J. Membrane Biol. 117, 223–231 (1990)

The Journal of
Membrane Biology

Reversible Activation of the ATP-Dependent Potassium Current with Dialysis of Frog Atrial Cells by Micromolar Concentrations of GDP

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Summary. We studied the effects of internal and external solutions on potassium currents in frog atrial cells. Experiments were carried out in whole cell recording in the presence of tetrodotoxin and cobalt in the bath to suppress the inward currents. In the absence of pyruvate and glucose in the external solution, a time-independent current increased progressively in a few minutes till the death of the cell. This current had the properties of the ATP-sensitive potassium current IK(ATP) in mammalian cells. In the presence of pyruvate and glucose in the external solution, the membrane current stayed low for 30 min. Addition of guanosine monophosphate (GMP, 40 μM), guanosine triphosphate (GTP, 40 to 1000 μM), adenosine diphosphate (ADP, 40 μM) or adenosine triphosphate (ATP, 3000 μM) to the internal solution had no major effect on the current amplitude. In contrast, addition of GDP (20 or 40 μM) produced a loss of rectification in a few minutes. The current activated by GDP was time independent as was the current observed in the absence of glucose and pyruvate. It was sensitive to cesium and barium, it was blocked when ATP was added to GDP in the internal solution, and it was suppressed by the sulphonylurea glibenclamide (1 μM). We suggest that GDP produced a local depletion of ATP, by displacement of the equilibrium between ATP, GDP, ADP and GTP. This hypothesis is supported by the fact that the current activated by GDP was rapidly suppressed when adding GTP in excess to the internal solution.

Key Words frog atrial cells · ATP · GTP · GDP · IK(ATP)

Introduction

Potassium currents play an important role in the regulation of the cardiac electrical activity.

A potassium current is known to activate in the absence of glucose (Vereecke et al., 1981) in the external (extracellular) solution, or under depletion of adenosine triphosphate (ATP) on the internal (intracellular) solution (Noma, 1983). This current, called IK(ATP), is also regulated by ADP (Dunne & Petersen, 1986*a*; Findlay, 1988), GTP and GDP (Dunne & Petersen, 1986*a*; Findlay, 1987), and other pyridine nucleotides (Dunne, Findlay & Petersen, 1988). It is specifically blocked by a class of hyperglycemic agents, the sulphonylureas (Fosset et al., 1988).

The background conductance, IK1, has also been reported to be regulated by internal ATP (Trube & Hescheler, 1984; Trube, 1985).

We studied in frog cardiocytes the effects of the composition of internal and external solutions on potassium currents, and we focused on the internal effects of nucleotides. We observed a pronounced increase of conductance when micromolar concentrations of guanosine diphosphate were present in the cells. The present paper describes this effect and our hypothesis about its mechanism.

Materials and Methods

Cell Preparation

Single-cells from *Rana esculenta* were obtained as described by Bonvallet (1987), except that collagenase from *Clostridium histolyticum* was purchased from Boehringer (*see below*). Cells were kept overnight in nominally calcium-free solution (*see* solutions). We used the patch-clamp technique in the whole cell recording mode.

Solutions

Concentrations are in millimolar, except where otherwise stated.

Control external solution contained: NaCl, 110; KCl, 2.5; $MgCl_2$, 2; $CaCl_2$, 1.8; $CoCl_2$, 3; tetrodotoxin, 1 μM; glucose, 10; pyruvic acid, 5; HEPES buffer, 10; pH adjusted to 7.2 with NaOH; CsCl (20 mM) and $BaCl_2$ (1.8 mM) were added to this solution when needed.

In one series of experiments, glucose and pyruvate were omitted. The other concentrations were unaffected.

Nominally calcium-free solution contained: same solution as above, but without $CaCl_2$, $CoCl_2$, and tetrodotoxin.

Control internal solution filling the patch pipettes contained: KCl, 20; K aspartate, 130; KH_2PO_4, 1; $MgCl_2$, 1; K-EGTA, 5; HEPES buffer, 10; pH adjusted to 7.3 with KOH.

