]_i$ rise is necessary for muscarinic modulation or that free Ca^{2+} itself is the diffusible cytoplasmic messenger. Although Schroeder et al. (1998) show an effect of intracellular cAMP on KCNQ2/KCNQ3 channels, the magnitude and direction of the effect (increased cAMP makes the current larger) make cAMP or protein kinase A unlikely mediators of this signaling pathway. In addition, several tests with cyclic nucleotides and kinase and phosphatase activators and inhibitors in sympathetic neurons have yielded only negative results (Hille, 1994; Marrion, 1997), as did the tests with staurosporine in this paper. Because the muscarinic signaling pathway is present in tsA cells, it cannot be a uniquely neuronal mechanism and may generalize to many different types of cells. Our laboratory and others have advanced much correlative evidence that the same unidentified messenger modulating the M current can modulate L- and N-type Ca^{2+} channels in sympathetic and central neurons (Beech et al., 1991; Bernheim et al., 1991; Shapiro et al., 1994a; Stewart et al., 1999). The development of the expression system presented here that reconstitutes muscarinic modulation in a mammalian cell line should facilitate the pursuit of this unidentified intracellular messenger with its wide-ranging actions and make possible the use of biochemical and genetic approaches. We expect that the capability of investigating this problem on a molecular level will uncover the interactions among the various proteins that act together to regulate this signaling system.

REFERENCES

Barhanin J, Lesage F, Guillemare E, Fink M, Lazdunski M, Romey G (1996) KvLQT1 and IsK (minK) proteins associate to form the I_{Ks} cardiac potassium current. Nature 384:78–80.

Bean BP (1989) Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. Nature 340:153–156.

Beech DJ, Bernheim L, Mathie A, Hille B (1991) Intracellular Ca^{2+} buffers disrupt muscarinic suppression of Ca^{2+} current and M current in rat sympathetic neurons. Proc Natl Acad Sci USA 88:652–656.

Bernheim L, Beech DJ, Hille B (1991) A diffusible second messenger mediates one of the pathways coupling receptors to calcium channels in rat sympathetic neurons. Neuron 6:859–867.

Bernheim L, Mathie A, Hille B (1992) Characterization of muscarinic receptor subtypes inhibiting Ca^{2+} current and M current in rat sympathetic neurons. Proc Natl Acad Sci USA 89:9544–9548.

Biervert C, Schroeder BC, Kubisch C, Berkovic SF, Propping P, Jentsch TJ, Steinlein OK (1998) A potassium channel mutation in neonatal human epilepsy. Science 279:403–406.

Brown DA (1988) M currents. In: Ion channels (Narahashi T, ed), pp 55–94. New York: Plenum.

Brown DA, Adams PR (1980) Muscarinic suppression of a novel voltage-sensitive K^+ current in a vertebrate neurone. Nature 283:673–676.

Brown DA, Abogadie FC, Allen TG, Buckley NJ, Caulfield MP, Delmas P, Haley JE, Lamas JA, Selyanko AA (1997) Muscarinic mechanisms in nerve cells. Life Sci 60:1137–1144.

Charlier C, Singh NA, Ryan SG, Lewis TB, Reus BE, Leach RJ, Leppert M (1998) A pore mutation in a novel KQT-like potassium channel gene in an idiopathic epilepsy family. Nat Genet 18:53–55.

Choi S, Lovinger DM (1996) Metabotropic glutamate receptor modulation of voltage-gated Ca^{2+} channels involves multiple receptor subtypes in cortical neurons. J Neurosci 16:36–45.

Constanti A, Brown DA (1981) M currents in voltage-clamped mammalian sympathetic neurones. Neurosci Lett 24:289–294.

Coucke PJ, Hauwe PV, Kelley PM, Kunst H, Schatteman I, Velzen DV, Meyers J, Ensink RJ, Verstreken M, Declau F, Marres H, Kastury K, Bhasin S, McGuirt WT, Smith RJ, Cremers CW, Heyning PV, Willems PJ, Smith SD, Camp GV (1999) Mutations in the KCNQ4 gene are responsible for autosomal dominant deafness in four DFNA2 families. Hum Mol Genet 8:1321–1328.

Cruzblanca H, Koh DS, Hille B (1998) Bradykinin inhibits M current via phospholipase C and Ca^{2+} release from IP_3-sensitive Ca^{2+} stores in rat sympathetic neurons. Proc Natl Acad Sci USA 95:7151–7156.

