Contribution of BK channels to action potential repolarization at minimal cytosolic Ca^{2+} concentration in chromaffin cells

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ABSTRACT

BK channels modulate cell firing in excitable cells in a voltage dependent manner regulated by fluctuations in free cytosolic Ca^{2+} during the action potentials. Indeed, Ca^{2+} -*independent* BK channel activity has ordinarily been considered not relevant for the physiological behaviour of excitable cells. We recorded K^+ channels from bovine chromaffin cells at minimal intracellular and extracellular Ca^{2+} concentration. Despite the low open probability of BK channels in the absence of Ca^{2+} ($V_{50}=+146.8$ mV), they are responsible of more than 25% of the total K^+ efflux during the first millisecond of step depolarisations to +20 mV. Moreover, BK channels activate ~30% faster ($\tau=0.55$ ms) than the average of the rest of K^+ channels. The other main source of fast voltage-dependent K^+ efflux at such a low Ca^{2+} is a transient K^+ current (I_A -type) activating with a V_{50} of -14.2 mV. To determine the role of BK currents in the absence of Ca^{2+} rises, we recorded BK currents and action potentials in perforated-patch configuration in the presence and in the absence of external Ca^{2+} , and tested the effect of specific BK channels blockers. Our results show that BK channels activate during action potentials and modulate the spike shape, at minimal Ca^{2+} concentration, and suggest that they could even do so in the presence of extracellular Ca^{2+} , before Ca^{2+} entering the cell enhances their activity.

INTRODUCTION

Upon splanchnic nerve stimulation, chromaffin cells release catecholamines, opioids and ATP to the bloodstream, thus contributing, as the endocrine arm of the sympathetic nerve system, to body homeostasis during stressful situations [1]. Adenomedullary hormone release is regulated by K^+ channels through the control of multiple aspects of cellular excitability [2-3]. Five different K^+ currents have been identified in bovine chromaffin cells: a high conductance voltage- and Ca^{2+} -dependent K^+ current (BK current; [4-5]), a delayed rectifier K^+ current [5-6], a small conductance Ca^{2+} -activated K^+ current [7], a G protein-activated K^+ current [8] and an M-type current [9]. Despite the similarities of chromaffin cells with neurons, an A-type K^+ current that regulates the interspike intervals [10-11] has not been reported in bovine chromaffin cells. A-currents typically activate at subthreshold voltages and rapidly inactivate. Since chromaffin cells, like many neurons, are able to fire trains of action potentials, this current might be involved in the regulation of the firing frequency during these trains.

The K^+ current that has been more thoroughly studied in relation to action potential firing in chromaffin cells is the high conductance voltage- and Ca^{2+} -dependent K^+ current (BK current). BK channels shape the initial phase of repolarisation of Na^+ - and Ca^{2+} -dependent action potentials (APs) [12-14]. BK channels activate in a voltage-dependent fashion, but the increase of cytosolic Ca^{2+} (Ca^{2+}_i) greatly enhances their voltage sensitivity by an allosteric mechanism [15-18]. BK channels are composed of two distinct subunits, α and β , which are arranged in a 1:1 stoichiometry. Each channel exists as a tetramer formed by four α subunits either alone or in combination with β subunit pairs. The α subunit is the pore-forming unit whereas the β subunit is the regulatory unit. While only one α subunit has been identified until now, 4 types of β subunits (β_1 - β_4) have been reported [19]. The different β subunits

modify the gating and pharmacological properties of BK channels [20-27]. Thus, channels containing the α subunit alone or those with β_1 or β_4 subunits produce sustained currents that do not inactivate [28-29]; on the contrary, those consisting of the α subunit and the β_2 or β_3 give rise to inactivating BK currents [28, 30]. Likewise, the various β -subunit phenotypes also affect the sensitivity to pore-blockers like charybdotoxin and iberiotoxin (ChTx and IbTx). Coexpression of α and β_2 or β_3 confers a reduced sensitivity to these toxins whereas BK channels formed by α and β_4 are resistant [29, 31-32].

BK channels from chromaffin cells are kinetically and molecularly heterogeneous. A functional variant exhibiting rapid inactivation (BK_i) and presumably formed by mixtures of α and β_2 or β_3 has been well characterized in rat and bovine chromaffin cells [30, 33-35]; this variant coexists with a non-inactivating counterpart (BK_s), which may be composed by the association of α and β_4 subunits [36]. Interestingly, BK_i and BK_s channels from rat chromaffin cells have been assigned to different roles in cellular excitability. Thus, cells expressing mostly BK_i are capable to produce more sustained AP firing than those expressing mainly BK_s channels [34, 37]. This differential behaviour has been attributed to the slower deactivation kinetics of BK_i channels relative to that of BK_s channels.

The physiological relevance of BK channels has been normally related with their sensitivity to intracellular calcium rises. Although it is well established that BK channels can be maximally activated by extreme depolarisations in the absence of intracellular Ca^{2+} [15-17, 24, 30, 36, 38], the relative contribution of this current to the total K^+ efflux and, therefore, their relevance for the electrical behaviour of the cells at minimal Ca^{2+}_i has not been investigated. Here, we explore the biophysical properties of two K^+ currents –BK and A

types- and investigate the role of BK channels in modulating AP shape at low intracellular Ca^{2+} .