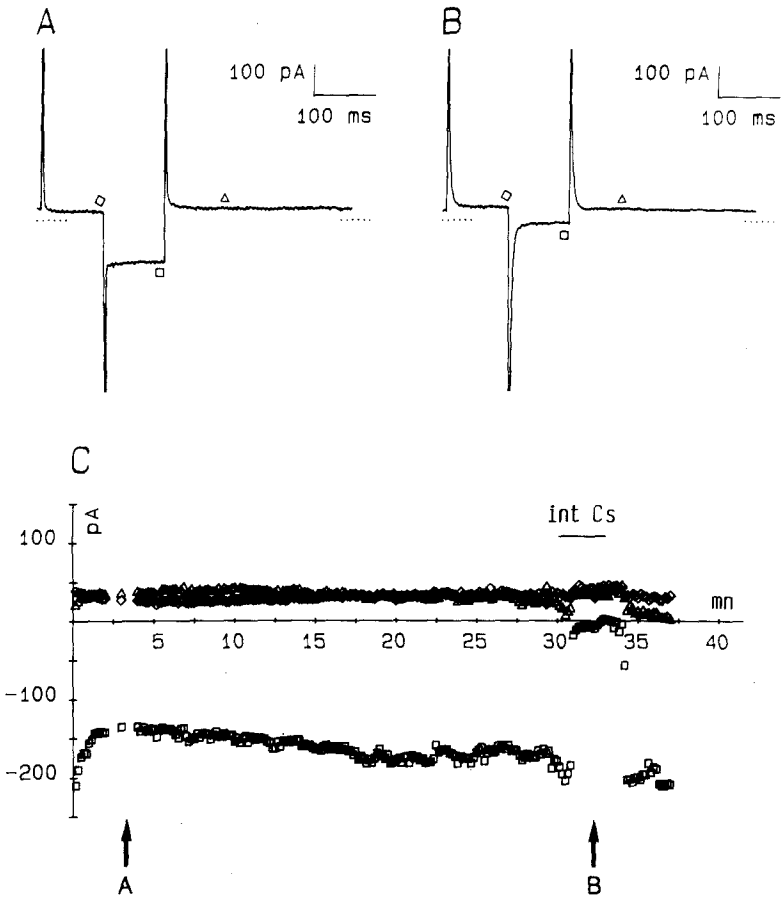

Fig. 1. Membrane current recorded from an atrial myocyte. The zero current level is indicated by the dots at the right and at the left ends of the traces. The potential is held at −70 mV (triangles), close to the potential at which the inward rectifying potassium current is maximally outward. The potential was stepped to −30 mV (lozenges), then to −110 mV (squares). (*A*) Control current. The steady-state current, less outward at −30 mV than at −70 mV, and the large inward current at −110 mV, reflect the inward rectification. (*B*) Current recorded from the same cell dialyzed with 30 mM cesium chloride. (*C*) Steady-state current measured at −70 mV (triangles), −30 mV (lozenges) and −110 mV (squares) function of the time. *int Cs* indicates the time during which the CsCl solution was applied. The arrows indicate the traces displayed in *A* and *B*. Note the rapidity of the change of current, and its reversibility, which reflect a satisfactory dialysis

When needed, CsCl (30 mM), $MgCl_2$ (9 mM) or LiCl (20 μM to 2 mM), and adenosine tri-, diphosphate (respectively ATP, ADP), guanosine tri, di- and monophosphate (respectively GTP, GDP, GMP) were added to this solution as K_2ATP (3 mM), KHADP (40 μM), Na_2GTP (1 mM), Li_2GDP (20 or 40 μM), Na_2GMP (40 μM). The nonhydrolyzable and nonphosphorylable analogue of GDP, GDP-β-S (40 to 80 μM) was also added to this solution in one series of experiments.

The GDP concentrations used were three to sixfold higher than the actual concentration measured in heart (6 μM), cerebral cortex and muscle (10 μM), or liver (12 μM). Values were calculated from De Azeredo et al. (1979), assuming an average of 1 mg of protein per 4.7 mg wet wt.

Pronase was purchased from Sigma (St. Louis, MO). Other chemicals were purchased from Boehringer Mannheim France (Meylan, France).

Electrophysiological Experiments

Experiments were carried out at room temperature (21–22°C). The bath solution was continuously flowing in the experimental chamber. The pipette solution was continuously perfused by the use of a capillary tube close to the tip of the patch electrode. A constant depression of 15 cm of water was applied to the inside of the pipette and ensured a constant flow rate of about 0.2 μl/min through the capillary and the pipette assembly. Patch pipettes were made from borosilicate glass (Clark Electromedical, Reading, UK). Their impedance was between 2–5 MΩ when filled with the internal solution, and the seals between the pipettes and the cells ranged from 1 to 10 GΩ.

A RK300 amplifier (Biologic, Echirolles, France) was used for whole cell voltage-clamp experiments. Voltage command and simultaneous signal recording were performed by the pCLAMP software (Axon Instruments, Burlingame, CA) on a PC microcomputer. Programs for measurements, computations and plots from pCLAMP files were developed in our laboratory.

After the rupture of the membrane patch, a standard double voltage-steps protocol was used at 0.1 Hz: from a holding potential of −70 mV, symmetrical pulses of ±40 mV were applied, setting the membrane potential to −30 mV for 100 msec, then to −110 mV for 100 msec. Dots on both ends of the current traces in the figures indicate the zero current level.

