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Hypoxic Regulation of Ca^{2+} Signaling in Cultured Rat Astrocytes

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ABSTRACT Acute hypoxia modulates various cell processes, such as cell excitability, through the regulation of ion channel activity. Given the central role of Ca^{2+} signaling in the physiological functioning of astrocytes, we have investigated how acute hypoxia regulates such signaling, and compared results with those evoked by bradykinin (BK), an agonist whose ability to liberate Ca^{2+} from intracellular stores is well documented. In Ca^{2+}-free perfusate, BK evoked rises of $[Ca^{2+}]_i$ in all cells examined. Hypoxia produced smaller rises of $[Ca^{2+}]_i$ in most cells, but always suppressed subsequent rises of $[Ca^{2+}]_i$ induced by BK. Thapsigargin pre-treatment of cells prevented any rise of $[Ca^{2+}]_i$ evoked by either BK or hypoxia. Restoration of Ca^{2+} to the perfusate following a period of acute hypoxia always evoked capacitative Ca^{2+} entry. During mitochondrial inhibition (due to exposure to carbonyl cyanide p–trifluromethoxyphenyl hydrazone (FCCP) and oligomycin), rises in $[Ca^{2+}]_i$ (observed in Ca^{2+}-free perfusate) evoked by hypoxia or by BK, were significantly enhanced, and hypoxia always evoked responses. Our data indicate that hypoxia triggers Ca^{2+} release from endoplasmic reticulum stores, efficiently buffered by mitochondria. Such liberation of Ca^{2+} is sufficient to trigger capacitative Ca^{2+} entry. These findings indicate that the local O_2 level is a key determinant of astrocyte Ca^{2+} signaling, likely modulating Ca^{2+}-dependent astrocyte functions in the central nervous system.

Rapid fluctuations in local O_2 levels exert numerous, physiologically important effects, most of which are designed to reverse local O_2 deficits. For example, hypoxia excites chemoreceptors to enhance ventilation (Gonzalez et al., 1992), causes pulmonary vasoconstriction to improve ventilation-perfusion matching (Weir and Archer, 1995), and causes systemic vasodilation to enhance local blood flow (Franco-Obregon et al., 1995). Most, if not all, of these responses involve modulation of ion channel activity (Lopez-Barneo, 1994; Peers, 1997). For example, chemoreceptor K^+ channels are inhibited by hypoxia, causing cell depolarization (Peers, 1997; Peers and Kemp, 2001) and transmitter release, and in systemic blood vessels hypoxic inhibition of Ca^{2+} channels mediates vasodilation (Franco-Obregon et al., 1995). However, the effects of hypoxia on other cell functions, such as Ca^{2+} signaling, remain largely unexplored.

It is increasingly clear that astrocytes serve numerous important roles in brain function (Bezzi and Volterra, 2001; Bezzi et al., 2001; Ransom et al., 2003), modulating synaptic activity and matching local blood supply to neuronal activity (Zonta et al., 2003). Key to astrocytic signaling is the regulation of $[Ca^{2+}]_i$. Astrocytic activation is usually manifest as a rise of $[Ca^{2+}]_i$ due to release of Ca^{2+} from internal stores, as well as influx (Kang et al., 1998; Deitmer et al., 1998; Grosche et al., 1999; Bezzi et al., 2001). A rise of $[Ca^{2+}]_i$ in one astrocyte can initiate Ca^{2+} waves that propagate across significant distances via adjacent astrocytes (Giaume and Venance, 1998; Araque et al., 2001; Pasti et al., 2001). This intercellular signaling in the brain represents a form of astrocyte "excitability" that parallels and modulates neuronal synaptic communication and is of fundamental importance to central activity. Such

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activities include glutamate uptake and release and control of substrate requirements for neurones (see Ransom et al., 2003) and associated articles, for detailed reviews).

