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Bradykinin inhibits a potassium M-like current in rat pheochromocytoma PC12 cells

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We studied the action of bradykinin (BK) on ionic currents in fused pheochromocytoma PC12 cells under voltage-clamp in whole-cell mode, and on intracellular calcium using fura-2. BK induced the development of an outward current associated with an increase in intracellular calcium, followed by inhibition of an M-like current. The outward current was blocked by (+)-tubocurarine, and prevented when the calcium chelator BAPTA or high concentrations of inositol 1,4,5-triphosphate were introduced into the cell, whereas the M-like current and its inhibition by BK remained unaffected. The protein kinase activator phorbol 12,13 dibutyrate partially reduced the M-current. M-current density did not substantially change after prolonged treatment with nerve growth factor.

M-current; Ca^{2+} activated potassium current; Ca^{2+} imaging; Protein kinase C; Bradykinin; Muscarine; (PC12 cell)

1. INTRODUCTION

Activation of bradykinin (BK) receptors in rat PC12 cells causes an increase in phosphatidylinositol (PI) turnover and a cytosolic calcium rise, which are associated with membrane hyperpolarization followed by depolarization [1]. Similar BK actions on membrane potential have been described in NG108-15 hybrid [2] and NIE-115 neuroblastoma cells [3]. In all these cell types, the hyperpolarization has been attributed to activation of a calcium-dependent potassium current. However, the subsequent depolarization has been ascribed to inhibition of the voltage-dependent potassium current I_M in NG108-15 [2] and NIE-115 cells [4], or activation of a probable non-selective cationic current in PC12 cells [1] and NIE-115 [3].

The M-current is a non-inactivating, voltage- and time-dependent potassium current first described in bullfrog sympathetic neurons [5]. Its inhibition by muscarinic agonists [5] has been demonstrated to be coupled via a pertussis toxin-insensitive G protein by several laboratories [6–8]. Roles for protein kinase C (PKC) and inositol 1,4,5-trisphosphate (IP_3) have been suggested in NG108-15 cells [2] and in rat hippocampal cells [9], respectively, although it has been argued that neither IP_3 [10,11], nor PKC [12] mediate I_M inhibition in frog sympathetic ganglion cells.

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2. MATERIALS AND METHODS

2.1 *Cell culture*

PC12 cells were maintained in DMEM supplemented with 10% horse serum (Hyclone), 5% fetal calf serum (Hazleton), 100 μg/ml streptomycin and 100 U/ml penicillin. At least one day prior to recording, cells were fused with 50% polyethylene glycol 1500 (BDH) as described [13], and treated with 50 ng/ml nerve growth factor (NGF) (Collaborative Research) to induce BK receptor expression [14].

2.2 *Electrophysiology*

Currents were recorded in whole-cell mode at room temperature (22–25°C) with 3–5 MΩ electrodes and voltage-clamped with an Axoclamp2 amplifier in discontinuous mode. Potentials were corrected for junction potential by subtracting 5 mV. Currents were recorded at a holding potential of −35 mV and in response to 1 s hyperpolarizing test pulses. The current was quantified by measuring the amplitude of the repolarizing

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outward current relaxation at −35 mV after a 1 s step to −55 mV. Capacitance was estimated by integrating the capacitative transient elicited by a 10 mV hyperpolarizing pulse from a holding potential of −65 mV. Results reported are average values ± standard deviation of the mean (n = number of experiments).

2.3 *Chemicals*

Potassium 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetate (BAPTA), fura-2 and fura-2-ester (fura-2-AM) were obtained from Molecular Probes. Bradykinin (BK), phorbol 12,13-dibutyrate (PDBu), 4-α-phorbol, 12,13-didecanoate and inositol 1,4,5-trisphosphate (IP_3) were supplied by Sigma.

2.4. *Solutions*

Cells were continuously perfused with a flow rate of 3 ml/min in a solution containing (in mM): 140 NACl, 1 $MgCl_2$, 3 KCl, 10 HEPES, 2 $CaCl_2$, 10 glucose, and 0.2% Phenol red; pH 7.5. The composition for the standard intracellular solution was (in mM): 150 K-aspartate, 1 $MgCl_2$, 5 Hepes, 0.5 EGTA, 1 Na_2ATP, 0.2 Na_3GTP, 3 NaCl, pH 7.2. The BAPTA-containing intracellular solution differed from the standard solution in that it had 125 mM K-aspartate, 10 mM K_4BAPTA and no EGTA. In later experiments, 0.2 mM Leu-peptin was included [6]. Drugs were bath or puff applied. For puff dispensation, 1 mM Fast green was added to monitor access of the applied solution to the cell.

