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THE COEXISTENCE IN RAT MUSCLE CELLS OF TWO DISTINCT CLASSES OF Ca^{2+}-DEPENDENT K^+ CHANNELS WITH DIFFERENT PHARMACOLOGICAL PROPERTIES AND DIFFERENT PHYSIOLOGICAL FUNCTIONS

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SUMMARY : Ca^{2+}-dependent K^+ channels responsible for the long-lasting after-hyperpolarization in rat muscle cells in culture are not those extensively studied by the patch-clamp technique. The first ones are blocked by apamin, a bee venom polypeptide, and they are unaffected by tetraethylammonium (TEA) whereas the second ones are blocked by TEA and unaffected by apamin. These two Ca^{2+}-dependent K^+ channels coexist in rat muscle cells in culture but also probably in many other cellular types.

INTRODUCTION : Ca^{2+}-dependent K^+ channels have been found in a variety of excitable and non excitable cells (1-3). In rat muscle cells in culture, the action potential is followed by a long-lasting after-hyperpolarization (a.h.p.) (4). This a.h.p. have been demonstrated to be mediated by the activation of a Ca^{2+}-dependent slow K^+ conductance caused by the intracellular increase of Ca^{2+} concentration during the action potential (4). Since the development of patch-clamp techniques (5), single Ca^{2+}-dependent K^+ currents have been recorded from rat myotubes membrane patches (6-8). These channels show a large conductance (100-250 pS) and a high selectivity for K^+ ions. The opening frequency and the life time of these channels depend on both membrane potential and internal Ca^{2+} concentration. It was tempting to explain the a.h.p. by the activation of such a high conductance Ca^{2+}-dependent K^+ channel. We show here by a pharmacological approach that, in the case of rat myotube but also probably in a more general way, the a.h.p. is not linked to the activation of this high conductance channel.

MATERIALS AND METHODS :

Cell cultures. Primary culture of thigh muscle of new born rats were prepared as described previously (9). After one week of growth in Ham's F12 culture medium supplemented with 10 % fetal calf serum and 10 % horse serum, muscle

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cell cultures were transferred to Dulbecco modified Eagle's medium supplemented with 5 % fetal calf serum. Under these conditions the fusion of myoblasts into myotubes occurs within 3-4 days. Myosacs were prepared by exposure of 2-day-old myotubes to 0.1 µM vinblastine.

Electrophysiological experiments. Culture dishes were used directly after the culture medium had been replaced by the physiological medium containing 140 mM NaCl, 5.4 mM KCl, 1.6 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose, buffered by 20 mM Hepes-Tris at pH 7.4. The culture dish was placed on the warm stage of an inverted microscope (Leitz-Diavert). Voltage clamp experiments were performed with the use of a suction pipette method as described previously (10). Single- channel currents were recorded from outside-out membrane patches using patch-clamp techniques (5). The pipette was filled with a solution containing 150 mM KCl buffered with 10 mM Hepes-KOH to pH 7.1. We added 1 mM EGTA and 0.83 mM $CaCl_2$ to this solution to obtain a free Ca^{2+}-concentration of about 1 µM (8).

RESULTS AND DISCUSSION : Fig. 1A is a confirmation of the results of Barrett et al. (1981) showing that action potentials from rat myotubes in cultures are generally followed by an a.h.p. mediated by the activation of a Ca^{2+}-dependent slow K^+ conductance. It shows superimposed a.h.p. records for various levels of hyperpolarizing current. Reversal of a.h.p. occurs at about -80 mV, which is the expected value for a K^+ system. Fig. 1B shows the effect of 20 mM TEA on