To date, most studies of astrocyte function have employed procedures conducted at ambient, room-equilibrated O_2 levels (~150 mmHg). These are significantly greater than those experienced by the central nervous system, where local pO_2 levels are 20–40 mmHg (Hoffman et al., 1999). We have recently shown that prolonged hypoxia dramatically alters Ca^{2+} signaling in primary cultures of cortical astrocytes (Smith et al., 2003a). This hypoxia causes mitochondrial Ca^{2+} loading and inhibition of Na^+/Ca^{2+} exchange, and in so doing alters Ca^{2+} signaling in response to the well-characterized agonist, bradykinin (BK) (Smith et al., 2003a). In the present study, we describe the effects of acute hypoxia on such signaling.

Preparation and culture of primary astrocyte cultures (>95% pure, as confirmed by positive immunostaining with an anti-glial fibrillary acidic protein [GFAP] antibody) was exactly as described previously (Smith et al., 2003a). Culture medium was exchanged every 3–4 days, and cells were grown for ≤14 days. All recordings were made from cells at days 5–12. To measure cytosolic $[Ca^{2+}]$, cells were incubated in 2 ml of control solution containing 4 μM Fura-2AM for 1 h at 21–24°C in the dark, as previously described (Smith et al., 2003a,b). The control solution was composed of: NaCl 135 mM, KCl 5 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 2.5 mM, Hepes 5 mM, and glucose 10 mM (pH 7.4; 300 mOsm, 21–24°C). Following incubation, fragments of coverslips were transferred into an 80-μl recording chamber on the stage of an inverted microscope, and cells were continuously perfused (1–2 ml min^{-1}). Solutions were made hypoxic by bubbling with N_2 for at least 30 min prior to perfusion. This produced no shift in pH. pO_2 was measured (at the cell) using a polarized carbon fiber electrode (Mojet et al., 1997) at 20–25 mmHg. Ca^{2+}-free perfusate contained 1 mM EGTA and no added Ca^{2+}. $[Ca^{2+}]_i$ was determined with an Openlab System (Image Processing & Vision Co. Ltd, Coventry, UK). Excitation was via a xenon arc lamp (75-W) and excitation wavelengths (340 nm and 380 nm) were selected by a monochromator (Till Photonics, Planegg, Germany). Emission was collected through the objective and a 510-nm filter (40-nm bandwidth). Digital images were sampled at 14-bit resolution by an intensified charge-coupled device camera (Hamamatsu Photonics, Hertfordshire, UK). Fura-2 was excited alternately at 340 and 380 nm; ratios of the resulting images were collected every 4s. Regions of interest (ROI) were used to restrict data collection to individual cells. Mitochondrial Ca^{2+} levels were examined in cells loaded with Rhod-2 (by incubation of cells with 1.5 μM Rhod-2AM for 1 h at 21–24°C in the dark, followed by a 1-h period of maintaining cells in control perfusate solution for further dye de-esterification). Rhod-2 was excited at 543 nm, and emission light was collected at 570-nm filter (40-nm bandwidth). All imaging was controlled by Improvision software (Image Processing & Vision Company Ltd, UK) on a Macintosh PowerPC. Drugs and agonists were applied to cells as indicated in the Results by switching the perfusion reservoir with a 6-way Hamilton tap. Data are presented as ratio signals, and integrated for quantification (Smith et al., 2003a). Results are expressed as means ± SEM, together with example traces, and statistical comparisons made using ANOVA.