METHODS

Isolation and culture of bovine chromaffin cells

Bovine chromaffin cells were isolated from adrenal glands of adult cows and kept in culture following standard methods [39-40]. Cells were plated in 1 cm-diameter glass coverslips at a density of 5×10^4 cells per coverslip and were used for 1-4 days after isolation.

Electrophysiological methods and data analysis

Pipettes of approximately 3 M Ω were fabricated from borosilicate glass (Kimax 51, Witz Scientific, Holland, OH, USA) with a Narishige PP83 puller (Japan), and subsequently fire-polished and coated with dental wax. An EPC-9 patch-clamp amplifier and PULSE software (HEKA elektronik, Lambrecht, Germany) were used for data acquisition and stimuli generation. Data were acquired through a combination of the high time resolution PULSE software and the lower time resolution X-Chart plug-in module to the PULSE software. In the high resolution mode, whole-cell or perforated patch currents or voltages were filtered at 2 KHz and sampled at 8.33 KHz (120 μ s), whereas currents from excised-patches were filtered at 2.9 KHz and sampled at a frequency of 10 KHz. Low frequency acquisition was at 3 Hz irrespective of the recording configuration. For voltage-clamp experiments, after breaking-in or after the perforation had been stabilised, cells were held at a voltage (V_h) of -70 mV, and those with holding currents larger than 20 pA were rejected. Series resistances in whole-cell and perforated patch were always kept below 10 M Ω and 25 M Ω , respectively, and were compensated by 90%. A p/n protocol for subtracting leak and capacitive currents was routinely applied except for tail current measurements. Experiments were conducted at room temperature ($\sim 25^\circ\text{C}$).

Activation curves were built by using tail-current measurements upon repolarisation from different potentials. To avoid capacitive transient contamination, monoexponential functions starting 500 μ s after the beginning of the repolarisation were fitted to the deactivation phase of the currents. Peak-current amplitudes were extrapolated to the beginning of the repolarizing step from the resulting exponential fits and subsequently plotted against membrane potential during the activation pulse. The following Boltzmann equation was then fitted to the plotted data:

$$(1) \quad I = 1/[1+\exp(V_{50}-V_m)/K]$$

where V_{50} is the voltage at which tail current amplitude (I) is half of its maximum, V_m is the membrane potential, and K is the reciprocal of the slope at the inflexion point of the activation curve.

To generate steady-state inactivation curves, a double-pulse Cole-Moore protocol was used and the current responses to the second pulse plotted against the potential of the first-conditioning pulse. The following Boltzmann equation was fitted to that relation:

$$(2) \quad I/I_{\max} = [1+\exp(V_{50}-V_c)/K]^{-1}$$

where V_c is the potential during the conditioning pulse, V_{50} is the voltage at which conductance is inactivated by 50% –with respect to the response elicited in the absence of a conditioning pulse–, and K is the reciprocal of the slope at the inflexion point of the inactivation curve. The fitting was always performed by the least squares method.

The minimum number of channels in outside-out patches (n) was estimated as

$$(3) \quad n = n' / p$$

where n' is the maximum number of opening levels observed at a given voltage, and p is the BK channel open probability at the same voltage, obtained from activation curves.

Ensemble averages of BK and non-BK channel activity from patches containing mixtures of K^+ channels were performed by (i) identifying BK channel unitary currents from sweeps showing unequivocal BK channel openings, (ii) disregarding openings with amplitudes lower than those of BK channels and plotting the minimum number of points required to describe an idealized BK activity, (iii) interpolating among these points to build traces describing the idealized BK channel openings, and (iv) subtracting these traces from actual traces to get the non-BK channel activity. Averages of both idealized (BK) and subtracted (non-BK) traces reflect the relative contribution of purely voltage-dependent BK current to total K^+ current at physiological voltages.

Solutions

The external solution had the following composition (in mM): 140 NaCl, 2.8 KCl, 2 MgCl₂, 10 glucose and 10 HEPES; pH 7.3 adjusted with NaOH. To measure K^+ tail currents, an extracellular solution of the following composition was used: 140 KCl and 10 HEPES; pH 7.3 adjusted with KOH. The solution bathing the cytoplasmic side of the membrane always contained (in mM): 140 KCl and 10 HEPES; pH 7.3 adjusted with KOH. BAPTA (10 mM) was also added to this solution to buffer cytosolic Ca^{2+} concentration (Ca^{2+}_i) to around 10 nM [5]. In some experiments, 10 mM EGTA, instead of BAPTA, was added to the internal

solution, without producing significant differences. The internal solution for perforated patch recordings was (in mM): 140 KCl, 10 EGTA, 10 HEPES and 0.5 Amphotericin B.

Drug application and analysis of pharmacological data

Drugs were applied onto the cell under investigation by means of a gravity-driven perfusion system with 5 independent lines controlled by electronic valves (The Lee Company, Westbrook, CO, USA). This system allowed us to exchange the medium surrounding a cell in less than 1 s. The drug concentration that produces 50% of the maximum inhibition (IC_{50}) was calculated according to the following equation:

$$(4) \quad F = [D]/IC_{50}+[D]$$

where F is the fractional inhibition produced by the drug, and [D] is the drug's concentration.

