ROLE OF "M-TYPE" K^+ CHANNELS IN THE STABILIZATION OF NEURONAL FIRING AND REGULATION OF PRESYNAPTIC NEUROTRANSMITTER RELEASE

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The photo shows a co-worker of P.G. Kostyuk: Zaika O.

Introduction

The M-type K^+ current was first described in sympathetic ganglia cells as a voltage-gated non-inactivating conductance which is strongly suppressed by stimulation of muscarinic acetylcholine receptors (Brown and Adams, 1980; Constanti and Brown, 1981).

The voltage-threshold of gating near typical neuronal resting potentials and the slow kinetics of activation and deactivation of the M-current provides a powerful mechanism for the regulation of neuronal excitability. Modulation of the M-current exerts profound effect on the action potential initiation, firing fre-

quency, and general neuronal discharge properties (Cooper et al., 2001; Martire et al., 2004). However, although a number of investigators have characterized the impact of M-current activity on neuronal discharge properties, it has not yet been established if the resultant influence on release of neurotransmitter is also due to localized M-channel action at release sites which further tune the neurotransmitter released *per* arriving action potential. Studies using synaptosomes prepared from hippocampal nerve terminals and using slice recordings suggest such a direct link between the M-current and neurotransmitter release (Gu et al., 2005). Previous somatic recordings from hippocampal neurons have indicated that the KCNQ current is involved in determining several aspects of neuronal excitability, including the resting membrane potential (RMP), spike frequency adaptation, and burst suppression (Schwarz et al., 2006; Vervaeke et al., 2006; Yue and Yaari, 2006). These studies combined lead to the hypothesis that G-protein signaling pathways directly tune the release of neurotransmitter from nerve terminal via control of M-channel, and resultant regulation of pre-synaptic membrane potential. The knowledge of the role of M-channels in neuronal activity will allow a possible design of new modes of therapeutic intervention for multiple diseases.

Role of M channels in regulation of neuronal excitability in SCG neurons

Our first experiments showed the feasibility of our approach to assaying the functional role of M current, and the impact of its modulation, in sympathetic neurons of the rat superior cervical ganglion. Neurons are cultured in the presence of nerve growth factor, which allows cell survival and extension of normal processes in the culture dish. Fig. 1, *a* shows an experiment using XE991 (10 μM) and RTG (10 μM) from a SCG cell.

Under voltage clamp, typical M-type currents are recorded when the membrane potential is held at –25 mV and a hyperpolarizing step is to -60 mV, Fig.2. When studying in whole-cell mode under current-clamp, we can examine the relationship between injected current and firing properties under conditions of varying M-channel activity. Fig. 1, *b* shows an experiment in which two drugs that affect M-current activity were assayed for their effects on the firing properties of SCG neurons. They are XE991 and RTG, a selective KCNQ/M-channel blocker and an opener, respectively (Rundfeldt and Netzer, 2000; Zaczek et al., 1998). Under current clamp, a family of current pulses was applied from –100 pA to 100 pA (inset). The negative current pulses are used as an assay of cellular input resistance. A stimulatory current of 100 pA is usually sufficient to cause discharge in these cells, and in this case, it evoked four APs.

Further APs are not observed during the pulse, because of the accumulating activation of M channels by the initial APs. Application of XE991 greatly increases neuronal excitability, spike-train duration, the decrease in the threshold cur-

