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Toxin pharmacology of the ATP-induced hyperpolarization in Madin-Darby canine kidney cells

Michel Tauc, Monique Gastineau and Philippe Poujeol

Département de Biologie cellulaire et moléculaire, Service de biologie cellulaire, CEN Saclay, Gif sur Yvette (France)

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The effects of *Leiurus quinquestriatus hebraeus* (LQH) venom, mamba venom, *Buthus tamulus* (BT) venom, purified apamin and synthetic charybdotoxin on the membrane hyperpolarization induced by extracellular ATP were examined in Madin-Darby canine kidney cells. For this we used a membrane potential probe (bisoxonol) to determine the potential variations. The relation between bisoxonol fluorescence and membrane potential was established by treating Madin-Darby canine kidney cells suspended in solutions containing various external sodium concentrations with gramicidin. Extracellular ATP induced a rapid hyperpolarization that was blocked by LQH venom and synthetic charybdotoxin. BT venom also blocked the response but at a much higher concentration than that of LQH. Mamba venom (*Dendroaspis polylepis*) and apamin did not modify the ATP-induced hyperpolarization. We concluded that the ATP induced hyperpolarization was due to the augmentation of the potassium conductance probably through Ca^{2+}-activated K^+ channels sensitive to charybdotoxin but not to mamba venom. The interaction previously described between charybdotoxin and dendrotoxin (the main toxin of mamba venom) was not observed in our case.

Introduction

Charybdotoxin, a toxin purified from venom of the scorpion *Leiurus quinquestriatus hebraeus* is a potent inhibitor of the high conductance Ca^{2+}-activated K^+ channel [1,2]. It has been shown to block other types of K^+ channel [3–5] but very little is known about the pharmacology of the Ca^{2+}-activated K^+ channels localized in the renal epithelium. However, an apparent cross-reactivity has recently been shown [6,7] in binding studies between charybdotoxin and dendrotoxin, a toxin extracted from mamba venom, suggesting an analogy of structure between the Ca^{2+}-activated K^+ channel and the voltage-activated K^+ channel. To test the action of charybdotoxin and dendrotoxin on epithelial K^+ channels, we determined the membrane potential of suspended Madin-Darby canine kidney cells by using a potential probe (bisoxonol). The openings of the Ca^{2+} activated K^+ channels were elicited by extracellular ATP application. The data demonstrate that the Ca^{2+}-activated K^+ channel, present in the plasma membrane of Madin-Darby canine kidney cells, is completely blocked by charybdotoxin (total inhibition of the ATP-induced hyperpolarization) but unaffected by dendrotoxin. Moreover, the venom of the scorpion *Buthus tamulus* also inhibited the ATP response but with less efficiency than that of *Leiurus quinquestriatus hebraeus*.

Correspondence: M. Tauc, Département de Biologie cellulaire et moléculaire, Service de biologie cellulaire, CEN Saclay, 91191 Gif-sur-Yvette Cedex, France.

Material and Methods

Culture conditions

Madin-Darby canine kidney cells were obtained from the American Type Culture Collection and used from passage 65 to 70. Cells were seeded on culture flasks at a concentration of $2 \cdot 10^4$ cells/cm^2 in MEM (Gibco) medium containing 15 mM $NaHCO_3$, 20 mM Hepes (pH 7.4), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 ml/l 100X nonessential amino acid medium (Flow Laboratories) and 10% fetal calf serum (Eurobio). After the cells had become confluent, they were trypsinized and resuspended at $(10–20) \cdot 10^6$ cells/ml in NaCl buffer (in mM, $NaCl$ 140, KCl 1, $CaCl_2$ 1, $MgCl_2$ 1, glucose 5, Hepes 20 (pH 7.4)).

