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Experimental Physiology – Hot Topic Review

Festschrift for R. G. O'Regan – Sensing and adaptation to alterations in respiratory gases:
oxygen and carbon dioxide

Interactions of chemostimuli at the single cell level: studies in a model system

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The responses of afferent chemosensory fibres of the carotid body to individual chemostimuli have long been established. However, the mechanisms underlying the multiplicative interactions of these stimuli (i.e. how the combined effects of hypoxia and hypercapnia exert a greater effect on afferent nerve discharge than the sum of their individual effects) have not been elucidated. Using the membrane hypothesis for carotid body chemoreception, in which chemostimuli inhibit type I cell K^+ channels, leading to depolarization, voltage-gated Ca^{2+} entry and hence the triggering of exocytosis, this article considers data acquired in isolated type I carotid body cells and model chemoreceptor (PC12) cells to attempt to explain stimulus interactions. Whilst stimulus interactions are not clearly evident at the level of K^+ channel inhibition or rises of $[Ca^{2+}]_i$, they are apparent at the level of transmitter release. Thus, it is clear that individual chemoreceptor cells can sense multiple stimuli, and that interactions of these stimuli can produce greater than additive effects in terms of transmitter release.

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Introduction

For decades the carotid body has been recognized as the major peripheral arterial chemoreceptor, sensing changes in blood gas and pH levels and responding, when appropriate, by altering the firing frequency of afferent chemoreceptors (Gonzalez *et al.* 1994; Gonzalez *et al.* 1992). In this way, the carotid body informs the central respiratory centres of arterial blood gas status, and allows the initiation of corrective cardiorespiratory reflexes (Marshall, 1994; Lopez-Barneo, 1996). Afferent chemosensory recordings, performed over many years, have established the closely controlled relationship between stimulus intensity and afferent nerve activity, and have shown that excitation of these nerves can be brought about by each physiological stimulus (hypoxia, hypercapnia and acidity) applied independently. More interestingly, perhaps, these stimuli can interact at the

level of afferent chemosensory discharge in a multiplicative manner (Fitzgerald & Parks, 1971; Lahiri & DeLaney, 1975). In other words, the effects of hypoxia and hypercapnia applied together are greater than the sum of these two stimuli when applied separately to the carotid body. This is illustrated in Fig. 1, which plots the frequency of discharge of afferent chemoreceptor fibres as a function of CO_2 levels, whilst the background O_2 levels are varied from hyperoxia to severe hypoxia. Clearly, as the O_2 levels decline, the relationship between nerve activity and CO_2 becomes increasingly steep. This is indicative of multiplicative stimulus interaction, as if these stimuli were simply additive, the slope of the relationship between nerve discharge and CO_2 would not be altered.

Carotid body stimulus interactions have intrigued physiologists since their discovery, yet a mechanistic explanation has proved elusive. However, our understanding of the events which occur in chemosensory type I cells within the carotid body has advanced markedly over the past 15 years. On the basis of these studies, and those performed in model chemosensory cells, this

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article is aimed at reviewing available evidence in order to examine a cellular basis for this phenomenon of stimulus interactions.

Chemoreception in single type I cells

Since the development of viable preparations of isolated type I cells, numerous groups have sought to provide an answer to the question of how type I cells sense and respond to chemostimuli. Several lines of evidence had indicated that chemosensing by the carotid body was totally dependent on the presence of type I cells (reviewed by Gonzalez *et al.* 1994), and that neurotransmitter release from these cells was an absolute requirement for excitation of afferent nerves of the carotid sinus nerve. The nature of the neurotransmitters involved has long been contentious, but the most compelling evidence published in recent times would indicate that hypoxia releases (amongst others) excitatory transmitters acetylcholine and ATP (Zhang *et al.* 2000), along with dopamine (Obeso *et al.* 1992). The role of dopamine in chemoreception remains to be fully established, but it is clear that stimulus evoked transmitter release from type I cells on to afferent nerve endings is a key step in carotid body excitation. This generally accepted mechanism begs the question of how stimuli cause type I cells to release neurotransmitters, a question that several groups have approached since the late 1980s. Whilst there remain some points of contention (which are outside the scope of this article), it seems reasonable to assume today that the general consensus of

opinion is that hypoxia, hypercapnia and acidosis evoke transmitter release according to what has become known as the membrane hypothesis for chemotransduction.