The currents were measured at the end of each step: −70 mV (triangles in the figures), −30 mV (lozenges) and −110 mV (squares), and plotted as function of time. Time zero referred to the rupture of the membrane patch. The control recording was taken a few tens of seconds after breaking the membrane patch. Some points were often removed from the plots in order to show the different symbols.

In some experiments, the conductance changed continuously, and the current voltage relation could not be accurately measured with a series of voltage steps. The currents appeared, however, time independent. All current-voltage relations were then obtained with a ramp protocol (1 V/sec) applied at 0.1 Hz (*see* Fig. 6).

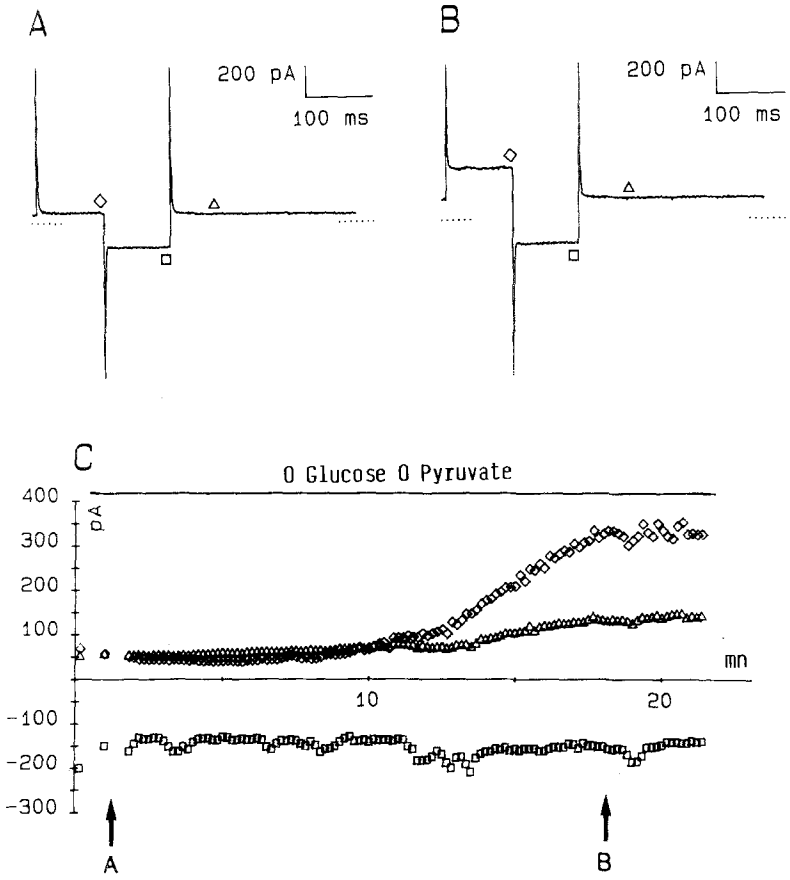

Fig. 2. Membrane current recorded in the absence of glucose and pyruvate in the bath. (*A*) Control current. (*B*) Current recorded after 18 min in this solution. Note the loss of inward rectification at the end of the experiment. (*C*) Steady-state current function of the time, with the same symbols as in Fig. 1. The arrows indicate the traces displayed in *A* and *B*

Results

All voltage-clamp experiments on frog atrial heart cells were carried out in the presence of 3 mM cobalt and 1 μM tetrodotoxin, which block the inward currents carried by calcium and sodium.

Control Internal and External Solutions

A typical record obtained under control conditions is shown in Fig. 1*A*. Outward membrane currents are positive and are shown as upwards deflections of the current trace. The membrane currents recorded appeared time independent after the capacitive transient. The current was maximum outward at -70 mV, the current was much larger when it flowed in the inward direction, and the current-voltage relation showed a strong inward rectification (*see* Fig. 6*B*). The zero current potential was close to the expected value of the potassium equilibrium potential, E_K.

After an initial phase of stabilization, the currents remained stable for more than 30 min. Similar results were obtained without pipette perfusion. Half an hour after the beginning of the experiment, the pipette solution was changed by addition of 30 mM cesium. The inward current at -110 mV was then completely suppressed (Fig. 1*B*). Switching the internal solution back to control restored this current to its control value.

The current at rest (triangles), at the end of the depolarizing step (lozenges) and at the end of the hyperpolarizing step (squares) were plotted as a function of the duration of the experiment in Fig. 1*C*. The time course of the effect of internal cesium and of its reversal indicates a rapid exchange between the pipette and the cytosol.