Procedure for potential measurement by fluorescence

All experiments were made with a Perkin-Elmer LS-5 spectrofluorimeter connected to a recorder. 1.5 ml of buffer was added to a quartz cuvette maintained at 37°C. Bis(1,3-diethylthiobarbituric)trimethine oxonol (bisoxonol, Molecular Probes) was prepared from a

stock solution (15 mM in ethanol) at a concentration of 0.15 mM in H_2O and was added to the cuvette to give a final concentration of 1.5 μM. For each measurement 10^6 cells were added and continuously stirred with a magnetic stirrer. The fluorescence signal was recorded with excitation at 540 nm (5 nm slit width) and emission at 580 nm (10 nm slid width). External calibration was made by adding 1 μM gramicidin D (Sigma) to cells suspended in isotonic media containing various ratios of NaCl and choline chloride. The membrane potential was calculated assuming that the rates of Na^+ and K^+ permeation through the ionophore were similar using the equation $E_m = RT/F \cdot \log[Na_o]/[Na_i + K_i]$ [8]. $Na_i + K_i$ was determined by the null-point method.

Determination of intracellular calcium

Intracellular calcium was determined by fluorescent video-microscopy. Cultures seeded on petri dishes were loaded with 3 μM fura-2/AM (Molecular Probes) in NaCl buffer for 2 h at room temperature. After rinsing, the cultures were observed on an inverted microscope (Zeiss ICM 405) coupled to a low light video camera (Lhesa, France). The excitation beam was filtered at 340 and 380 nm and emitted light above 520 nm was analyzed. Fluorescent images were digitized and analyzed with an image processing system as previously described (Biocom, France) [9]. Intracellular calcium concentrations were determined according to the method of Grynkiewicz et al. [10].

Results

Addition of micromolar concentrations of ATP to Madin-Darby canine kidney cells elicited a transient increase in intracellular calcium and a transient hyperpolarization. Fig. 1 shows the time course of changes of intracellular calcium and membrane potential of Madin-Darby canine kidney cells exposed to 50 μM extracellular ATP in the presence and then the absence of 1 mM extracellular calcium. Calculated intracellular calcium concentrations were 48.79 nM at rest and 664.39 nM at maximal stimulation 15 s after ATP application in 1 mM calcium (Fig. 1A) after which the concentration returned to the basal level. Simultaneously to the increase in intracellular calcium, the bisoxonol fluorescence decreased under ATP stimulation (Fig. 1B). Bisoxonol, a lipophilic anion, has physicochemical properties that induce a change in fluorescence proportional to changes in membrane potential [11]. ATP induced a transient hyperpolarization followed by a return towards the resting membrane potential. To measure the degree of this hyperpolarization we performed experiments in which artificial potentials were generated in the presence of 1 μM gramicidin D at various external sodium concentrations. Fig.

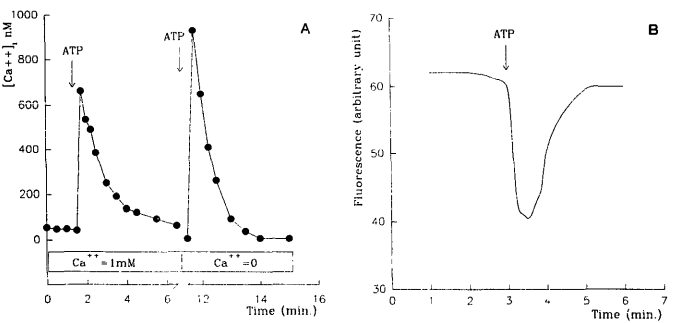

Fig. 1. Effect of extracellular ATP on intracellular calcium and membrane potential in Madin-Darby canine kidney cells. (A) Intracellular calcium determination: Madin-Darby canine kidney grown on petri dishes were loaded with 3 μM fura-2/AM in NaCl buffer for 2 h. After rinsing, cells were placed on the stage of an inverted microscope, the emitted light analyzed by an image-processing system and the 340/380 nm ratio in Grinkiewic's equation used to determine the calcium concentration. ATP (50 μM final) was added in a medium containing, or not, 1 mM external calcium. (B) Membrane potential variation: Isolated Madin-Darby canine kidney cells ($1 \cdot 10^6$) were suspended in the quartz cuvette of a spectrofluorimeter in 1.5 ml NaCl medium containing 1.5 μM bisoxonol, excited at 540 nm and emitted light at 580 nm was recorded. ATP (50 μM final) was added at the arrow. The figure is representative of three experiments.