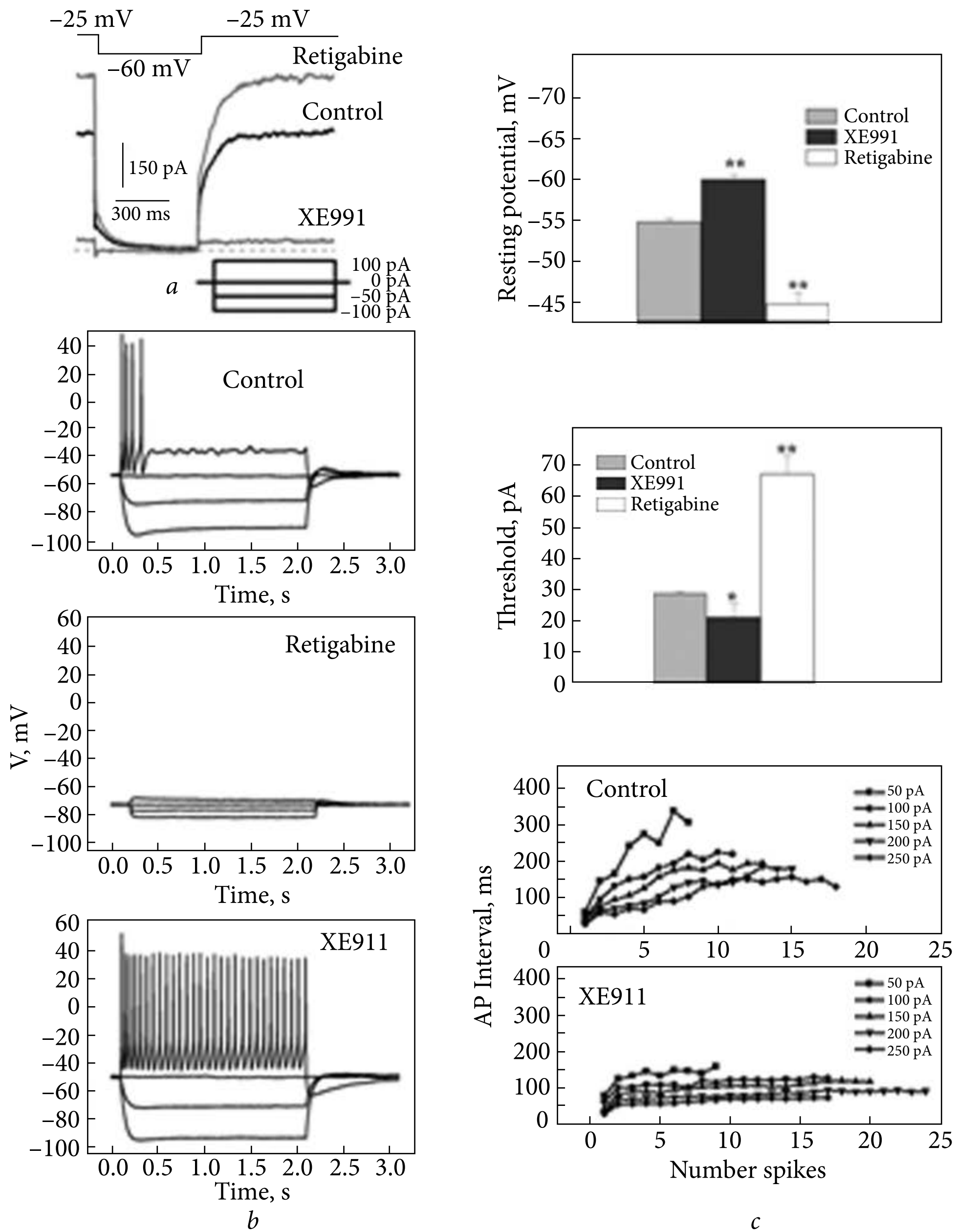

Fig. 1. The M-currents were recorded using the classical pulse protocol (*a*). Current-clamp records showing changes in membrane potential during depolarizing and hyperpolarizing current steps with gradual increase of amplitude of a current from −100 to 100 pA in control and upon application of the M channel openers Retigabine (RT 10 mM) or blocker XE991 (10 mM) (*b*). Analysis of electrophysiological parameters action potential in control and after application XE991 and retigabine: resting membrane potential, the amplitude of the current required to reach threshold for action potential generation and accommodation characteristics of the APs (*c*)

rent, and the depolarization of the resting potential in SCG cells. XE991 also influences spike frequency adaptation, measured as the increasing interval between action potentials (APs). The slopes of the AP interval *vs* spike number relations were considerably shallower in the presence of XE at all injected currents. On the other hand, application of RTG caused a strong decrease in excitability, decrease in input resistance, increase in the threshold currents, and hyperpolarization of the resting potential. For example, in this experiment the 100 pA stimulatory current failed to elicit any APs at all, and the negative pulses hyperpolarized the cell much less than in control. All of these effects are consistent with reduction or augmentation of M-channel activity, respectively.