Delmas P, Brown DA, Dayrell M, Abogadie FC, Caulfield MP, Buckley NJ (1998) On the role of endogenous G-protein beta gamma subunits

in N-type Ca^{2+} current inhibition by neurotransmitters in rat sympathetic neurones. J Physiol (Lond) 506:319–329.
Dolphin AC (1998) Mechanisms of modulation of voltage-dependent calcium channels by G-proteins. J Physiol (Lond) 506:3–11.
Ehrlich BE, Kaftan E, Bezprozvannaya S, Bezprozvanny I (1994) The pharmacology of intracellular Ca^{2+}-release channels. Trends Pharmacol Sci 15:145–149.
Felder CC (1995) Muscarinic acetylcholine receptors: signal transduction through multiple effectors. FASEB J 9:619–625.
Freeman LC, Kass RS (1995) Cholinergic inhibition of slow delayed rectifier K^+ current in guinea pig sino-atrial node is not mediated by muscarinic receptors. Mol Pharmacol 47:1248–1254.
Garcia DE, Li B, Garcia-Ferreiro RE, Hernandez-Ochoa EO, Yan K, Gautam N, Catterall WA, Mackie K, Hille B (1998) G-protein β-subunit specificity in the fast membrane-delimited inhibition of Ca^{2+} channels. J Neurosci 18:9163–9170.
Grove EA, Caulfield MP, Evans FJ (1990) Inhibition of protein kinase C prevents phorbol ester- but not muscarine-induced depolarizations in the rat superior cervical ganglion. Neurosci Lett 110:162–166.
Haley JE, Abogadie FC, Delmas P, Dayrell M, Vallis Y, Milligan G, Caulfield MP, Brown DA, Buckley NJ (1998) The α-subunit of G_q contributes to muscarinic inhibition of the M-type potassium current in sympathetic neurons. J Neurosci 18:4521–4531.
Hamilton SE, Loose MD, Qi M, Levey AI, Hille B, McKnight GS, Idzerda RL, Nathanson NM (1997) Disruption of the M_1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice. Proc Natl Acad Sci USA 94:13311–13316.
Heginbotham L, MacKinnon R (1992) The aromatic binding site for tetraethylammonium ion on potassium channels. Neuron 8:483–491.
Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA (1996) Modulation of Ca^{2+} channels by G-protein beta gamma subunits. Nature 380:258–262.
Hille B (1994) Modulation of ion channel function by G-protein-coupled receptors. Trends Neurosci 17:531–536.
Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits. Nature 380:255–258.
Inesi G, Sagara Y (1992) Thapsigargin, a high affinity and global inhibitor of intracellular Ca^{2+} transport ATPases. Arch Biochem Biophys 298:313–317.
Jakobs KH, Lasch P, Minuth M, Aktories K, Schultz G (1982) Uncoupling of α-adrenoceptor-mediated inhibition of human platelet adenylate cyclase by *N*-ethylmaleimide. J Biol Chem 257:2829–2833.
Jones S, Brown DA, Milligan G, Willer E, Buckley NJ, Caulfield MP (1995) Bradykinin excites rat sympathetic neurons by inhibition of M current through a mechanism involving B_2 receptors and G alpha q/11. Neuron 14:399–405.
Kavanaugh MP, Hurst RS, Yakel J, Varnum MD, Adelman JP, North RA (1992) Multiple subunits of a voltage-dependent potassium channel contribute to the binding site for tetraethylammonium. Neuron 8:493–497.
Kubisch C, Schroeder BC, Friedrich T, Lutjohann B, El-Amraoui A, Marlin S, Petit C, Jentsch TJ (1999) KCNQ4, a novel potassium channel expressed in sensory outer hair cells, is mutated in dominant deafness. Cell 96:437–446.
Liman ER, Tytgat J, Hess P (1992) Subunit stoichiometry of a mammalian K^+ channel determined by construction of multimeric cDNAs. Neuron 9:861–871.
MacKinnon R, Yellen G (1990) Mutations affecting TEA blockade and ion permeation in voltage-activated K^+ channels. Science 250:276–279.
Marrion NV (1997) Control of M current. Annu Rev Physiol 59:483–504.
Marrion NV, Zucker RS, Marsh SJ, Adams PR (1991) Modulation of M current by intracellular Ca^{2+}. Neuron 6:533–545.
Meggio F, Donella Deana A, Ruzzene M, Brunati AM, Cesaro L, Guerra B, Meyer T, Mett H, Fabbro D, Furet P, Dobrowolska G, Pinna LA (1995) Different susceptibility of protein kinases to staurosporine inhibition. Kinetic studies and molecular bases for the resistance of protein kinase CK2. Eur J Biochem 234:317–322.
Milligan G (1988) Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. Biochem J 255:1–13.
Neyroud N, Tesson F, Denjoy I, Leibovici M, Donger C, Barhanin J, Faure S, Gary F, Coumel P, Petit C, Schwartz K, Guicheney P (1997) A novel mutation in the potassium channel gene *KvLQT1* causes the Jervell and Lange-Nielsen cardioauditory syndrome. Nat Genet 15:186–189.