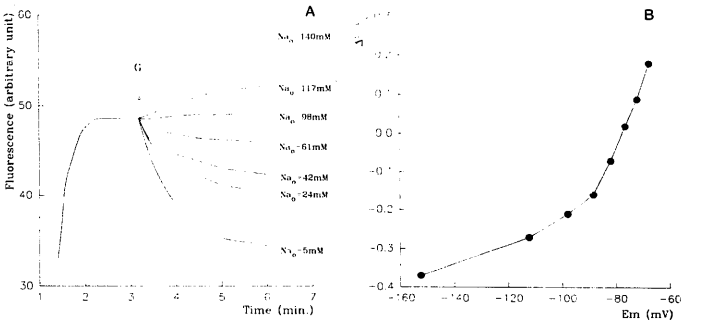

Fig. 2. Correlation between emitted fluorescence and membrane potential. (A) Isolated Madin-Darby canine kidney cells ($1 \cdot 10^6$) were suspended in the cuvette of a spectrofluorimeter in 1.5 ml of a solution containing 1.5 μM bisoxonol in which the sodium was to varying degrees isoosmotically replaced by choline. Emitted fluorescence at 580 nm (excitation 540 nm) was recorded and gramicidin D (1 μM final) added (G). Fig. 2A represents recordings obtained at different external sodium concentrations. (B) The relative fluorescence variations recorded at various sodium concentrations were plotted against the membrane potential generated and calculated according to the following equation: $E_m = RT/F \cdot \log[Na_0]/[Na_i + K_i]$. $Na_i + K_i$ was determined by the null-point method. It was equal to $[Na_0]$ when addition of gramicidin did not produce a fluorescence change. Each point was the mean of two experiments.

2A shows the relative fluorescence as a function of external sodium concentration. Isoosmolarity was maintained with choline replacement. The membrane potential was calculated from the equation described in material and methods. Fig. 2B shows the ratio of the variation of fluorescence to the initial fluorescence before the addition of gramicidin as a function of the generated membrane potential. According to this calibration, the peak hyperpolarization induced by ATP was -19.76 ± 0.09 mV, $n = 16$ (S.E. values) and was found to be ATP dose-dependent. Fig. 3 shows a dose response curve in which the induced hyperpolarization was plotted against the ATP concentration. Half-maximal effect was obtained at $1.5 \cdot 10^{-7}$ M ATP. For all the following experiments a dose of 50 μM ATP was used. Addition of ATP did not produce hyperpolarization in Madin-Darby canine kidney cells suspended in a K^+ solution (data not shown), suggesting that an outwardly directed K^+ gradient is necessary for the potential change: a suggestion consistent with the opening of K^+ channels by an increase in intracellular calcium.

We tested the effects of various venoms and purified toxins known to act on potassium conductances on the ATP-induced hyperpolarization. Fig. 4 represents the bisoxonol fluorescence recordings of ATP-induced hy-

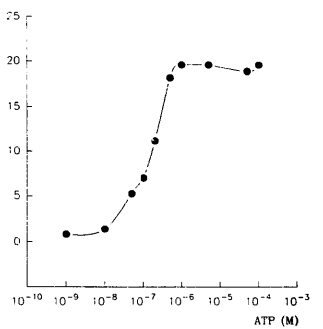

Fig. 3. ATP dose-response curve of the induced hyperpolarization. For each value, $1 \cdot 10^6$ cells were suspended in the cuvette of a spectrofluorimeter containing 1.5 ml NaCl buffer and 1.5 μM bisoxonol. cells were excited at 540 nm and the emitted light recorded at 580 nm. ATP was added at various concentrations between 10^{-9} and 10^{-4} M and the maximum fluorescence variation obtained used to determine the induced hyperpolarization from the calibration curve. Each point represents the mean of two experiments.