Chemicals

Dulbecco modified Eagle's medium (DMEM), fetal bovine serum, penicillin, and streptomycin were purchased from Gibco (Madrid, Spain). Bovine serum albumin, 5-Fl-2'-deoxyuridine, cytosine arabinoside, L-leucine methyl ester, dimethylsulfoxide, HEPES, EGTA, BAPTA, and tetraethylammonium (TEA) were from Sigma Aldrich Quimica (Spain); iberiotoxin (IbTx) and charybdotoxin (ChTx) were from Peptide Institute (Osaka, Japan). Collagenase A from *Clostridium histolyticum* was obtained from Boehringer-Mannheim, and Percoll from Pharmacia. The rest of reagents were from Panreac (Barcelona, Spain)

Statistical analysis

Results are expressed as means $\pm$ standard error of the mean (SEM). Statistical differences between means were assessed by the Student's *t*-test. $p < 0.05$ was taken as the limit of significance.

RESULTS

Multiple voltage-activated K^+ currents with different inactivation kinetics and pharmacological sensitivities are expressed in bovine chromaffin cells

We have studied K^+ currents from bovine chromaffin cells dialysed in the whole-cell configuration with an internal solution containing 10 mM BAPTA and superfused with a bath solution devoid of added Ca^{2+} . Under these conditions the occupancy of Ca^{2+} binding sites in BK channels is thought to be negligible [15]. Step depolarisations from a V_h of -70 mV elicited inactivating outward currents (Fig. 1A, *left panel*) that were partially blocked by the BK channel blocker ChTx (150 nM; [41]; Fig. 1A, *right panel*, and 1B). The remaining ChTx-resistant component was mostly due to a fast inactivating K^+ current (henceforth named I_A) that, nevertheless, was progressively masked by slowly inactivating (V_i around 10 s^{-1}) current at potentials positive to +5 mV (Fig. 1A, *right panel*, and 1C). These results suggest the existence of at least three types of voltage-activated K^+ currents in bovine chromaffin cells differing in their inactivation kinetics and sensitivity to ChTx: i) a ChTx-sensitive current, namely the BK current, ii) a fast inactivating ChTx-resistant current, that we have designated as I_A , and iii) a non- or slow-inactivating ChTx-resistant current that could correspond, at least in part, to a delayed rectifier K^+ current [6].

To record BK currents in isolation, cells were clamped at 0 mV for over 2 min, to inactivate the delayed rectifier K^+ current [6, 42] and I_A . Step depolarisations from a V_h of 0 mV activated outward currents that were ~80% reduced by ChTx (150 nM, 10 min; Fig. 2A and B). The current component sensitive to ChTx, likely the BK current, exhibited voltage-dependent inactivation (Fig. 2C). Due to the lack of inactivation of ChTx-resistant currents, the rate of inactivation of K^+ currents in control conditions was similar to that of the ChTx-sensitive component, thus allowing the study of BK current inactivation in the absence of

channel blockers (Fig. 2C). The ChTx-resistant current component could be attributed to either delayed rectifier K^+ channels still active despite a sustained depolarisation at 0 mV, or to non-inactivating BK channels not blocked by the toxin. To distinguish between these two possibilities, we studied the effect of ChTx on unitary BK channel currents recorded from outside-out patches (Fig. 3). Multiple BK channels were easily detected and identified by their large conductance (~ 190 pS; [4-5]). Moreover, BK channels were the only type of channel observed in more than 95% of the patches analysed ($n = 50$). The ensemble averages of unitary BK channel currents during test pulses from a V_h of 0 mV to +100 mV showed inactivating kinetics. The application of ChTx (150 nM) to the external face of the patches blocked ~ 70 % of BK channel openings and unmasked a non-inactivating ChTx-resistant BK component (Fig. 3A and C). Interestingly, the contribution of this non-inactivating and ChTx-resistant component ($\sim 30\%$; Fig. 3B) to the averaged BK current in excised patches is similar to that observed in whole-cell recordings of BK current at identical voltages (Fig. 2B). These results point toward the coexistence of inactivating and non-inactivating BK channels with differential sensitivity to ChTx, and suggest that most of the ChTx-resistant current recorded in the whole-cell configuration from a V_h of 0 mV can be attributed to non-inactivating BK channels (BK_s channels). In agreement with these observations, it has been reported the presence in the adrenal medulla of β_4 subunits that produce non-inactivating and ChTx-resistant BK currents [36].

The effects of TEA, a broad spectrum blocker of K^+ channels, on K^+ currents were consistent with those observed with selective toxins. Extracellular TEA produced a concentration dependent reduction of K^+ currents evoked by long depolarising pulses (Suppl. Fig. 1A-B) and caused pronounced changes in their rate of inactivation (Suppl. Fig. 1C). In this latter respect, TEA (30 mM) accelerated inactivation in cells depolarised from a V_h of -70 mV to

potentials positive to +40 mV, thus suggesting a preferential block of BK current with respect to I_A (Suppl. Fig. 1C, *left panel*). TEA (30 mM) slowed the inactivation of K^+ currents activated from a V_h of 0 mV (Suppl. Fig. 1C, *right panel*), possibly because it affects predominantly the BK_i channels, the only inactivating channels active at that V_h [5].