We can interpret the effects of RTG as ones due to increased opening of M-type channels at all potentials and shifts of their voltage dependence to more hyperpolarized potentials. Thus, M channels, which normally begin to open at –50 mV, are now open at substantially more negative potentials, causing a hyperpolarization of the resting potential and much less hyperpolarization caused by the injected negative currents. The increase in M-channel opening and the left-shift in channel activation prevent the 100 pA injected current from depolarizing the cell to threshold for firing, preventing APs. Application of XE991, on the other hand, strongly increased somatic excitability, and slightly depolarized the resting potential, due to complete M-channel closure. Thus, the 100 pA injected current now elicits a continued train of action potentials, since the M channels have been closed by the drug. XE991 does not affect the response to the negative injected currents, since the M channels are almost all closed at potentials negative to rest, both in the presence and absence of the drug. These data are summarized in Fig 1, *c*.

Role of M channels in regulation of exocytosis in PC12 cells

The sympathetic nervous system activates both the cardiovascular and the adrenal catecholaminergic systems which are critical for rapid homeostasis. However, although a number of investigators have thus characterized the impact of M-current activity on neuronal discharge properties, it has not yet been established if the resultant influence on the release of neurotransmitter is also due to localized M-channel actions at release sites that further tune the neurotransmitter released per arriving action potential. PC12 cells possess all the necessary components for studying catecholaminergic systems (such as chromaffin cells, which play important physiological roles in the coordinated response to stress, heart rate and blood pressure): catecholamine synthesis, storage, release and reuptake. Fig. 3 shows an experiment using XE991 (10 μM) and RTG (10 μM) from a PC12 cell. Under voltage clamp, typical M-type currents are recorded when the membrane potential is held at –25 mV and a hyperpolarizing step is given to –60 mV.

Furthermore, we performed experiments demonstrating NE release from PC12 cells using carbon fiber amperometry (CFA). Amperometric measurements

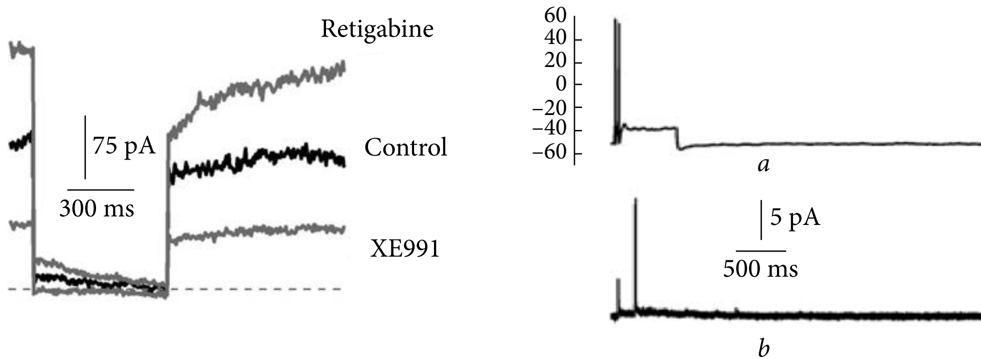

Fig. 2. The M-currents were recorded from PC12 cells using the classical pulse protocol

Fig. 3. Current-clamp records showing voltage responses and AP firing elicited by 100 pA depolarizing step (*a*); Amperometry recording from a PC12 cell (*b*)

were made with polyethylene-insulated carbon fiber electrodes fabricated according to published techniques and the techniques in use in our laboratory (Chow *et al.*, 1992). The electrode potential used was +600mV. These recordings were made with NE release triggered by electrical stimulation (20 Hz, 0.07–0.1 mA strength, and 1 ms duration with a 50-ms interval). We showed what M-channel activity increases the amount of NE released by each evoked action potential (AP). A simultaneous current clamp and amperometry experiment on a PC12 cell are shown in Fig. 3.

A 100 pA depolarizing pulse elicits a pair of APs, and, in parallel, two amperometric spikes which report exocytosis of NE-containing vesicles. We also assayed the effect of M channels on NE release using XE991, a selective KCNQ/M-channel blocker.

Fig. 4 shows representative experiments. In control, the rate of release, reflected by the number of amperometric spikes, increases sharply after application of XE991.