2.5. *Calcium measurements*

Cells plated on laminin-polylysine-coated glass coverslips (no. 1) were incubated with fura-2-AM (5 μM) for 45 min at room temperature. The cells were then washed with extracellular solution at a flow rate of 10 ml/min for approximately 20 min. Phenol red was omitted from the extracellular solution. Fluorescent images were taken with excitation wavelengths alternating between 340 and 380 nm at a rate of 10 Hz for 2 s, and emission filtered with a 480-nm-long pass filter. After subtraction of background images, the brightest image was used to create a template. Images were then ratioed and the template superimposed to remove extraneous background noise. The final image, therefore, represented an average of 10 frames. Calculations of Ca^{2+} concentration were by means of a calibration curve using a K_D of 224 nM for calcium-fura-2 binding [15], which was constructed by loading dissociated bullfrog sympathetic neurons with known Ca-EGTA/EGTA mixtures to determine R_{min}, R_{max} and intermediate (340 nm)/(380 nm) fluorescence ratio values.

3. RESULTS AND DISCUSSION

3.1. *M-current in PC12 cells*

In the whole-cell configuration, PC12 cells showed a resting membrane potential of −62 ± 14 mV (n = 38). In cells voltage-clamped at a depolarized potential, a non-inactivating time- and voltage-dependent outward current was recorded in 93 out of 106 cells (fig.1). The inward relaxations elicited during 1 s voltage steps became faster with hyperpolarization (fig.1A), but contamination by

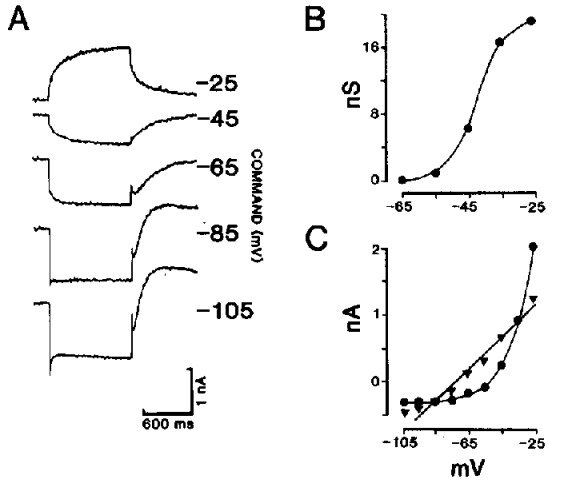

Fig.1. M-like current in PC12 cells. (A) Currents recorded during 1-s voltage jumps to the various potentials shown to the right of each trace. (B) Conductance measured on repolarization to −35 mV. (C) Plot of the instantaneous current against the command potential (▼); and of the current amplitude at the end of a 1-s pulse (●).

other currents precluded adequate estimation of kinetic constants. The conductance estimated from the repolarizing current tails after the test pulse showed a sigmoidal voltage dependence between −65 and −25 mV (fig.1B). The instantaneous I/V relationship was roughly linear, whereas the I/V constructed from the current measured at the end of the pulse showed a strong outward rectification, as a consequence of activation of this current (fig.1C). The reversal potential shifted from around −90 mV to around −40 mV when the external potassium concentration was changed from 3 mM to 27 mM, close to the expected Nernstian shift for a potassium electrode, indicating that potassium was a major charge carrier for this current. Bath application of barium (4 mM) produced a rapid reduction of the relaxation measured on repolarization (90 ± 7%, n = 5) along with a decrease in conductance, a reduction of the outward rectification and an inward shift of holding current. This current is analogous to the M-current first described in frog sympathetic neurons [5], but it differs in that the relaxations appeared to be slower and they were sensitive to the potassium channel blocker TEA. TEA reduced more than 60% of the relaxation on repolarization at concentrations above 2 mM, and more than 95% at 10 mM. Similar M-current sensitivity to TEA has been

found in guinea pig olfactory cortex [16], and rat hippocampal neurons [17].

On repolarization following large hyperpolarizing steps (>40 mV), a slow outward current that slowly inactivated, contaminated the M-current relaxation (fig.1A). This current presumably resulted from removal of inactivation of a potassium current termed I_Z by Hoshi and Aldrich [18].