External Solution Containing Neither Pyruvate Nor Glucose

A similar experiment is described in a solution containing neither pyruvate nor glucose (Fig. 2). In Fig. 2*A*, the control current appeared similar to the previous one. However, the current was not stable and increased with time (Fig. 2*B* and *C*). The current remained time independent at any potential. A similar current has been described in frog atrial trabecu-

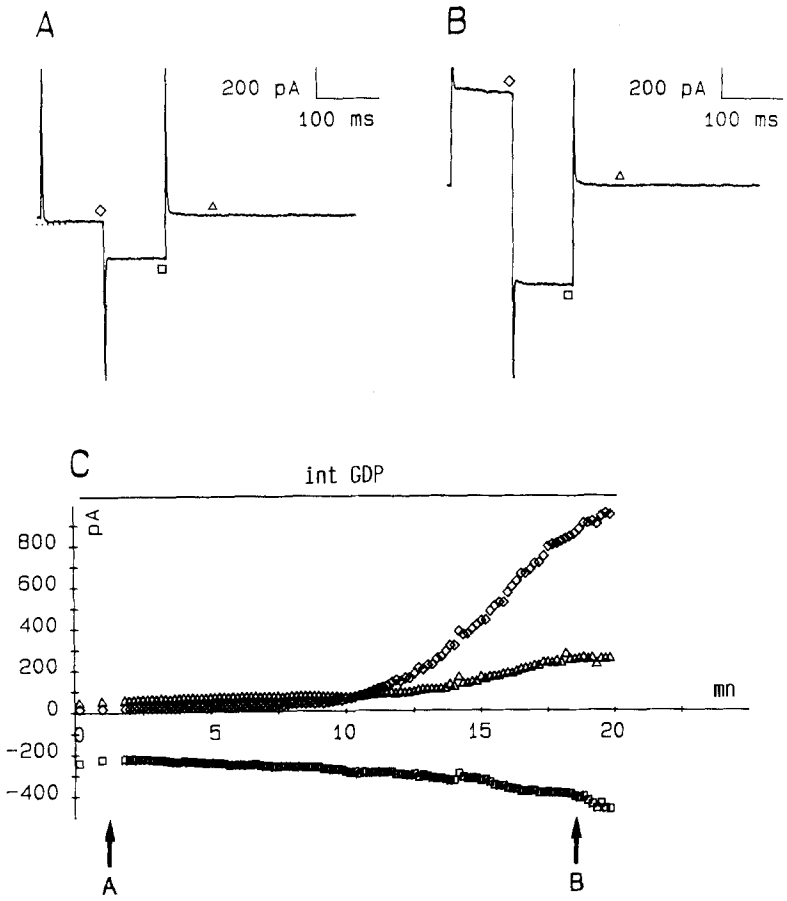

Fig. 3. Effect of a steady concentration of 40 μM GDP in the internal solution. (*A*) Control current. (*B*) Current recorded after 17 min, reflecting the loss of rectification. (*C*) Steady-state current function of the time, with the same symbols as in Fig. 1. The arrows indicate the traces displayed in *A* and *B*

lae (Nargeot, 1976) and in mammalian ventricular muscle (Vleugels, Vereecke & Carmeliet, 1980; Isenberg et al., 1983) under intoxication by dinitrophenol (DNP), or in single cells (Belles, Hescheler & Trube, 1987). This current has been shown to flow through ATP-sensitive potassium channels (Noma & Shibasaki, 1985).

Effects of Nucleotides in the Internal Solution

In the control external solution (containing pyruvate and glucose), the current remained stable when we added to the internal solution either ATP (3 mM), ADP (40 μM), GMP (40 μM) or GTP (40 to 1000 μM). The conditions of these experiments are summarized in the Table.

Conversely, with addition of 20 to 40 μM of GDP, a time-independent current similar to IK(ATP) activated in every cell from 29 experiments (3 cells with 20 μM GDP and 26 with 40 μM). The following experiments focused on this current and its mode of activation.

Table 1. Conditions of experiments not showing an effect on the membrane currents

Nucleotide(s) in the internal solution	Concentration (μM)	Duration of experiment (min)	Number of cells tested
ADP	40	21–36	4
ATP	3000	16–48	5
GMP	40	13–35	3
GTP	40	13–35	3
	100	14–35	13
	300	15–22	2
	1000	16–43	13
GTP + ATP	1000 + 1000	20–42	12
GMP + GTP	40 + 1000	35, 50	2

Glucose and pyruvate were present in the bath in all experiments. The "Concentration" column represents the respective concentration of the two components when both were present. The "Duration of experiment" column reports the shortest to the longest duration from a series of experiments.