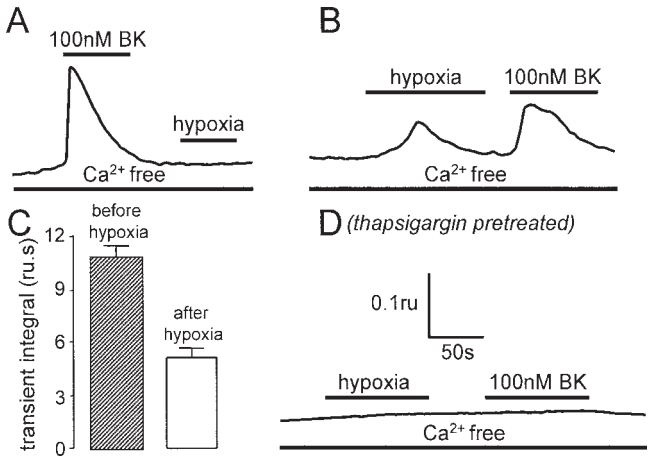

Fig. 1. **A:** Representative recording of $[Ca^{2+}]_i$ from a cortical astrocyte. Throughout the recording, Ca^{2+} was omitted from the perfusate and replaced with 1 mM EGTA. For the periods indicated by horizontal bars, the cell was exposed to 100 nM bradykinin (BK), or to hypoxia (pO_2 ~20–25 mmHg). **B:** Same as A, except that hypoxia was applied and removed before exposure to BK. **C:** Bar graph (with SEM bars) quantifying the integrated responses to BK either before (hatched bar; n = 24) or after (open bar; n = 54) exposure to hypoxia. **D:** Same as B, except that the cells were pretreated with 1 μM thapsigargin for 20 min prior to recording. ru, ratio units. Scale bars in D apply to A,B,D.

As previously shown (Smith et al., 2003a), application of BK (100 nM) to astrocytes in the absence of extracellular Ca^{2+} caused a rapid, transient rise of $[Ca^{2+}]_i$ due to release of Ca^{2+} from the endoplasmic reticulum (ER) (Fig. 1A). On washout of BK, exposure of astrocytes to a hypoxic solution (pO_2 ~20 mmHg) always (n = 26) failed to evoke detectable changes in $[Ca^{2+}]_i$ (Fig. 1A). By contrast, when astrocytes were exposed to hypoxia before application of BK, small, transient rises of $[Ca^{2+}]_i$ were commonly (84% of cells tested) observed (e.g., Fig. 1B). When O_2 was restored, BK evoked rises of $[Ca^{2+}]_i$, but these were significantly smaller than those seen in cells that were not previously exposed to hypoxia (Fig. 1C). This suggested that hypoxia depleted the same intracellular Ca^{2+} pool liberated by BK. In support of this idea, subsequent exposure to hypoxia or to BK always failed to elicit any changes of $[Ca^{2+}]_i$ in cells that were pretreated with thapsigargin (TG; 1 μM) for 15 min (Fig. 1D, representative of 25 recordings).

During perfusion with Ca^{2+}-free solution, depletion of intracellular stores (either with agonists such as BK, or the Ca^{2+} -ATPase inhibitors TG or cyclopiazonic acid) activates capacitative Ca^{2+} entry (CCE), seen as a rise of $[Ca^{2+}]_i$ when Ca^{2+} is readmitted to the perfusate