I_M can be inhibited by agonists able to stimulate PI turnover [for example, 19]. BK has been shown to mobilize PI in PC12 cells [1,14]. We found that BK (tested in the range of 50 nM–1 μM) produced a transient outward current accompanied by an increase in conductance (fig.2A) which was associated with a rise in intracellular calcium (not shown). The outward current was blocked by 200 μM (+)-tubocuraine (not shown), and was prevented in all cells tested when the rapid calcium chelator BAPTA (10 mM) was included in the solution surrounding the electrode (fig.2B) manifesting the calcium dependency of this current. This was followed by an inward shift of the holding current with reduction in conductance, due to I_M inhibition (figs 2B and 3). I_M was reduced in response to BK in all the cells recorded with BAPTA-containing electrodes ($67 \pm 16\%$, $n = 7$), indicating that BK can inhibit I_M in an apparently calcium-independent manner.

The effect of muscarine on this current was studied. Muscarinic receptor activation in PC12 cells causes PI mobilization [20], but application of 10 μM muscarine reduced I_M in only 2 of 11 cells tested (3 not NGF treated, with no effect). In these cells an outward current preceded I_M inhibition, but not in cells with no M-current inhibition.

In NG108-15 cells transfected with different muscarinic receptor sybtypes, the receptors were able to induce mobilization of calcium-mediated M-current inhibition [21]. We simultaneously imaged cytosolic calcium in several fura-2-AM-loaded cells at different time points. The estimated resting intracellular calcium concentration was 41 ± 20 nM ($n = 59$), lower than approx. 100 nM which was reported by others [1,22]. The difference could be due to the methods used. In the other studies, the resting levels were measured in cell suspensions at 37°C, and it is possible that fura-2 leakage from the cells [22], or presence of dead cells with high calcium levels, or the difference in temperature, contributed to the higher estimated resting concentration.

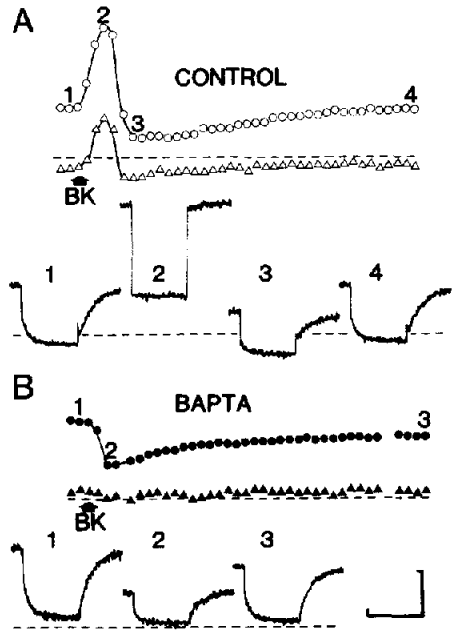

Fig.2. Effects of a 10-s puff application of 1 μM BK on membrane currents measured at −35 mV in control (○) and BAPTA-loaded cells (●), at the end of a 1-s pulse to −55 mV in control (△) and BAPTA-loaded cells (▲). Voltage pulses were delivered every 10 s. Broken lines indicate 0 current. (A) Control: top, slow time base; bottom, selected traces on expanded timebase. The increased instantaneous current on repolarization in trace 3 could be related to activation of a non-specific cationic current [1]. (B) 10 mM BAPTA included in the recording electrode. Same layout as in A. Calibration bars: vertical, 625 pA for A, 1000 pA for B; horizontal, 62 s for top traces, 600 ms for expanded traces.

The calcium levels augmented more than 2-fold in 3 out of 16 cells challenged with 10 μM muscarine, and in 15 cells bathed afterwards in 1 μM BK. The average increase in intracellular calcium was 3.8 ± 0.4-fold ($n = 3$) and 5.3 ± 1.8-fold ($n = 15$) in cells treated with muscarine or BK, respectively (table 1). These data illustrate the heterogeneity of the response to muscarine in these cells, and are compatible with the possibility that the infrequent inhibiton of I_M by muscarine was due to inadequate receptor density.

3.2. *NGF does not affect M-current density*

PC12 is a cell line derived from a rat pheochromocytoma that acquires a neuron-like

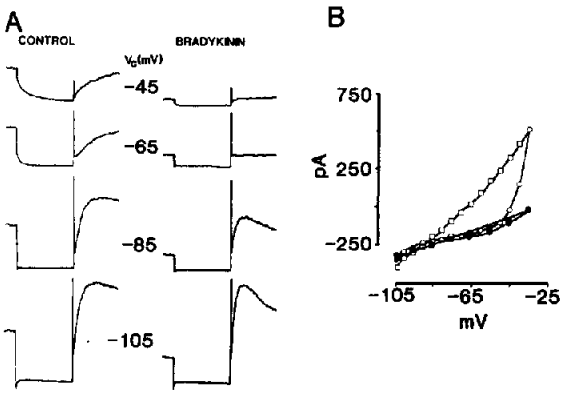

Fig.3. Effects of bath application of 100 nM BK on I_M recorded with a BAPTA-containing electrode. (A) Current traces in control (left) and during BK treatment (right). Command potentials are shown between traces. (B) Currents at the end of the pulse against command potential (○,●), and instantaneous currents (□,■). Open symbols, control before BK; filled symbols, during BK treatment.