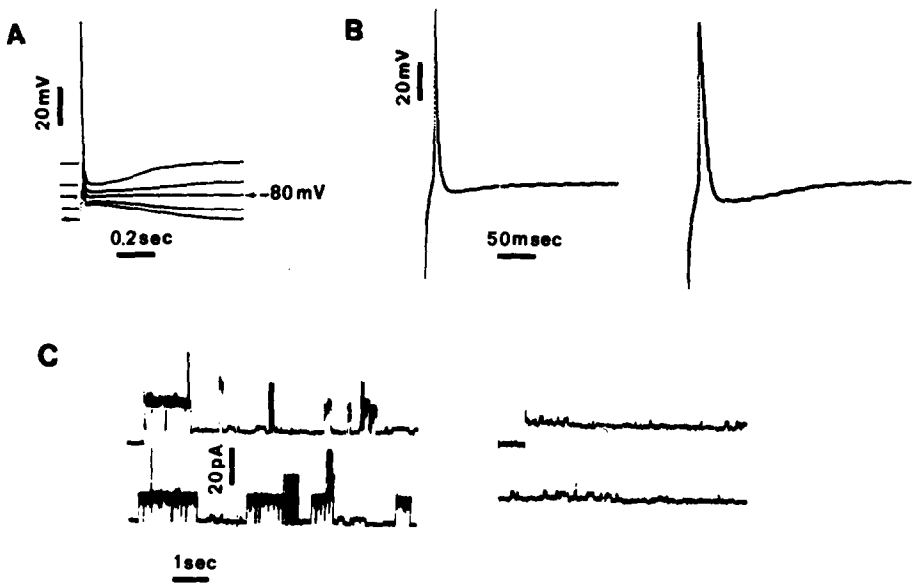

Fig. 1. A. Reversal of the slow a.h.p. The superimposed traces show action potentials and after-potentials evoked in a rat myosac during the passage of a steady hyperpolarizing current of increasing intensity. The a.h.p. reverses at -80 mV (arrow). B. Action potentials and a.h.p. evoked by anodal break stimulation. (Left) : control. (Right) : after a 5 min exposure to 20 mM TEA. Single channel recordings of Ca^{2+}-dependent K^+ currents from an outside-out membrane patch of a rat myotube. (Left) : control. (Right) : 5 min after the addition of 10 mM TEA.

the electrical activity of a rat myotube. The falling phase of the action potential is prolonged and the a.h.p. amplitude is increased. These two effects of TEA can be attributed to the known blockade by TEA of the delayed voltage-dependent K^+ conductance. The slight decrease of the spike amplitude is probably due to the partial blockade of the fast Na^+ conductance by a high concentration of TEA (20 mM). The main result which comes from Fig. 1B is the absence of blockade of the a.h.p. by TEA even at the high concentration. Patch-clamp experiments on rat myotube have identified high conductance Ca^{2+}-dependent K^+ channels (6-8). Methfessel and Boheim (1982) have indicated that a concentration of 5 mM TEA blocks the channels reversibly. We have used the outside-out configuration in order to study the effects of different externally applied drugs. Fig. 1C left shows a typical current record during a depolarizing voltage step to + 30 mV from a holding potential of -80 mV. In addition to the high conductance Ca^{2+}-dependent K^+ channels, membrane patches often contain other channels with smaller conductance values. External application of 10 mM TEA leads in a few minutes to a complete block of the large unitary channels (Fig. 1C right). Even after a large number of patch-clamp experiments we have been unable to record single channel activity of TEA-insensitive K^+ channels which would have a large conductance.

Apamin a bee venom toxin of 18 amino acids with 2 disulfide bridges is a recently discovered blocker of Ca^{2+}-dependent K^+ channels (2). However K^+ flux experiments have shown that the toxin blocks Ca^{2+}-dependent K^+ efflux in mammalian hepatocytes but not in red blood cells (2) in pancreatic cells (11). There are two possible explanations to these observations : (i) there are several types of Ca^{2+}-dependent K^+ channels, (ii) there is only one type of channel which can be either apamin-sensitive or apamin-resistant depending on the cellular system just as voltage-dependent Na^+ channels involved in the generation of action potentials can be either tetrodotoxin-sensitive or tetrodotoxin-resistant (9).

Electrophysiological experiments have shown that apamin specifically blocks the slow Ca^{2+}-dependent K^+ conductance which mediates the a.h.p. in neuroblas-

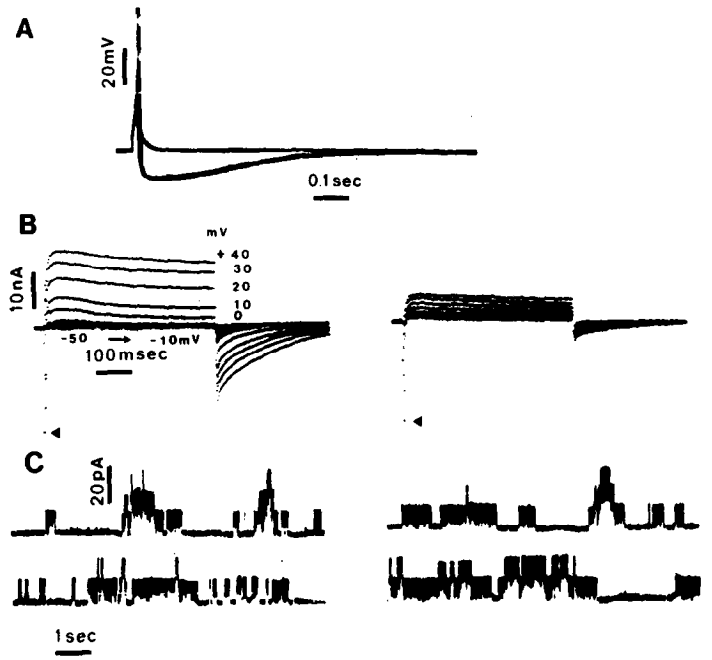

Fig. 2. A. Selective block of the a.h.p. after a 10 min incubation with 10 nM apamin. B. Voltage-clamp analysis of the effect of apamin on rat myosacs. Families of membrane currents associated with different step depolarizations from a holding potential of -90 mV. (Left) : control. (Right) : 10 min after the addition of 10 nM apamin. The fast inward current is not affected (◀) ; the slow outward current is strongly depressed. This experiment was done in the presence of 20 mM TEA in order to block TEA-sensitive K^+ currents. C. Single channel recordings of Ca^{2+}-dependent K^+ channel at +30 mV. (Left) : control. (Right) : same patch after a 10 min incubation with 10 nM apamin.

toma cells (10). On the other hand apamin receptors have been biochemically characterized with ^{125}I-apamin in rat muscle cells cultures. The dissociation constant of the apamin-receptor is 36-60 pM and the number of apamin-receptors per cell is 7,000 (3.5 fmol/mg of protein)(12). This is a low density as compared to that of Na^+ channels (13).