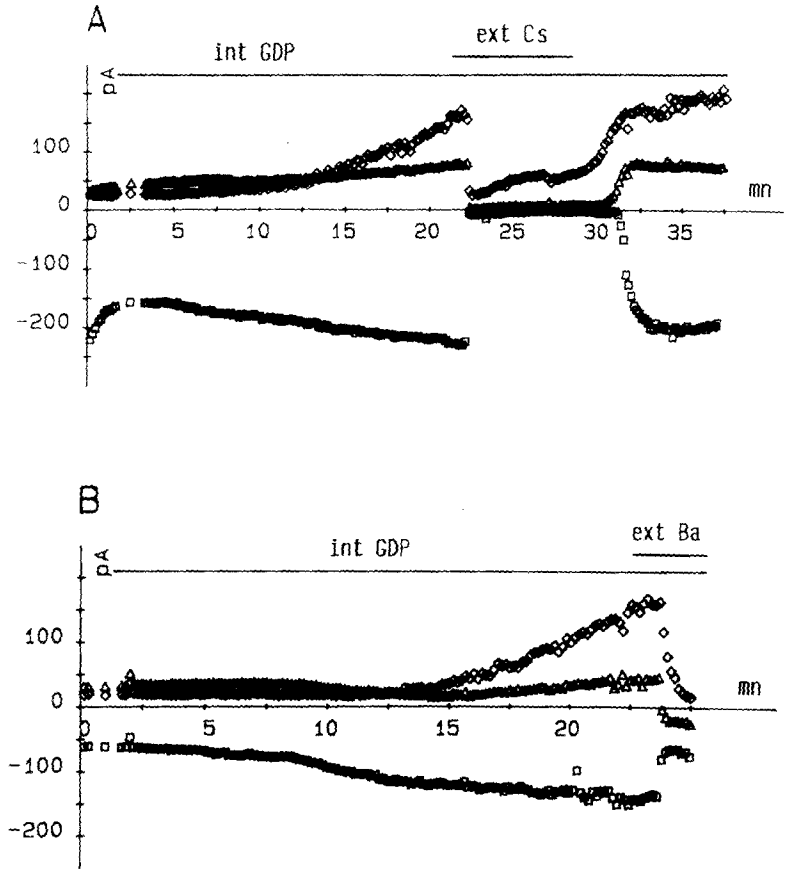

Fig. 4. Effect of potassium channel blockers on the current evoked by internal GDP. GDP (40 μM) was present throughout the experiment. Steady-state current function of the time, with the same symbols as in Fig. 1. (*A*) Effect of externally applied 20 mM CsCl (upper bar). Upon return to control, the inward and the outward current returned rapidly to their preceding value. (*B*) Effect of externally applied 1 mM $BaCl_2$ (upper bar). Note the complete suppression of the outward current

The standard three-step voltage protocol was applied in the presence of GDP in the internal solution, and the control currents were reported in Fig. 3*A*. They indicate a rectification in the inward direction as described earlier. After 12 min, quite different current responses were recorded (Fig. 3*B*). The inward rectification of the membrane was progressively suppressed (Fig. 3*B* and *C*). The currents still appeared time independent. They increased much more in the outward direction for potentials positive to E_K than in the inward direction for potentials negative to E_K. The current-voltage relation indicated that the reversal potential of the extra current was indeed close to E_K (*see* Fig. 6*B*). The currents increased progressively (Fig. 3*C*) until the cell became round in a few seconds. This event is referred to in the following as the death of the cell.

These results suggest that the ATP-dependent potassium current was activated by the presence of GDP in the internal solution, as in the absence of metabolites in the bath. In order to check this hypothesis, first we used potassium channel blockers to ensure the ionic nature of the current, then we tested the effect of internal addition of ATP and of IK(ATP) blockers.

Effects of Cesium and Barium in the Presence of GDP

The experiments shown in Fig. 4 were carried out to determine the effects of potassium channel blockers upon the membrane current induced by internal GDP. The addition of 20 mM CsCl to the bath solution dramatically reduced both inward and outward membrane currents (Fig. 4*A*). The effect of cesium was rapidly reversed on return to the control bath solution.

In another experiment, the addition of 1.8 mM $BaCl_2$ to the bath also dramatically reduced the GDP-activated current (Fig. 4*B*). The effect of barium was reversible (*not illustrated*).

Effects of Internal ATP and of Sulphonylureas

40 μM GDP was applied in the internal solutions throughout the experiment displayed in Fig. 5. As described, GDP induced a loss of rectification (Fig. 5*A* and *B*). The effect of GDP could be rapidly reversed by addition of ATP (3 mM) in the internal solution (Fig. 5*C* and *D*).