Role of M channels in regulation of neuronal excitability end exocytosis in hippocampus CA1 pyramidal neurons

Clinical, pathological, and physiological studies of epilepsy have historically emphasized the role of the hippocampus. In CA1 pyramidal neurons, native M-channels seem to be composed largely of KCNQ2/3 heteromultimers. Fig. 5 shows an experiment using the M-channel blocker, linopirdine (LP, 10 μM), in CA1 region hippocampal neurons. Under current clamp, we see typical M-type currents when the membrane potential is held at –25 mV (Fig. 5, *a*). Under current clamp, a family of current pulses was applied from –100 pA to 100 pA (Fig. 5, *b*). A stimulatory current of 100 pA is usually sufficient to cause discharge in these cells, and in this case, it evoked several APs. Further APs are not observed during the pulse due to the accumulating activation of M channels by the initial APs. Application

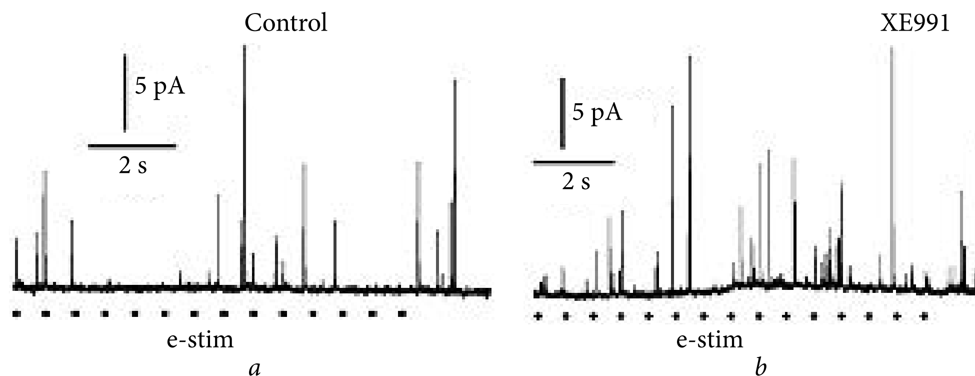

Fig. 4. NE secretion from PC12 cells detected by carbon-fiber electrode (CFE)

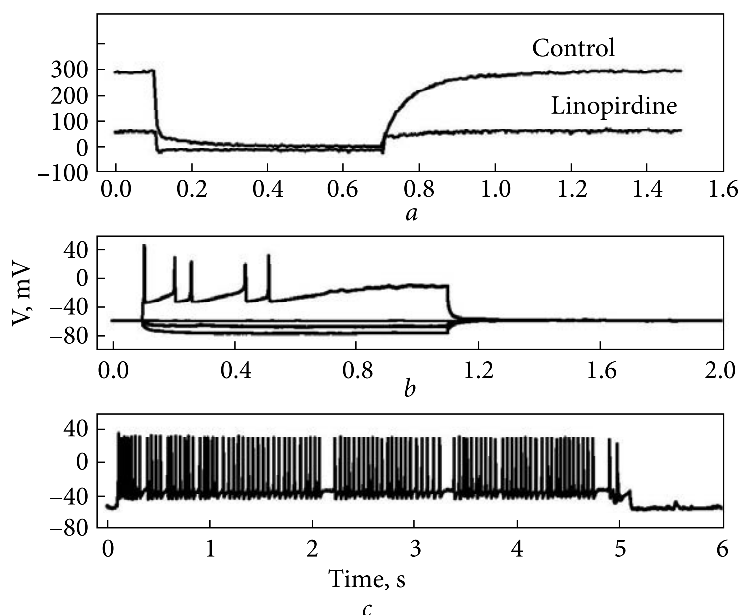

Fig. 5. Typical M-type currents in CA1 hippocampal neurons produced under voltage clamp, using a classical M-current voltage protocol (*a*); Current-clamp records showing voltage responses and AP firing elicited by depolarizing and hyperpolarizing current steps from -100 to 100 pA (*b*); Linopirdine (LP. 10 μM) strongly increased somatic excitability and slightly depolarized the resting potential in CA1 pyramidal neurons (*c*)

of linopirdine strongly increased somatic excitability and slightly depolarized the resting potential (Fig. 5, *c*). The injected 100 pA current now elicits a continued train of action potentials.