Fast activation of BK channels in outside-out patches

The concealment of I_A by BK current described in Fig. 1 is indicative of a fast activation of BK channels even at low Ca^{2+}_i , similar to the reported for slo channels associated with $\beta 3$ subunits in channels expressed in *Xenopus* oocytes [30]. Simultaneous activation of BK and other K^+ channels could also be observed in outside-out patches depolarized from -70 mV to +20 mV in the nominal absence of Ca^{2+} (Fig. 4A). Due to their large conductance, BK channels were easily distinguished from other K^+ channels (Fig. 4A, *left panel*), that disappeared when the patch was clamped at 0 mV (Fig. 4A, *right panel*). Moreover, the high number of BK channels existing in our patches (39 channels/patch, on average) facilitated the observation of their activity under our recording conditions. The percentage of traces with BK channel openings during the first 5 ms of the pulse was $29 \pm 3\%$. Thus, considering the high density of BK channels in chromaffin cells (~ 550 channels/cell; [5]), similar depolarisations should evoke BK channel activity in 100% of the trials assayed in the whole-cell configuration.

The estimation of the time course of BK channel activation (during the first 5 ms of the pulse) was based on the idealisation of BK channel openings in outside-out patches (Fig. 4A, *left and middle panels*). Such idealisation was performed manually after determining the BK unitary current amplitude. Although it was not possible to do the same for each type of non-BK channels, as these channels had fairly similar amplitudes, we could model the activation

of non-BK currents by subtracting idealised BK channel activity from the original current traces (see Methods). The ensemble averages for both types of channels are shown in Fig. 4B. An exponential function fitted to the data indicated that BK channels activated faster (τ of 0.55 ms) than non-BK channels (τ of 0.69 ms). These results also indicate that BK channels represent ~25% of the total K^+ efflux during, at least, the first millisecond of a depolarisation under our recording conditions (test pulses to +20 mV and ~10 nM Ca^{2+} on the cytoplasmic side of the membrane).

Voltage dependence of activation of K^+ currents from bovine chromaffin cells

Tail current measurements using symmetrical K^+ solutions (140 mM) were performed to study the voltage-dependence of activation of the different K^+ currents. For BK currents, we clamped the cells at 0 mV and applied 10 ms-long step depolarisations to a maximum of +215 mV (Suppl. Fig. 2A, *left panel*). A Boltzmann function fitted to the estimated tail current amplitudes gave a V_{50} of +146.8 mV and a K of 31.71 mV (Suppl. Fig. 2A, *right panel*). To build the activation curve of I_A , tail currents were generated in the presence of IbTx (150 mM; 5 min), another specific blocker of BK currents [41]. The current resistant to IbTx should mainly correspond to I_A because delayed rectifier K^+ channels activate too slowly to significantly contribute to whole-cell K^+ currents during a 10 ms depolarisation [5-6, 43-44]. IbTx reduced by 60% the amplitude of tail currents, in good agreement with the proportion of BK channels blocked by specific toxins in excised patches (Fig. 5) and whole-cell recordings in asymmetric K^+ (Fig. 1). Fitting a Boltzmann function to tail current amplitudes in the presence of IbTx gave a V_{50} of -14.2 mV, and K of 11.94 mV, in the range of typical A-type currents.

Voltage dependence of inactivation of K^+ currents from bovine chromaffin cells

To study the voltage dependence of BK current inactivation, we held the cells at 0 mV and applied 15 mV-step depolarisations during 600 ms (long enough to get steady-state inactivation of BK currents). Immediately after these pulses, a 600 ms pulse to +105 mV was applied (Fig. 5A). Only the inactivating component of the current elicited by the latter pulse was considered to build the steady-state inactivation curve. A Boltzmann function fitted to the data gave a V_{50} of +45.25 mV and K of -15.55 mV (Fig. 5C). The steady-state inactivation of I_A was studied in the presence of ChTx by using depolarising prepulses to a maximum of +15 mV from a V_h of -90 mV, which were followed by pulses to +20 mV (Fig. 5B). A Boltzmann function with V_{50} of -42.83 mV and K of -7.13 mV was fitted to the experimental data (Fig. 5C). Steady-state BK channel availability has also been examined in rat chromaffin cells at 0 Ca^{2+} [36], showing similar values to those presented here for bovine cells. These observations validate the use of 0 mV holding potential to isolate BK currents. In essence, at 0 mV there is little steady-state inactivation of BK current.

The time course of recovery from inactivation of I_A and BK current was also studied (Suppl. Fig. 3). BK currents were elicited by 600 ms-long paired pulses to +100 mV from a V_h of 0 mV separated by variable intervals. Ratios between pairs of peak currents were plotted against intervals between pulses. A double exponential function fitted to the data gave two rate constants ($k_{fast} = 23 \text{ s}^{-1}$ and $k_{slow} = 0.77 \text{ s}^{-1}$), reflecting two different kinetic components with similar amplitudes (Suppl. Fig. 3A). The recovery from inactivation of I_A was characterized by using paired pulses to +20 mV from a V_h of -70 mV, and was also described by a double exponential function with a k_{fast} of 27 s^{-1} accounting for 85% of the recovery, and a k_{slow} of 1.84 s^{-1} (Suppl. Fig. 3B).