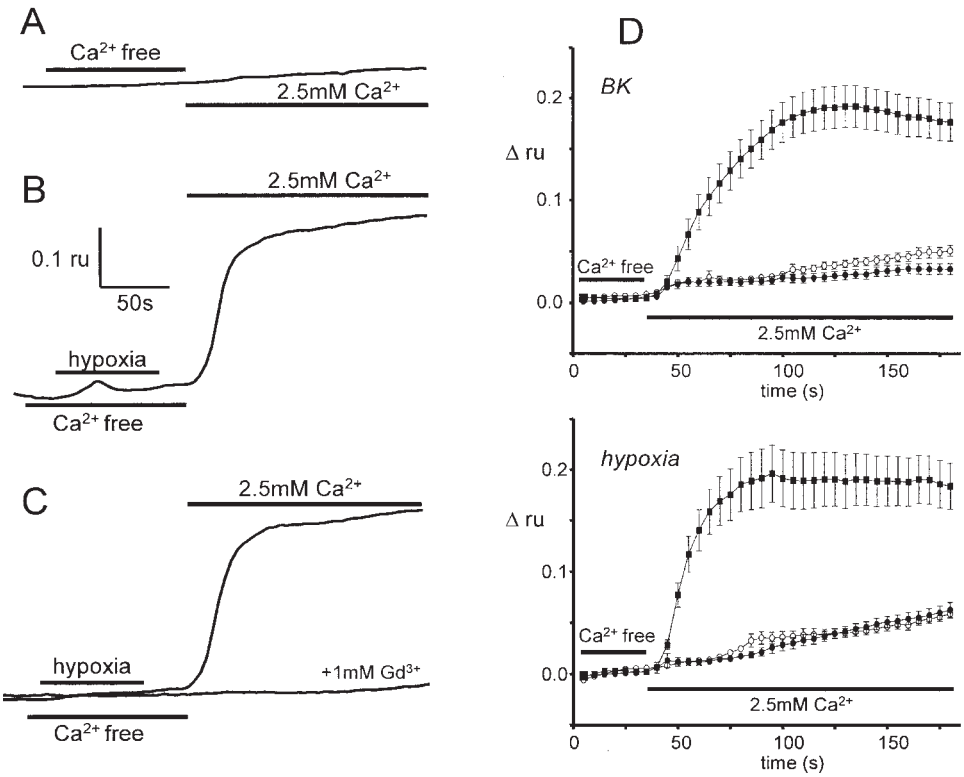

Fig. 2. **A:** Example recording of $[Ca^{2+}]_i$ during perfusion with Ca^{2+}-free solution, which was then switched to Ca^{2+} -containing (2.5 mM), as indicated by bars. **B:** Example recording illustrating a modest effect of hypoxia on $[Ca^{2+}]_i$ during perfusion with Ca^{2+}-free solution. At the point indicated by the bars, perfusate was switched to one containing 2.5 mM Ca^{2+}. Note the rise due to capacitative Ca^{2+} entry. **C:** Example of hypoxia apparently failing to raise $[Ca^{2+}]_i$ during perfusion with Ca^{2+}-free perfusate, yet still triggering subsequent capacitative Ca^{2+} entry when Ca^{2+} was restored to the perfusate, as indicated by horizontal bars. Also shown is the result of an identical experiment, except that 1 mM Gd^{3+} was included with the Ca^{2+}, completely preventing a rise of $[Ca^{2+}]_i$. **D:** Mean (with SEM bars) rises in $[Ca^{2+}]_i$ due to capacitative Ca^{2+} entry. Prior to the onset of the traces, cells were exposed to either 100 nM bradykinin (BK) (upper plot) or hypoxia (lower plot) in the absence of Ca^{2+}. For the periods indicated by the bars in each case, 2.5 mM Ca^{2+} was restored to the perfusate either alone (solid squares; n = 31 for BK, and 23 for hypoxia) or together with 1 mM Gd^{3+} (solid circles; n = 23 for BK, and 13 for hypoxia) or 1 mM La^{3+} (open circles; n = 26 for BK, and 22 for hypoxia). ru, ratio units. Scale bars apply to all traces in A–C.

(Smith et al., 2003b). If stores are not depleted, no such rise in $[Ca^{2+}]_i$ is observed (e.g., Fig. 2A, representative of 23 cells). When cells were exposed to hypoxia under Ca^{2+}-free conditions, subsequent restoration of normoxia and readmission of Ca^{2+} consistently triggered CCE, regardless of whether hypoxia evoked a clear rise of $[Ca^{2+}]_i$ (e.g., Fig. 2B), or even when no detectable rise of $[Ca^{2+}]_i$ was observed (e.g., Fig. 2C). That this observed rise of $[Ca^{2+}]_i$ was due to CCE was confirmed by the observation that Gd^{3+} (1 mM) completely blocked the rise (e.g., Fig. 2C). Mean rises of $[Ca^{2+}]_i$ due to CCE are shown in Figure 2D, following exposure (under Ca^{2+}-free conditions) to either BK (upper plot, solid squares) or hypoxia (lower plot, solid squares). Also shown are the inhibitory effects of Gd^{3+} (1 mM; solid circles) and La^{3+} (1 mM; open circles), two known blockers of CCE. Thus, hypoxia was able to cause sufficient release of Ca^{2+} from BK-sensitive intracellular stores to trigger capacitative Ca^{2+} entry in cortical astrocytes.