Ruegg UT, Burgess GM (1989) Staurosporine, K-252, and UCN-01: potent but nonspecific inhibitors of protein kinases. Trends Pharmacol Sci 10:218–220.
Sanguinetti MC, Curran ME, Zou A, Shen J, Spector PS, Atkinson DL, Keating MT (1996) Coassembly of KvLQT1 and minK (IsK) proteins to form cardiac I_{Ks} potassium channel. Nature 384:80–83.
Schroeder BC, Kubisch C, Stein V, Jentsch TJ (1998) Moderate loss of function of cyclic-AMP-modulated KCNQ2/KCNQ3 K^+ channels causes epilepsy. Nature 396:687–690.
Selyanko AA, Brown DA (1996) Intracellular calcium directly inhibits potassium M channels in excised membrane patches from rat sympathetic neurons. Neuron 16:151–162.
Selyanko AA, Brown DA (1999) M channel gating and simulation. Biophys J 77:701–713.
Selyanko AA, Stansfeld CE, Brown DA (1992) Closure of potassium M channels by muscarinic acetylcholine receptor stimulants requires a diffusible messenger. Proc R Soc Lond [Biol] 250:119–125.
Selyanko AA, Hadley JK, Wood IC, Abogadie FC, Delmas P, Buckley NJ, London B, Brown DA (1999) Two types of K^+ channel subunits, Erg1 and KCNQ2/3, contribute to the M-like current in a mammalian neuronal cell. J Neurosci 19:7742–7756.
Shapiro MS, Wollmuth LP, Hille B (1994a) Angiotensin II inhibits calcium and M current channels in rat sympathetic neurons via G-proteins. Neuron 12:1319–1329.
Shapiro MS, Wollmuth LP, Hille B (1994b) Modulation of Ca^{2+} channels by PTX-sensitive G-proteins is blocked by *N*-ethylmaleimide in rat sympathetic neurons. J Neurosci 14:7109–7116.
Shapiro MS, Zhou J, Hille B (1996) Selective disruption by protein kinases of G-protein-mediated Ca^{2+} channel modulation. J Neurophysiol 76:311–320.
Singh NA, Charlier C, Stauffer D, DuPont BR, Leach RJ, Melis R, Ronen GM, Bjerre I, Quattlebaum T, Murphy JV, McHarg ML, Gagnon D, Rosales TO, Peiffer A, Anderson VE, Leppert M (1998) A novel potassium channel gene, KCNQ2, is mutated in an inherited epilepsy of newborns. Nat Genet 18:25–29.
Stansfeld CE, Marsh SJ, Gibb AJ, Brown DA (1993) Identification of M channels in outside-out patches excised from sympathetic ganglion cells. Neuron 10:639–654.
Stewart AE, Yan Z, Surmeier DJ, Foehring RC (1999) Muscarine modulates Ca^{2+} channel currents in rat sensorimotor pyramidal cells via two distinct pathways. J Neurophysiol 81:72–84.
Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M, Tomita F (1986) Staurosporine, a potent inhibitor of phospholipid/Ca^{2+}-dependent protein kinase. Biochem Biophys Res Commun 135:397–402.
Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP (1990) Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc Natl Acad Sci USA 87:2466–2470.
Tones MA, Bootman MD, Higgins BF, Lane DA, Pay GF, Lindahl U (1989) The effect of heparin on the inositol 1,4,5-trisphosphate receptor in rat liver microsomes. Dependence on sulfate content and chain length. FEBS Lett 252:105–108.
Viana F, Hille B (1996) Modulation of high voltage-activated calcium channels by somatostatin in acutely isolated rat amygdaloid neurons. J Neurosci 16:6000–6011.
Wang HS, McKinnon D (1995) Potassium currents in rat prevertebral and paravertebral sympathetic neurones: control of firing properties. J Physiol (Lond) 485:319–335.
Wang HS, Pan Z, Shi W, Brown BS, Wymore RS, Cohen IS, Dixon JE, McKinnon D (1998) KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M channel. Science 282:1890–1893.
Wanke E, Ferroni A, Malgaroli A, Ambrosini A, Pozzan T, Meldolesi J (1987) Activation of a muscarinic receptor selectively inhibits a rapidly inactivated Ca^{2+} current in rat sympathetic neurons. Proc Natl Acad Sci USA 84:4313–4317.
Wickman KD, Clapham DE (1995) G-protein regulation of ion channels. Curr Opin Neurobiol 5:278–285.
Yang WP, Levesque PC, Little WA, Conder ML, Ramakrishnan P, Neubauer MG, Blanar MA (1998) Functional expression of two KvLQT1-related potassium channels responsible for an inherited idiopathic epilepsy. J Biol Chem 273:19419–19423.
Yu SP, Kerchner GA (1998) Endogenous voltage-gated potassium channels in human embryonic kidney (HEK293) cells. J Neurosci Res 52:612–617.