The role of BK currents in spike shape modulation in the absence of Ca^{2+}

Our results show that BK channels activated during square pulses to +10 mV contribute to ~25% of the total fast K^+ current at minimal Ca^{2+} concentration. We therefore investigated BK current activation during APs in the absence of extracellular Ca^{2+} (Fig. 6A). APs were recorded in perforated patch and then used as AP-waveforms (APWF) in voltage-clamp to stimulate chromaffin cells. APWFs activated efflux currents that reached their maximal amplitude at the peak of the APWF (Fig. 6A). These currents were partially sensitive to IbTx (150 nM, 3 min), suggesting that BK currents are indeed importantly activated (~37% I_K) during APs in the absence of Ca^{2+}_o (Fig. 6B). The IbTx-resistant current is likely to correspond mainly to I_A . We also recorded currents in response to long square pulses to +10 mV from V_h of -70 mV and tested the effect of IbTx (150 nM, 3 min) in perforated patch for comparison with our whole-cell data from Fig. 1. In the presence of 2 mM Ca^{2+}_o , IbTx blocked $41 \pm 3\%$ of the total current and, in the absence of Ca^{2+}_o , the toxin blocked $34 \pm 2\%$ of the total efflux current (Suppl. Fig. 4).

Next, we investigated if BK currents activated by real APs independently of Ca^{2+}_o had any role in controlling the spike shape. To address this question we compared APs recorded in the absence and in the presence of Ca^{2+}_o in perforated patch (Fig. 7A-B). APs in the absence of Ca^{2+}_o were ~43% longer than in the presence of 2 mM Ca^{2+}_o , mainly due to the expected smaller amplitude of BK currents at zero Ca^{2+}_o ($p < 0.01$, 14 cells). IbTx significantly increased AP half duration either in the presence (~36%) or in the absence of Ca^{2+}_o (~20%) mainly due to a slower repolarizing phase in the presence of the blocker (Fig. 7A-B). Interestingly, IbTx significantly reduces the amplitude of APs in the absence of Ca^{2+}_o and also has a tendency to reduce the overshoot of AP recorded in the presence of Ca^{2+} , suggesting an early activation of BK current during APs (histograms, Fig. 7). Taken together,

these results suggest an important role for BK channels in AP repolarization in the absence of Ca^{2+} entry. Indeed, data from Pancrazio et al [14] suggest that a large proportion of BK current activated by APWF in the presence of 2 mM Ca^{2+}_o occurs earlier than the onset of Ca^{2+} current. Our results in perforated patch using pre-recorded APWFs and APWFs built up with voltage-ramps to stimulate the cells are fully consistent with this (Suppl. Fig. 5). APWFs activated fast K^+ currents that are partially sensitive to IbTx. In some cells, Ca^{2+} currents peaks were clearly distinguished from BK currents. In these cells BK currents reached a maximum more than 2 ms earlier than Ca^{2+} currents. Interestingly, in some cells depolarised with a slow APWF, we observed a second peak of delayed BK current, suggesting that in these cells Ca^{2+} entry activate BK currents in a substantially delayed manner (Suppl. Fig. 5). These evidence further supports the hypothesis that, during an AP, some BK channels could be activated even before Ca^{2+} entering the cell increases their open probability in the presence normal external Ca^{2+} .

DISCUSSION

Characterisation of K^+ currents in bovine chromaffin cells

In the present study we have characterised voltage-gated K^+ currents from cultured bovine chromaffin cells when both sides of their plasma membrane were exposed to minimal Ca^{2+} concentrations (no added external Ca^{2+} and Ca^{2+}_i buffered to about 10 nM). In these conditions the occupancy of Ca^{2+} binding sites for the activation of BK channels is thought to be negligible [15]. Two main conclusions arise from our results from this section: (i) even during the first millisecond of a voltage step to physiological potentials (from 0 to +20 mV) BK current contributes to approximately one third of the K^+ efflux at minimal Ca^{2+}_i , (ii) like many neurones, chromaffin cells are endowed with fast-inactivating $-I_A$ -like K^+ currents.

Throughout this study we have used a V_h of 0 mV to isolate BK currents. Several experimental evidence support the suitability of this V_h for that purpose: (i) at this V_h , only 2 out of 50 outside-out patches showed unitary currents arising from channels different to the BK ones; moreover, these other channels contributed minimally to the ensemble average current in those 2 patches; (ii) the relative density of ChTx-resistant BK_s channels in outside-out patches was similar to the fractional contribution of ChTx-resistant BK_s current to the whole-cell outward K^+ current (Fig. 2 and 3), thus suggesting that BK channels ($BK_i + BK_s$) underlie most of the outward current at the whole-cell level; (iii) although IbTx abolishes a noticeable percentage of K^+ currents elicited from -70 mV to 0 mV (Suppl. Fig. 2B), the toxin-sensitive component represents only a ~2% of the maximal BK current available from a V_h of 0 mV (Suppl. Fig. 2A-B).

The voltage-dependence of activation of BK currents varies among cell types, presumably due to the expression of different types of pore-forming α subunits and a variety of regulatory β subunits [22, 30, 32]. Thus, α and β subunits from human myometrium cells coexpressed in *Xenopus* oocytes show a V_{50} of +220 mV when Ca^{2+}_i is in the range from 10 to 100 nM [24]; in contrast, BK channels in rat supraoptic neurones show a V_{50} of +80 mV at a similar Ca^{2+}_i [38]. BK currents from bovine chromaffin cells activate with a V_{50} of +147 mV in 10 nM Ca^{2+}_i (Suppl. Fig. 2A). This value is similar to that reported for BK channels from rat chromaffin cells (V_{50} of +135 mV; [36]), which are known to express α as well as β_2 and β_3 subunits [26, 28, 30].