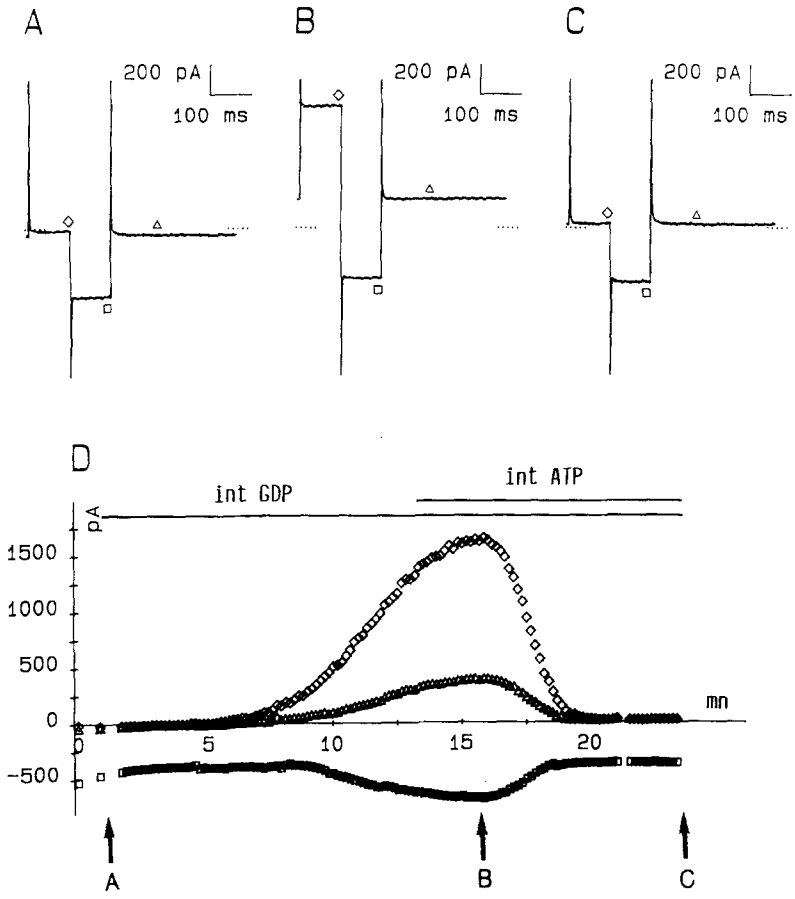

Fig. 5. Effect of ATP (3 mM) on the current evoked by internal GDP. GDP (40 μM) was present throughout the experiment. (*A*) Control current. (*B*) Current recorded after 15 min in the presence of GDP (40 μM), inducing a loss of rectification. (*C*) Steady-state current function of the time, with the same symbols as in Fig. 1. (*D*) Restoration of the initial current by addition of ATP (3 mM) to the internal solution (*see* the horizontal bar above the graph)

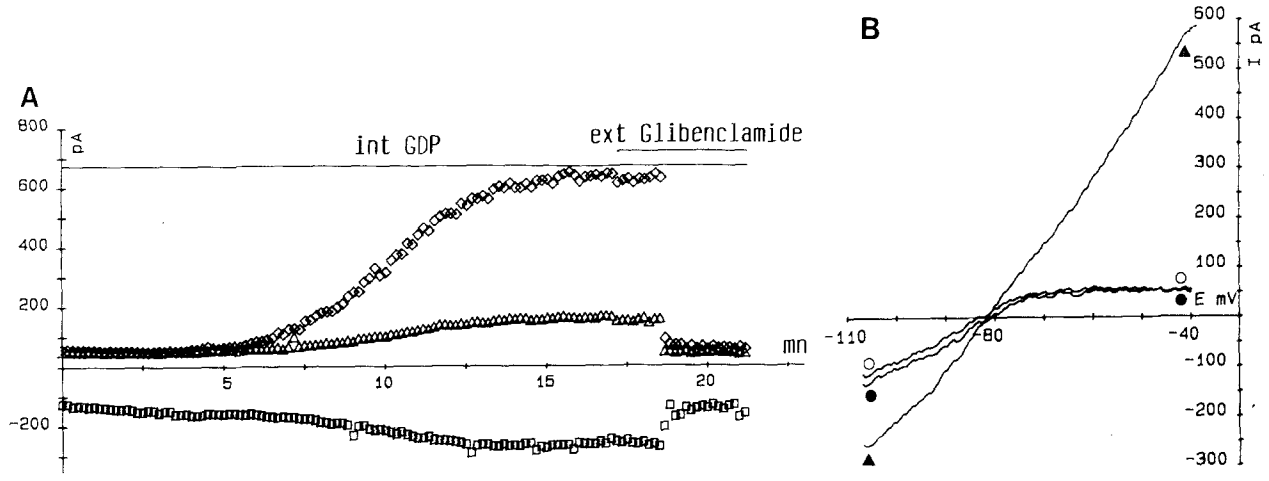

Fig. 6. Effect of glibenclamide (1 μM) on the current evoked by internal GDP. GDP (40 μM) was present throughout the experiment. (*A*) Steady-state current function of the time, with the same symbols as in Fig. 1. Effect of the application of external glibenclamide on the current recorded in the presence of GDP (40 μM) in the pipette. The effect of glibenclamide is reversible (*not illustrated*). (*B*) Current/voltage relation in control (open circle), after 15 min in the presence of GDP (filled triangle), and after addition of glibenclamide to the bath (filled circle)