Fig. 2A shows that the a.h.p. of rat myotubes is suppressed within a few minutes after an external application of 10 nM apamin. Voltage-clamp experiments using myosacs were carried out in the presence of 20 mM TEA. They show a slow outward current which is then insensitive to TEA and which is blocked by 10 nM apamin (Fig. 2B). Patch clamp experiments presented in Fig. 2C show that apamin is without effect on the large unitary currents.

The existence of these two classes Ca^{2+}-dependent K^+ channels, one being apamin-sensitive and the other one TEA-sensitive has also been seen in neuroblastoma cells (G. Romey, unpublished results).

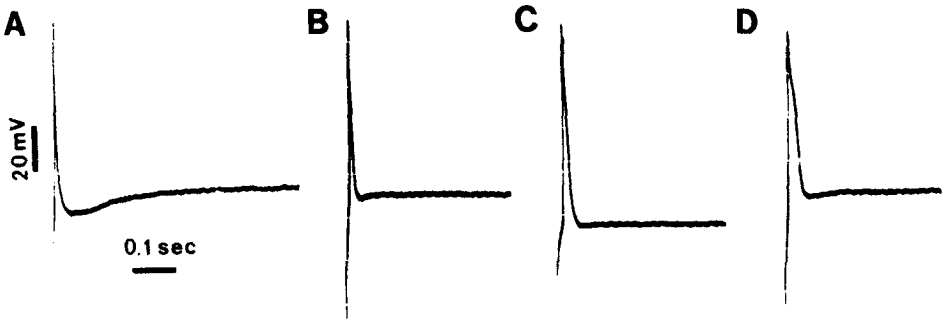

Fig. 3. Effect of A23187 on the electrical activity evoked by anodal break stimulation. A. Control. B. 4 min after the addition of 10 µM A23187 to the bath solution. The a.h.p. is blocked. C. 10 min after the addition of A23187. Note the increase of the membrane potential. D. Reversal of the A23187-induced hyperpolarization by 20 mM TEA.

The voltage dependence and the influence of the internal Ca^{2+} concentration on Ca^{2+}-activated K^+ channels identified by patch-clamp techniques in cultured muscle cells have been studied in great detail (7). The Ca^{2+} sensitivity of the channel is very voltage-dependent. Ca^{2+} concentrations of 1 µM produce nearly full activation of the channel at a membrane potential of + 50 mV, Ca^{2+} concentrations of 10 µM are necessary to obtain the same effect at a potential of + 20 mV and Ca^{2+} concentrations as high as 100 µM only partially activate these channels at negative voltage potentials near -50 mV.

To increase the internal Ca^{2+} concentration in rat muscle cells in culture we have used the classical Ca^{2+} ionophore A23187 which has been employed in many of the studies in which K^+ flux was used to identify the Ca^{2+}-dependent K^+ channel. Fig. 3 shows that the addition of A23187 to rat myotubes in culture has two effects : (i) a complete suppression of the a.h.p., (ii) a maintained membrane hyperpolarization which can be reversed by 10 mM TEA. The easiest interpretation of these results is the following : (i) the hyperpolarization induced by A23187 is due to the activation of the TEA-sensitive Ca^{2+}-dependent K^+ channels which have been identified by patch-clamp ; this activation occurs in spite of the polarized state of the membrane (initial membrane potential : ~ -60 mV) because of the massive influx of Ca^{2+} that enters the cell in the presence of A23187 ; (ii) the inhibition of the a.h.p. is due to the apamin-sensitive Ca^{2+}-dependent K^+ channels which are activated at low Ca^{2+} concentrations and inhibited at high internal concentrations of Ca^{2+}.

In conclusion, this paper demonstrates the existence of two classes of Ca^{2+}-dependent K^+ channels in rat muscle cells in culture, one which is blocked by TEA and is insensitive to apamin, the other one which is blocked by apamin and is insensitive to TEA. These two types of channels have different responses to variations of internal Ca^{2+} concentrations at membrane potentials between -50 and -80 mV. High Ca^{2+} concentrations which activate the TEA sensitive Ca^{2+}-dependent K^+ channel inhibit the apamin-sensitive Ca^{2+}-dependent K^+ channel. The function of the apamin-sensitive channel is to participate in the generation of after hyperpolarisation potentials whereas the physiological function of the TEA-sensitive Ca^{2+}-dependent K^+ channel is probably to prevent prolonged depolarizations of the cell that would be accompanied by increases of internal Ca^{2+} concentrations.

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