We have shown that mitochondria buffer the rises of cytosolic $[Ca^{2+}]$ evoked by BK, and that their inhibition enhances BK-evoked responses (Smith et al., 2003a). This is shown in Figure 3A, where BK-evoked rises of $[Ca^{2+}]_i$ are significantly enhanced in cells also exposed to FCCP (10 μM) and oligomycin (2.5 μg/ml). Responses to acute hypoxia were also dramatically enhanced (Fig. 3B). In the presence of FCCP and oligomycin, all cells responded to acute hypoxia, and these responses were significantly greater than those observed in the absence of mitochondrial inhibition (Fig. 3C). This strongly supports the supposition that hypoxia-evoked liberation of Ca^{2+} from the ER is normally markedly suppressed (as is the response to BK; see also Smith et al., 2003a) because of uptake into mitochondria. To support this statement, we found that Rhod-2 fluorescence increased during hypoxia (Fig. 3D, representative of 19 cells) a response indicative of an increase of intramitochondrial Ca^{2+} levels.

The major finding of this study is that acute hypoxia liberates Ca^{2+} from a BK-sensitive intracellular pool, presumably the ER. The degree of liberation was modest, and in some cases not detectable using the cytosolic Ca^{2+} indicator Fura-2 (Fig. 1). However, the ability of