phenotype after NGF treatment [23]. NGF increases both the density of several receptors [14,24], and the expression of type II sodium channels [25], but not of high-voltage-activated calcium currents [26]. To assess if NGF altered the M-current density on PC12 cells, we compared the current normalized for capacitance in control and in cells treated with NGF for more than 4 days. In order to compare cells of similar size, we included non-fused cells in the NGF-treated population. In both groups, the set of density values was normally distributed ($P \leq 0.001$, Kolmogorov-Smirnov test). The density of I_M in both treated and non-treated populations was comparable (control, 0.81 ± 0.34 pA/pF, $n = 16$; NGF-treated 0.93 ± 0.42 pA/pF, $n = 13$). The means (t-test) and variances (F-test) were equal for both groups at the 0.001 significance level, indicating that NGF did not induce an increase in M-current density. Furthermore, in 2 out of 4 control non-fused cells, a current similar to I_M that rapidly washed-out was observed. This suggests that the M-current recorded in the fused cells was not induced by the fusion, and that the bigger size of the fused and NGF-treated cells might have helped to replenish some factors diffusing out via the recording pipette.

3.3. *PKC is not involved in BK-induced* I_M *inhibition*

Based on the partial reduction of I_M produced by active phorbol esters known to activate PKC, and lack of action of inactive analogs, it has been proposed that PKC may participate in I_M inhibition [2]. However, it has been shown in *Rana pipiens* that inhibitors of PKC were able to prevent phorbol ester-induced I_M reduction, but not its inhibition by muscarine and other agonists [12].

Table 1

Changes in intracellular calcium levels at the times indicated, in response to a 60 s application of 10 μM muscarine and a subsequent (after 10 min washing) application of 1 μM BK, in cells that showed a larger than two-fold increase in intracellular calcium in a population of 16 cells

	Intracellular calcium concentration (nM)	
Time (s)	10 μM Muscarine ($n = 3$)	1 μM Bradykinin ($n = 15$)
Control (0)	21 ± 4	28 ± 4
40	75 ± 13	140 ± 38
90	42 ± 16	116 ± 38
130	32 ± 9	80 ± 41
300	24 ± 5	30 ± 7

We found that bath application of the active phorbol ester PDBu (1 μM) caused an average 36 ± 15% reduction of I_M, along with an inward shift in the holding current in 6 out of 8 cells. Compared to the action of BK, this effect was of smaller magnitude, developed more slowly, and did not reverse with washout (up to 15 min). Vehicle alone (0.01% ethanol) or the inactive 4-α-12, 13 phorboldidecanoate (1 μM) had little or no effect on I_M ($n = 5$). These effects were comparable to those reported in frog [10,12], rat superior cervical ganglia [7] and NG108-15 cells [2]. The differences between PDBu and BK actions, together with the findings in frog neurons employing PKC inhibitors [12] make it unlikely that PKC plays a primary role in BK-induced I_M inhibition.

In some PDBu-treated cells, other effects were seen, but not examined in detail. First, a net outward current slowly developed at both the holding and command potentials, along with an increase in conductance. Second, the time-dependent relaxations during hyperpolarizing pulses became dominated by slow inward ramps. The significance of these phenomena is presently unknown.

It has been reported that in rat hippocampal pyramidal cells, phorbol esters had no effect on M-current [9]. Instead, it has been proposed that IP_3

might mediate I_M inhibition in a calcium-independent manner, because there was a significant reduction of I_M in cells intracellularly recorded with IP_3-loaded electrodes [9]. Unlike rat hippocampal cells, in none of 5 PC12 cells did I_M significantly decline when recorded with IP_3-containing pipettes (100 μM) over more than 10 min. In all these cells, I_M was inhibited by 1 μM BK. In 3 cells, no outward current preceded I_M inhibition, whereas in all the control cells ($n = 16$) it did. In these cells the IP_3-mediated mechanism for calcium release could have desensitized, or the calcium stores could have been depleted [11,27]. Whatever the mechanism of action, this indicates that IP_3 reached the cell interior, suggesting that IP_3 plays no major role on I_M inhibition.

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