The hypothesis of the activation of IK(ATP) was further checked by the use of specific blockers of this current from the family of the sulphonylureas (Aschroft, 1988). The experiment shown in Fig. 6 was performed in the presence of GDP in the pipette. After the complete loss of rectification induced by 40 μM GDP, addition of 1 μM glibenclamide to the external solution reversed the current rapidly to its control value (Fig. 6*A*). The current/voltage relation in the presence of glibenclamide was the same as in

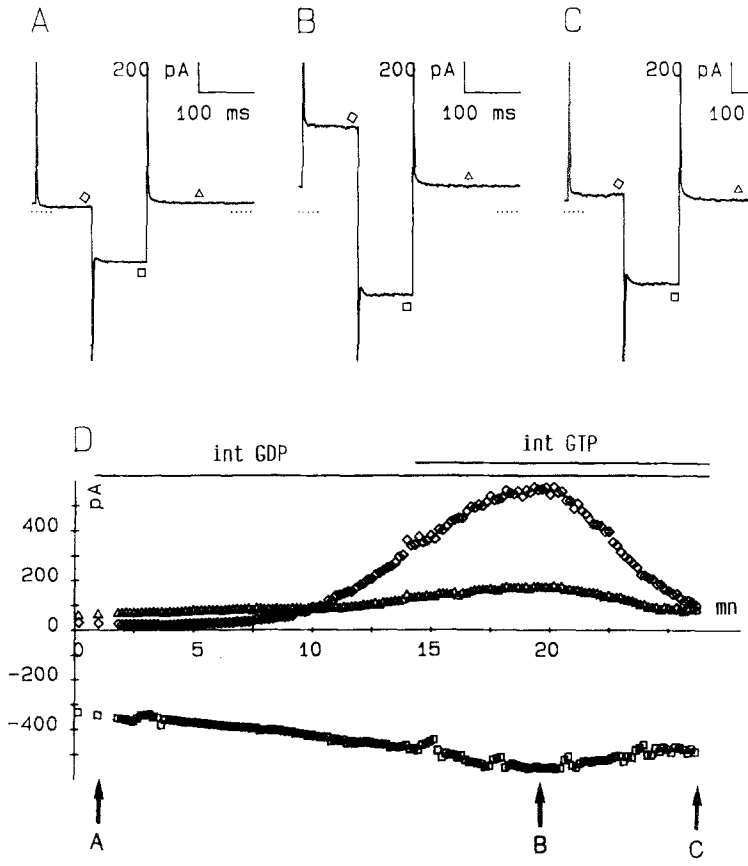

Fig. 7. Effect of GTP (1 mM) on the current evoked by internal GDP. GDP (40 μM) was present throughout the experiment. (*A*) Control current. (*B*) Current recorded after 18 min of dialysis with internal GDP. (*C*) Current recorded in the presence of GDP and GTP in the internal solution. (*D*) Steady-state current function of the time, with the same symbols as in Fig. 1. The arrows indicate the traces displayed in *A–C*. Note the dramatic decrease of the current in the inward and in the outward directions after addition of GTP

control, even in the continuous presence of GDP (Fig. 6*B*). Glibenclamide at this concentration could be applied either to the internal or to the external solution (*not illustrated*). It could also block the current more slowly at an external concentration of 1 nM. Tolbutamide (0.1 mM) had a similar effect, but was less potent (*not illustrated*).

The current that was activated in the presence of GDP in the internal solution was blocked by potassium channel blockers and was suppressed by addition of ATP or glibenclamide, so we can conclude that it was the ATP-dependent potassium current described by Noma (1983).

Mechanism of Activation of IK(ATP) by GDP

Several authors (Kakei, Noma & Shibāsāki, 1985; Dunne & Petersen, 1986*a*,*b*; Spruce, Standen & Stanfield, 1987; Findlay, 1988) reported the ability of different nucleotides, such as GDP, to open this current in excised membrane patches. In their experiments, the concentration necessary was higher than in ours. Furthermore, ADP, GTP and GDP act similarly, whereas only internal GDP could activate IK(ATP) in our whole cell experiments (*see* Discussion).

One possibility would be that GDP might convert into GTP in the cytosol, by a (direct or indirect) dephosphorylation of ATP into ADP. According to this hypothesis, once the effect of GDP was observed (Fig. 7*A* and *B*), an excess of GTP was expected to reverse it. The experiment is reported in Fig. 7. Addition of GTP (1 mM) to the internal solution completely suppressed the GDP-induced current (Fig. 7*C* and *D*).

Discussion

In a study on the stability of potassium currents in different situations, we made the initial observation that micromolar concentrations of GDP in the internal solution induced a dramatic increase of the membrane conductance of frog atrial myocytes. The aim of the present study was to investigate this conductance and the mechanism of its activation.