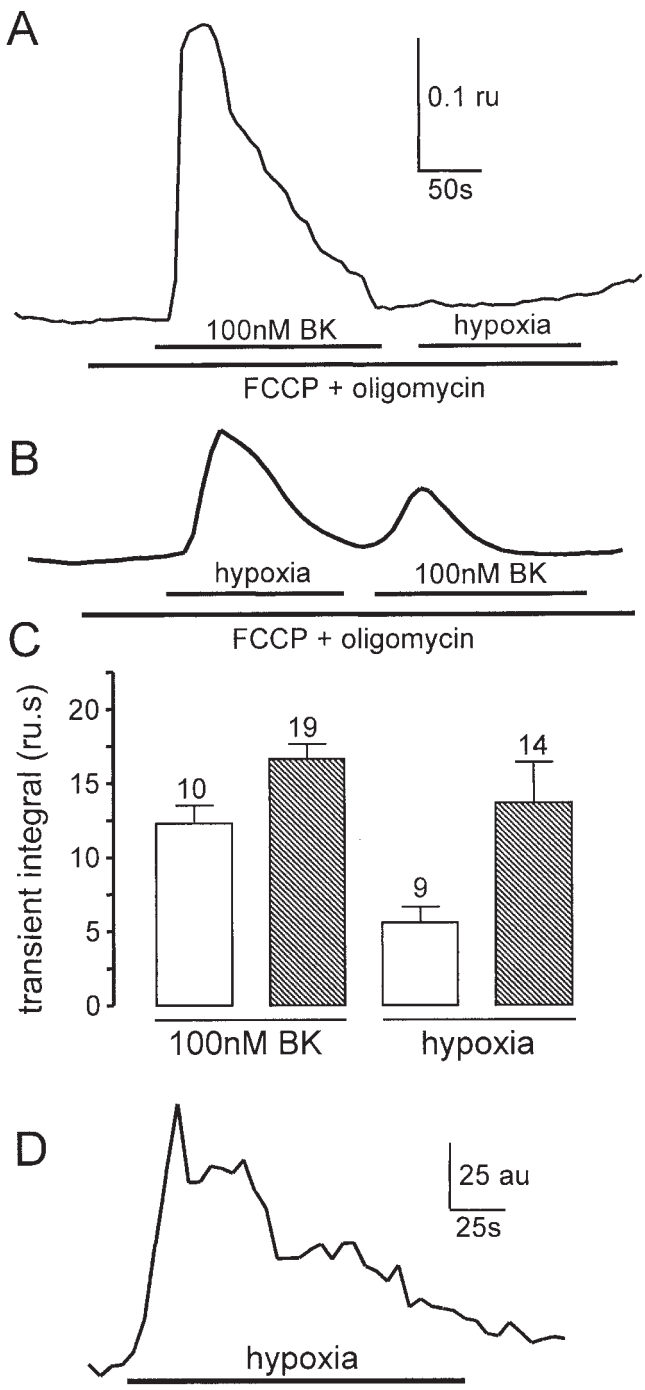

Fig. 3. **A:** Representative recording of $[Ca^{2+}]_i$ from a cortical astrocyte. Throughout the recording, Ca^{2+} was omitted from the perfusate and replaced with 1 mM EGTA. For the periods indicated by the horizontal bars, the cell was exposed to FCCP (10 μM) and oligomycin (2.5 μg/ml), either alone, or together with bradykinin (BK) (100 nM), or during exposure to hypoxia (pO_2 ~20–25 mmHg). **B:** Same as A, except that during mitochondrial inhibition, the cell was exposed to hypoxia before BK. **C:** Mean (with SEM bars) integrated responses evoked by BK and hypoxia in the absence (open bars) or presence (hatched bars) of mitochondrial inhibitors. Numbers above each bar indicate number of cells examined. **D:** Rhod-2 fluorescent signal, a rise of which indicates a rise of mitochondrial Ca^{2+} in response to acute hypoxia (applied for period indicated by horizontal bar). ru, ratio units; au, arbitrary unit. Scale bar in A applies to B.

hypoxia to mobilize Ca^{2+} from the ER is also evidenced by the fact that hypoxia consistently stimulated CCE when Ca^{2+} was restored to the perfusate (Fig. 2). Indeed, the magnitude of the CCE response was similar regardless of whether the stores were previously depleted with BK or hypoxia (Fig. 2D). The data in Figure 1 suggest strongly that hypoxia liberated Ca^{2+} from the same intracellular pool that was sensitive to BK, as BK-evoked responses were significantly smaller following hypoxia, suggesting partial pool depletion by hypoxia. Therefore, the question arises of why hypoxia-evoked rises of $[Ca^{2+}]_i$ were not consistently observed. Results presented in Figure 3 suggest that mitochondria are intimately involved in this process, acting as an efficient Ca^{2+} sink so that, in some instances, the movement of Ca^{2+} from the ER to the mitochondria must involve such a brief traverse of the cytosol as to be undetectable. This, in turn, suggests that tight spacial coupling between mitochondria and discrete regions of the ER are present, as described in various other cell types (Meldolesi, 2001; Rizzuto, 2001; Berridge, 2002; Ganitkevich, 2003). It is noteworthy that during mitochondrial inhibition, the magnitude of the responses to BK and hypoxia were similar (Fig. 3C). However, BK was always able to discharge additional Ca^{2+} following hypoxic exposure, whilst hypoxia produced no detectable rise of $[Ca^{2+}]_i$ following BK application. We cannot account for this observation at present, but it may imply either that BK is capable of releasing Ca^{2+} from an additional intracellular pool (which is not sensitive to hypoxia), or that hypoxia can only act as a partial agonist to release Ca^{2+} from the same pool.

The physiological implications of these findings are likely to be profound. On the one hand these effects of hypoxia may be considered as contributing to Ca^{2+} overload and consequent tissue damage seen following hypoxic or ischemic episodes in the central nervous system. Alternatively, and perhaps more plausibly, these findings may have unveiled a more physiological response of astrocytes: as mentioned in the introduction, brain pO_2 levels are normally much lower (20–40 mmHg) than arterial levels (90–100 mmHg). This implies our findings with hypoxia are actually reflecting what occurs normally i.e., the pO_2 levels of 20–40 mmHg represent normoxia for these and other central nervous system cells. This, in turn, implies that under physiological conditions, because of the local O_2 levels, intracellular Ca^{2+} stores are far more labile than previously thought, and Ca^{2+} flux between intracellular organelles may be much more dynamic. Finally, our findings suggest that current understanding of Ca^{2+} signaling in astrocytes (and perhaps in other cell types of the central nervous system) may require reassessment, given that most of these types of experiments are conducted with room-air equilibrated (or even O_2 supplemented) solutions, which would provide cells with a hyperoxic environment.

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