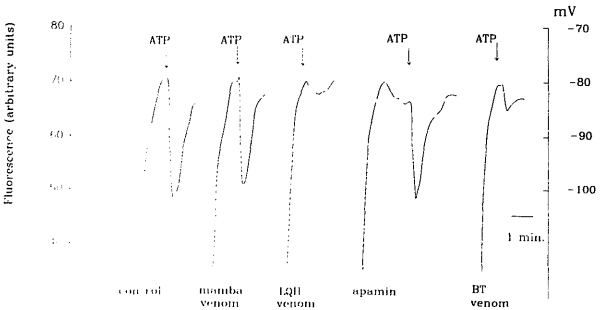

Fig. 4. Effect of various venoms or toxins on the ATP induced hyperpolarization. Isolated Madin-Darby canine kidney cells were suspended in 1.5 ml NaCl buffer containing 1.5 μM bisoxonol (control) or the same buffer containing mamba venom (20 μg/ml), *Leiurus quinquestriatus hebraeus* (LQH) venom (20 μg/ml), apamin ($5 \cdot 10^{-6}$ M) or *Buthus tamulus* (BT) venom (200 μg/ml). ATP (50 μM) was added and the emitted fluorescence at 580 nm (excitation 540 nm) recorded. The figure is representative of three experiments with each venom or toxin. The scale on the right gives the membrane potential calculated from calibration experiments using gramicidin.

perpolarization in the presence of these drugs. As compared with the control condition in NaCl buffer, addition of 20 μg/ml mamba venom (Latoxan, Rosans France) did not modify the response. On the other hand, addition of 20 μg/ml LQH venom (Latoxan) almost completely inhibited the hyperpolarizing phase induced by ATP. $5 \cdot 10^{-6}$ M apamin (Sigma) had no effect whereas 200 μg/ml BT venom (Sigma) modified the response. Since LQH and BT venoms inhibited hyperpolarization, we performed inhibition experiments at various concentrations of these two venoms. Figs. 5A and 5B show the dose inhibition curves ob-

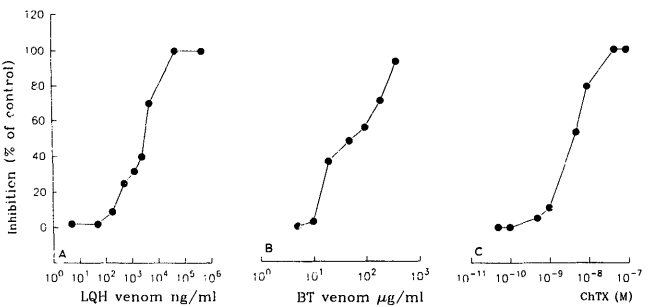

Fig. 5. Inhibition curves of the ATP-induced hyperpolarization by *Leiurus quinquestriatus hebraeus* (LQH) venom (A), *Buthus tamulus* (BT) venom (B) and synthetic charybdotoxin (ChTX) (C). Isolated Madin-Darby canine kidney cells were suspended in 1.5 ml NaCl buffer containing 1.5 μM bisoxonol and various concentrations of venom or toxin. ATP (50 μM) was added and the amplitude of the fluorescence change recorded. The inhibition was determined as a % of the control response in the absence of the effectors. Each point is the mean of two experiments.

tained. IC_{50} was 2 μg/ml for LOH venom and total inhibition was obtained between 20 and 50 μg/ml. Using BT venom, IC_{50} was 100 μg/ml and total inhibition was not obtained even at 400 μg/ml. LQH venom contains charybdotoxin, a potent inhibitor of the Ca^{2+}-activated maxi K^+ channels and it is therefore probable that its action is due to the charybdotoxin preventing hyperpolarization by blocking the increase in Ca^{2+}-activated K^+ conductance, as reported in other systems [1,3,12,13]. To confirm this hypothesis we performed inhibition experiments at various concentrations of synthetic charybdotoxin *. Fig. 5C shows the dose-response inhibition of the ATP-induced hyperpolarization by synthetic charybdotoxin. IC_{50} was $3 \cdot 10^{-9}$ M and total inhibition was obtained at $4 \cdot 10^{-8}$ M.