I_A current

A transient outward current, I_A , activating between -70 mV and +30 mV was apparent in the presence of ChTx and nanomolar Ca^{2+}_i . This current might have been previously unnoticed in whole-cell recordings because under physiological Ca^{2+} concentrations BK currents could have masked it. I_A behaves as a rapidly inactivating current with τ about 25 ms in the voltage range from 0 to +60 mV (Fig. 1C). Furthermore, recovery from inactivation was also fast, taking about 100 ms to develop by 80%. I_A had a moderate sensitivity to TEA and was resistant to high concentrations (150 nM) of ChTx. The ion channel responsible for I_A could correspond to the inactivating FK channel of 18 pS identified by [5]. Similarly to the transient current described here, FK current maximally activates between 20 and 40 mV and becomes inactivated in about 60 ms. At variance, the channel underlying I_A would differ from the fast-inactivating but TEA-resistant (40 mM) channel cloned by García-Guzmán et al. (1992, [45]) from a bovine chromaffin cell cDNA library.

I_A currents may contribute to the regulation of chromaffin cell excitability during trains of action potentials. Due to the partial overlapping (from -65 mV to -30 mV) of the voltage ranges for activation and steady-state inactivation, I_A would be steadily activated between consecutive AP, thus acting as a damper on the development of interspike depolarisations to reduce the frequency of firing. This frequency-encoding role is played also because of the inactivation properties of I_A , which inactivates during the spike depolarisation but quickly recovers over the hyperpolarisation phase that immediately follows each AP. I_A can be of importance to chromaffin cell physiology by determining firing rates in a way that reflects the stimulus intensity (e.g. acetylcholine output from splanchnic nerve terminals), but also by imposing an upper limit so that the exhaustion of the readily-releasable pool of secretory vesicles could be avoided [40, 46].

Physiological relevance of BK channels in the absence of Ca^{2+}

The high ionic conductance of BK channels (~ 190 pS at 140 mM K^+_i and 140 Na^+_o) and their elevated density in the membrane of bovine chromaffin cells (550 channels per cell; [5]) can explain the large proportion of K^+ efflux through BK channels in the absence of Ca^{2+} . This implies that, despite the low open probability of any individual channel under these activation conditions, given the number of BK channels in a typical chromaffin cell and the large single channel current, BK current of appreciable amplitude will be reliably activated.

Our results show that BK channels largely contribute to the total K^+ efflux during the first millisecond of a square pulse to physiological potentials (+20 mV) in the absence of Ca^{2+} . Moreover, we have shown that BK channels activate significantly during action potentials and contribute to shorten their duration in the absence of Ca^{2+} entry, at minimal Ca^{2+} concentration. This raises the possibility of an entirely voltage-dependent role for these

channels in repolarising the cell during APs even before Ca^{2+} entering the cell diffuses and reaches BK channels. The time window necessary for a strictly BK channel voltage-dependent modulation of membrane potential during an action potential in the presence of Ca^{2+} would start during the depolarising phase (phase 0) of the AP, and would end by the time Ca^{2+} enters through Ca^{2+} channels and diffuses reaching its binding sites at BK channels. In chromaffin cells, APs last for ~15 ms, and Ca^{2+} currents elicited under voltage-clamp by pre-recorded AP-waveforms start to activate soon after the peak of the AP [14], as in some neurons [47-48]. The fact that virtually every patch from chromaffin cells contains BK channels suggests that these channels are distributed rather homogeneously across the cell surface [49]. Additionally, although it has been found that BK and Ca^{2+} channels are colocalized in the frog neuromuscular junction [50] and the calyx synapse from the chick ciliary ganglion [51], in chromaffin cells Ca^{2+} channels and BK channels are poorly colocalized (50-160 nm away; [49]). Additionally, BK channels have been found not only in presynaptic sites, where colocalization is more likely to occur, but in several compartments of the neuron, such as postsynaptic sites and, in early development stages, in the axon, out of the synapses [52]. In the chromaffin cell, with a cell surface of more than 800 μm^2 , even if a few Ca^{2+} channels were open before the peak of the AP (and Ca^{2+} -independent BK current), it is unlikely that most of BK channels were exposed to Ca^{2+} ions entering in the cell, especially when Ca^{2+} entry diffusing from a Ca^{2+} channel would barely reach a few hundreds of nm [53-54].

In some neurons like the supraoptic nerve and in non-excitable cells, somatic BK channels show an activation curve shifted to negative potentials [38, 55]. Our results strongly suggest that BK channels can modify the membrane potential without large depolarisations or changes in cytosolic Ca^{2+} in the micromolar range and propose a role for these channels on

AP early repolarisation independently on Ca^{2+} entry, especially at subregions of the membrane with BK channels located far from Ca^{2+} channels.