In the absence of ATP or other nucleotides in the pipette, the membrane currents were stable for typically 30 min in a bath containing pyruvate and glucose. When pyruvate and glucose were omitted in the bath, a time-independent current, reversing at the potassium equilibrium potential, developed in a

few minutes in each of three experiments. When 20 or 40 μM GDP (lithium salt) was present in the pipette, with both pyruvate and glucose in the bath, a similar time-independent membrane current developed within a few minutes. Lithium alone (up to 2 mM LiCl) had no effect.

This current was suppressed or dramatically reduced by external cesium and barium ions and its reversal potential was close to the potassium equilibrium potential, so it must be carried by potassium ions. It was suppressed by addition of ATP in the cell and by application of glibenclamide in the bath or in the pipette; thus, this current could be identified as an ATP-dependent potassium current (for reviews, *see* Ashcroft, 1988; Stanfield, 1987).

The effects of nucleotides on the ATP-sensitive K channel have been extensively studied on excised membrane patches.

An activating effect of ADP has been reported in pancreatic (0.1 to 0.5 mM, Dunne & Petersen, 1986*b*) and cardiac cells (10 to 250 μM, Findlay, 1988). GDP (0.1 to 1 mM) and GTP (10 μM to 1 mM) can also activate this channel in pancreatic cells (Dunne & Petersen, 1986*a*).

A partial blockade by millimolar concentrations of GTP has been observed in skeletal muscle (Spruce et al., 1987) and in heart (Kakei et al., 1985).

In contrast to the excised membrane results reported by these authors, in our whole cell experiments IK(ATP) could be activated neither by ADP (40 μM) nor by GTP (40 μM to 1 mM). It was activated by GDP (20 to 40 μM) in every cell from 29 experiments. The concentrations of GDP we used were lower than the concentrations necessary to open the channels on excised patches of pancreatic cells (Dunne & Petersen, 1986*a*: 0.1 to 1 mM; Findlay, 1987: 1 mM). The cellular death occurred strikingly, usually in a few minutes if the effect of GDP was not antagonized by ATP or GTP. These observations suggest that the effect of GDP we report in the present paper differs from the one at the single channel level.

The mechanism by which this current was activated remains unclear. In insulinoma cells where a G-protein regulation has been described (de Weille et al., 1989), GTP is necessary to open the channels, whereas it blocks them in our experiments. We found that 80 μM GDP-β-S, an analog of GDP which binds irreversibly to G-proteins (*not illustrated*), produced the same effect as GDP. This concentration of GDP-β-S was checked to prevent any effect of carbachol on K^+ conductance. The effects of GDP-β-S were quickly reversed by internal addition of 3 mM ATP. The current activated again when ATP was withdrawn. The activation of the K^+ channels by GDP is then opposite to the usual G protein regulation.

We investigated the possibility of another mechanism, leading to a decrease in the ATP concentration. This could in turn modify the concentration of molecules contributing to the regulation of the channel, such as ADP, NAD, NADH, NADP, NADPH (Dunne et al., 1988). The redistribution of these compounds could ultimately also be lethal for the cell.

The ATP regulating this conductance is preferentially produced by glycolysis (Weiss & Lamp, 1989). One hypothesis is a GDP blockade of one of the glycolytic enzymes, which is prevented by GTP.

Another hypothesis is a GDP-induced decrease in the cytosolic ATP. The reaction

$$ATP + GDP \leftrightarrow ADP + GTP$$

is normally at quasi-equilibrium in the cell, by a direct conversion by the mitochondrial nucleoside diphosphokinase (NDPK) or by indirect cytosolic enzymes. An excess of GDP in the pipette might displace this equilibrium and decrease the concentration of ATP by interconversion of the phosphate group. We observed that the effect of GDP can be totally reversed by an excess of GTP, as expected from this schematic model. Although part of the mitochondria are located close to the sarcolemma, it is unclear whether the NDPK activity can sufficiently reduce the concentration of ATP close to the cell membrane.

In summary, we report the existence of a glibenclamide-sensitive potassium current activated in frog atrial cells in the presence of low concentrations of GDP.

A similar glibenclamide-sensitive current, which probably flows through the same ionic channels, can be activated in this preparation by micromolar concentrations of the potassium channel opener BRL34915 (cromakalim). When it was activated by the channel opener, this current could not be suppressed by addition of ATP (Pilsudski, Rougier & Tourneur, 1989), whereas it could when it was activated by internal GDP. GDP appears, therefore, as a tool to model the activation of IK(ATP) occurring in anoxic conditions.

We wish to thank Dr. I. Findlay for helpful discussions. We also thank J.L. Andrieu and J. Amsellem for skillful help. This study was supported by grant I.N.S.E.R.M. C.R.E. 885014.

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Received 19 January 1990; revised 4 April 1990