Discussion

In this study extracellular ATP was used to make active potassium channels in Madin-Darby canine kidney cells, an effect already described [14,15]. In our conditions, ATP induced an increase of the intracellular calcium within 15 s in both the presence and absence of 1 mM extracellular calcium. The present results are in close agreement with those of Paulmichl and Lang [16], confirming that ATP induced the liberation of calcium from an intracellular store. At the same time, ATP produced a rapid reduction of the fluorescence emitted by bisoxonol-loaded cells. Calibration experiments using gramicidin in isotonic media at various levels of sodium have shown that the fluorescence decline was due to an induced ATP hyperpolarization. This hyperpolarization was ATP dose-dependent and may be related to an increase in K^+ potassium permeability. An enhanced potassium permeability or activation of potassium channels after application of extracellular ATP has been reported in fibroblasts [17], cultured myoblasts and myotubes [18] and salivary glands [19]. To characterize the potassium conductance increase, we used venoms or toxins known to act on potassium channels. The transient hyperpolarization was blocked by LQH venom and partially blocked by BT venom. The ATP response was unchanged in the presence of mamba venom or apamin. LQH venom contains charybdotoxin, a toxin which is a potent inhibitor of high conductance Ca^{2+}-activated K^+ channels. Inhibition experiments using synthetic charybdotoxin gave a half inhibition of the ATP-induced hyperpolarization at a value of 3 nM. This agrees with the value obtained by Anderson et al. [20] using patch-clamp experiments, who found a half inhibition of the Ca^{2+}-activated K^+ channel at 10 nM charybdotoxin. It has recently been shown that BT venom contains a toxin named iberiotoxin [20] which acts with high affinity on Ca^{2+}-activated K^+ channels present in aortic smooth muscle. In our experiments, BT venom inhibited the ATP-induced hyperpolarization at a higher concentration than did LQH venom. The fact that the concentrations of charybdotoxin and iberiotoxin in their respective venoms are quite similar [12,21] suggests that charybdotoxin is a more potent inhibitor than iberiotoxin for the Ca^{2+}-activated K^+ channel involved in the ATP-induced hyperpolarization. Another interesting result of this study is that the ATP-induced hyperpolarization was not blocked by mamba venom. The principal toxin of this venom is dendrotoxin which is a potent inhibitor of the voltage-activated K^+ channels [22]. However, Harvey et al. [6] and Vazquez et al. [7] have recently demonstrated an interaction between dendrotoxin and charybdotoxin at binding sites on neuronal membranes at low toxin concentrations. Our results show that there was no effect of dendrotoxin on the blocking of the Ca^{2+} activated K^+ channel involved in the ATP response. In some experiments we incubated Madin-Darby canine kidney cells in the presence of mamba venom before the addition of LQH venom. In these conditions the ATP-induced hyperpolarization was still blocked by charybdotoxin (data not shown). There would thus appear to be no competition between charybdotoxin and dendrotoxin at the binding sites of charybdotoxin. Finally, apamin, a specific blocker of the small K^+ conductance channels [23], had no effect on the ATP response even at a high concentration.

It has already been proposed that in epithelial cells charybdotoxin inhibits a Ca^{2+}-activated K^+ channel of high conductance [24,25]. However, Friedrich et al. [15] have clearly shown that in Madin-Darby canine kidney cells ATP activates an inwardly rectifying K^+ channel of 65 pS. On the other hand, Bolivar et al. [26] have demonstrated that Madin-Darby canine kidney cells also possess a maxi K^+ channel activated by calcium. Thus it is probable that extracellular ATP stimulates at least two types of channel in Madin-Darby canine kidney cells. Since synthetic charybdotoxin inhibited the entire ATP response, we postulate that charybdotoxin is effective on both the intermediate and maxi epithelial K^+ channels and that dendrotoxin is ineffective.

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