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Abbreviations

AP: Action potential

BK_i: inactivating BK (current or channel)

BK_s: non-inactivating BK (current or channel)

Ca²⁺_i: Cytosolic Ca²⁺

ChTx: Charybdotoxin

I_A: fast inactivating potassium current

IbTx: Iberiotoxin

TEA: Tetraethylammonium

V_h: holding potential

FIGURE LEGEND

Figure 1. Voltage-activated K^+ currents in bovine chromaffin cells dialysed with 10 mM BAPTA and superfused with a solution devoid of added Ca^{2+}

A. Typical experiment showing K^+ currents activated by 600 ms-long step depolarisations from a V_h of -70 mV to different potentials ranging from -55 mV to +130 mV in the absence (*left panel*) and the presence of ChTx (150 nM for 5 min; *right panel*). B. Effect of ChTx on peak current amplitudes. C. Voltage dependence of the rate of K^+ current inactivation (V_i) in the absence and in the presence of ChTx. The toxin discloses a fast inactivating K^+ current, I_A , which is apparent between -40 and +100 mV. This current is eclipsed in the absence of the toxin by a non- or slow- inactivating K^+ current at voltages positive to +5 mV. Data are means $\pm$ SEM of 21 experiments. $P < 0.001$.

Figure 2. BK currents from bovine chromaffin cells at minimal Ca^{2+}_i

A. K^+ currents activated by 600 ms-long step depolarisations from a V_h of 0 mV to different potentials ranging from +20 up to +120mV, in 20 mV increments, in the absence (Control, *upper panel*) and the presence of ChTx (150 nM, 5 min; ChTx, *middle panel*); *lower panel*, BK currents resulting from the subtraction of traces in the presence of ChTx from control ones (Control-ChTx). B. Effect of ChTx on peak K^+ currents at different potentials. Amplitudes were measured 15 ms after the beginning of the pulse. An expanded view of the graph is shown as an inset. C. Voltage dependence of the rates of inactivation (V_i) of control and BK currents. Data are means $\pm$ SEM of 11 experiments. ** $P < 0.01$.

Figure 3. Effect of ChTx on BK channels in outside-out patches

A, *upper panels*. Representative current traces of BK channel openings in an outside-out membrane patch depolarised every 3 s to +100 mV from a V_h of 0 mV. ChTx (150 nM, 10

min) did not block all BK channel openings; *lower panel*. Ensemble averages of 30 sweeps from the patch above in the absence and the presence of ChTx. *B*. Effect of ChTx on the peak amplitude of the ensemble averages from 6 excised patches. *C*. Effect of ChTx on the rates of inactivation (V_i) of the ensemble averages. Data are means $\pm$ SEM. ** $P < 0.01$.

Figure 4. Fast activation of BK channels at minimal Ca^{2+}_i

A Left. Representative current traces (15 ms-long) of multichannel activity in outside-out patches depolarised to +20 mV from a V_h of -70 mV. BK channels are easily distinguishable among K^+ channels due to their large conductance. *Middle*. Idealized BK channel openings and non-BK channel activity (resulting from the subtraction of BK idealisation from the original sweeps; see Methods) over the first 5 ms of depolarisation from the recordings shown in the left panel. *Right*. Channel activity in the same representative patch at a V_h of 0 mV. Most of non-BK channels are inactivated at this V_h . *B*. Ensemble averages of 20-30 sweeps for BK or non-BK channels from 6 different patches during the first 5 ms of depolarisation. An exponential function was fitted to averaged data from each type of channel ($\tau_{BK}=0.55$ and $\tau_{non-BK}=0.69$ ms)

Figure 5. Steady-state inactivation of BK and I_A currents

A. BK currents were activated by 600 ms-long conditioning pulses to different potentials from a V_h of 0 mV, which were immediately followed 600 ms pulses to +105 mV (see adjoining voltage protocol). *B*. A similar stimulation paradigm was used to study inactivation of I_A currents (V_h of -90 mV; test pulse to +20 mV; see adjoining voltage protocol). *C*. Steady-state inactivation curves of BK and I_A currents. Only the inactivating component of the current responses to the second pulse was considered. Data were normalized with respect to currents elicited by pulses to +20 or +105 mV applied from the corresponding V_h (0 mV or

-90 mV). Boltzmann functions were fitted to the experimental data and gave the following values: $V_{50} = +45.25$ mV and $K = -15.55$ mV for the BK current, and $V_{50} = -42.83$ mV and $K = -7.13$ mV for I_A . Data are means $\pm$ SEM of 7 cells for each type of current.

Figure 6. Effect of IbTx on BK currents induced by pre-recorded action potential wave forms (APWF) at minimal extracellular Ca^{2+} concentration.

A, top panel. K^+ currents (black trace) induced by a fast APWF pre-recorded (dotted line) in perforated patch in the absence of Ca^{2+} . The blue trace corresponds to the remaining current after the administration of IbTx (150 nM, 5 min). The red line is the result of subtracting the effect of IbTx from the control current, corresponding then to BK current. *Lower panel,* Effect of IbTx on K^+ currents induced by a slower pre-recorded APWF from another cell. Note that Ca^{2+} currents are not appreciated in these recordings due to the absence of Ca^{2+} . *B,* Summary of the effect of IbTx in K^+ currents from experiments similar to the ones showed in panel A (* $p < 0.001$; data are means $\pm$ SEM from 14 cells)

Figure 7. Effect of IbTx on action potentials in the presence and in the absence of Ca^{2+}

A, Action potentials induced by a short current injection (20 pA, 20 ms) in the presence of 2 mM Ca^{2+}_o in perforated patch (black trace). The red trace corresponds to the AP after the perfusion of the cell with IbTx (150 nM, 5 min, red trace). Note that IbTx widens the APs preferentially affecting the repolarizing phase. *Lower panel,* summary of the effect of IbTx on AP overshoot and AP half width (data are means $\pm$ SEM from 14 cells). *B,* Effect of IbTx on APs recorded in the absence of Ca^{2+} . *Lower panel,* summary of the effect of IbTx on AP overshoot and AP half width. Note the widening effect of IbTx also in these conditions. (Data are means $\pm$ SEM from 14 cells. * $p < 0.05$, ** $p < 0.001$).