We also performed analysis of the activity-dependent destaining of the fluorescent styryl dye FM1-43 as a reporter of exocytosis. FM1-43 is taken up by synaptic vesicles in an activity-dependent manner (Cochilla et al., 1999; Gaffield

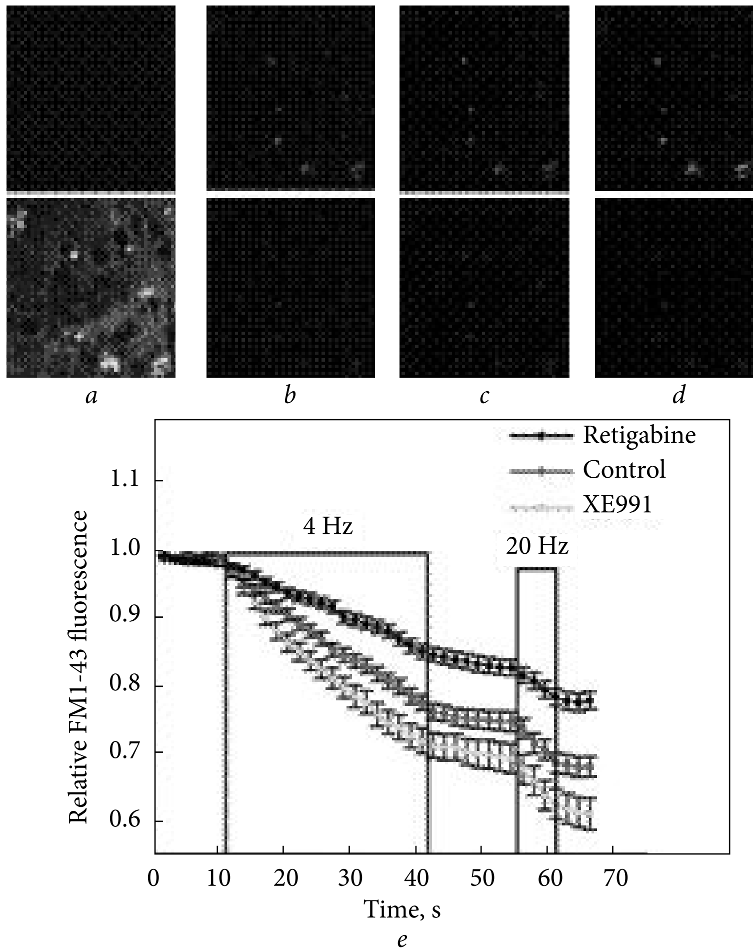

Fig. 6. Fluorescent imaging of cultures for uptake of vesicle dye FM1–43: *a* — without FM and FM loaded no wash; *b* — control at the beginning and at the end of the experiment; *c* — retigabine at the beginning and the end of the experiment; *d* — XE991 at the beginning and the end of the experiment; *e* — average relative FM1–43 fluorescence measured from 9 synapses

and Betz, 2006). FM1-43 becomes loaded into neurotransmitter-containing synaptic vesicles during endocytosis. In the presence of the dye in the bath (15 μM), cultured SCG or CA1 pyramidal neurons can be stimulated using a field electrode for 30-60 s (10-20 Hz) at a stimulation strength supramaximal for eliciting APs, causing robust exocytosis at release sites.

After the stimulation, the cells remain in the presence of FM1-43 for 1-2 min (optimal time to be determined empirically), to allow loading of the dye into the empty vesicles and subsequent endocytosis and re-filling with neurotransmitter. After this procedure, the release sites contain vesicles containing neurotransmitter and dye molecules, and subsequent excitation and exocytosis can be moni-

tored in real-time as the "destaining" of the dye, as it is released into the bath during exocytosis. The rate of destaining is thus proportional to the rate of exocytosis and release of neurotransmitter, and we canmonitor such release by taking time-lapse images of the cells using imaging equipment. We examined synaptic vesicle dynamics in neurons cultured from CA1 regions of the rat hippocampus. Field stimulation of hippocampal cultures in the presence of FM1-43 followed by washing of the dye loads vesicles corresponding to recently retrieved synaptic vesicle membrane at release sites. The time course of exocytosis can be studied by measuring the destaining during subsequent stimulation (Fig. 6, *b-d*). These results confirm that M-current opener RTG slowed, but the blocker XE991 increased the rates of destaining at each synapse. As expected, increasing the stimulus frequency to 20 Hz caused an increase in the relative rate of destaining (Fig. 6, *e*).

Basing on the publications (Zaika et al., 2006; Zaika et al., 2007) and on the data presented, we hypothesize that control of M channels regulates the release of neurotransmitter at synapses, most likely by control of membrane potential in pre-synaptic nerve terminals. These studies provide important information on the mechanisms underlying hyper-excitability in the nervous system.

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