Supplemental Figure 1. Effect of TEA on K⁺ currents from chromaffin cells.

A. Effect of TEA (30 mM) on K⁺ currents activated by 600 ms-long depolarisations to 0 mV up to +120 mV, in 20 mV steps, from a V_h of -70 mV. B, Effect of different concentrations of TEA (3, 10 and 30 mM) on peak currents evoked by the voltage protocol employed in A. C, *left panel*. Effect of TEA (30 mM) on the rate of inactivation (V_i) of K⁺ currents elicited as indicated in A; *right panel*. Effect of TEA (30 mM) on the rate of inactivation (V_i) of K⁺ currents elicited by 600 ms-long depolarisations to 0 mV up to +120 mV, in 20 mV steps, from a V_h of 0 mV. Data are means ± SEM of 8 experiments. *P < 0.05, ** *P* < 0.01.

Supplemental Figure 2. K⁺-tail currents from bovine chromaffin cells

A, *left panel*. Tail currents (arrow) observed upon repolarisation to -70 mV from different test potentials in cells held at a V_h of 0 mV (see the adjoined voltage protocol); tail current amplitudes were subsequently used to build the activation curve for BK channels (*right panel*). The Boltzmann function fitted to the experimental data gave a value of +146.8 mV for V₅₀, and 31.71 mV for K. Data were normalized with respect to the maximal current derived from curve fitting. B, *left panel*. Effect of IbTx on K⁺ currents elicited by graded depolarisations from a V_h of -70 mV (see the adjoined voltage protocol); *right panel*. Tail current amplitudes before and after treatment with IbTx were used to build the corresponding activation curves. A Boltzmann function fitted to tail currents in the presence of IbTx gave values for V₅₀ and K of -14.2 mV and 11.94 mV, respectively. Values were normalized with respect to the maximal current derived from curve fitting to data in the presence of IbTx. Data are means ± SEM of 8 and 7 cells for panel A and B, respectively.

Supplemental Figure 3. Time course of recovery from inactivation of BK and I_A currents

A, top panel. BK currents activated by two voltage depolarisations (V_1 and V_2), 600 ms-long to +100 mV from a V_h of 0 mV, were separated by variable intervals; *lower panel.* Peak BK currents in response to the first and second pulses were ratioed (I_2/I_1) and plotted against the interpulse interval. Experimental data were fitted by a double exponential function with two rate constants: $k_{fast} = 23 \text{ s}^{-1}$ and $k_{slow} = 0.77 \text{ s}^{-1}$; an expanded view of the faster recovery component is shown as an inset. *B, top panel.* I_A currents were elicited by two 600 ms-long voltage depolarisations to +20 mV from a V_h of -70 mV separated by variable intervals; *lower panel.* Peak I_A responses to the first and second pulses were ratioed (I_2/I_1) and plotted against the interpulse interval. The result of fitting a double exponential function with rate constants $k_{fast} = 27 \text{ s}^{-1}$ and a $k_{slow} = 1.84 \text{ s}^{-1}$, is shown; an expanded view of the faster recovery component is also shown as an inset. Data are means $\pm$ SEM of 7 cells for each type of current.

Supplemental Figure 4. Effect of IbTx on BK currents recorded in perforated patch induced by square pulses.

A. Typical experiments showing K^+ currents activated by 200 ms-long step depolarisations from a V_h of -70 mV to +10 mV in the presence (*left panel*) and the absence (*right panel*) of 2 mM Ca^{2+}_o and the effect of IbTx on both conditions (150 nM for 5 min; *right panel*). Red traces correspond to the subtraction of the control and IbTx currents. *B.* Summary of the effect of ChTx on peak current amplitudes ($n=14$ cells for each condition). Data are means $\pm$ SEM of 13 experiments for each condition. $*p < 0.001$.

Supplemental Figure 5. Relative occurrence of BK and Ca^{2+} currents induced by pre-recorded action potential wave forms (APWF) in the presence of 2 mM Ca^{2+}_o .

A, Example of Ca^{2+} currents induced by an APWF built up with voltage ramps in cells in the presence of 2 mM Ca^{2+}_o . B, top panel. Two examples of Ca^{2+} and K^+ currents (blue traces) induced by a fast pre-recorded APWF in cells held in perforated patch in the presence of 2 mM Ca^{2+}_o . The red traces correspond to the effect of IbTx (150 nM, 3 min). The green line is the result of subtracting the effect of IbTx from the control current. Note the prominent inward current (Ca^{2+} current) after the peak of BK current in these cells. B, Two examples of the effect of IbTx on currents induced by slower pre-recorded APWFs in the presence of 2 mM Ca^{2+}_o . Note the existence of 2 peaks of BK current: first a small one coinciding with the peak of the APWF and the second delayed peak occurring in the late repolarizing phase of the APWF. Grey arrow indicate $V_m=0$ mV

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