The key initial step of the membrane hypothesis for chemotransduction is the stimulus-induced inhibition of K^+ channels (Lopez-Barneo *et al.* 1988; Peers, 1990; Stea & Nurse, 1991; Buckler, 1997). These channels normally maintain a hyperpolarizing influence on resting membrane potential and thereby control cell excitability. Their inhibition therefore leads to cell depolarization. This in itself is sufficient to open voltage-gated Ca^{2+} channels, and the consequent rise of $[Ca^{2+}]_i$ (Buckler & Vaughan-Jones, 1994) is the trigger for transmitter release. Whilst this mechanism is not the only one to have been proposed to account for stimulus–secretion coupling in type I cells (Biscoe & Duchon, 1990; Rocher *et al.* 1991), it is clearly the best supported, and it is with this sequential series of events we can address possible mechanisms to account for stimulus interactions within the carotid body.

Effects of hypoxia and acidosis on K^+ channel activity

Whilst a number of groups have reported that hypoxia and hypercapnia/acidosis can inhibit K^+ channels and cause depolarization of type I cells, the question of whether an individual type I cell can respond to both stimuli remained unanswered until 1997. In that year, Pepper & Kumar (1997) reported that K^+ currents (most likely high conductance, Ca^{2+} sensitive (maxiK) currents) in individual rat type I cells could be inhibited both by hypoxia and by intracellular acidosis (in this case evoked by extracellular application of sodium propionate). This was the first report that a type I cell could respond to both stimuli. However, coapplication of both stimuli failed to cause channel inhibition that was quantitatively greater than either stimulus alone. Thus, stimulus interaction did not occur at the level of K^+ channel activity within type I cells. To date, this remains the only study aimed directly at addressing this issue. It will be of interest to examine whether TASK channels, also present in these cells (Buckler *et al.* 2000), or the O_2 sensitive, inactivating voltage-gated K^+ channels of rabbit type I cells (Lopez-Lopez & Peers, 1997), respond in a similar manner to those reported by Pepper and Kumar.

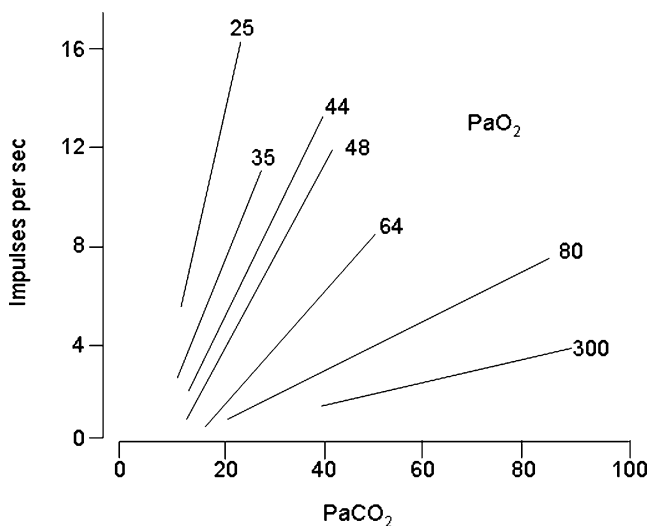


Figure 1. Plot of the relationship between afferent chemosensory nerve discharge and CO_2 levels, recorded from the cat carotid body sinus nerve preparation. CO_2 levels were varied at different background P_{O_2} levels, as indicated. Note the increased slope of the relationship as background O_2 levels decline. Reproduced with permission from Lahiri & DeLaney, 1975.

Effects of hypoxia and acidosis on $[Ca^{2+}]_i$ in type I cells

Following K^+ channel inhibition caused by hypoxia or hypercapnia/acidosis in type I cells, a rise of $[Ca^{2+}]_i$ is observed due to Ca^{2+} entry via voltage-gated Ca^{2+} channels. The question of whether rises of $[Ca^{2+}]_i$ display

any form of interaction was addressed in detail by Dasso *et al.* (2000). Under carefully controlled conditions, these workers examined the rises of $[Ca^{2+}]_i$ in response to graded hypoxia/anoxia with CO_2 levels maintained either at the normal, eucapnic level of 5% or when raised to hypercapnic levels of 10% or 20%. The key observations are summarized in Fig. 2. Whether or not one can conclude from these data that stimulus interactions occur at the level of $[Ca^{2+}]_i$ is not clear. Using 10% CO_2 as the additional stimulus (Fig. 2A), rises of $[Ca^{2+}]_i$ seen in response to hypoxia are generally elevated, but not in a manner that could be described as multiplicative. Indeed, some of the additional rises of $[Ca^{2+}]_i$ seen when hypoxia is applied together with hypercapnia are only slightly greater than those observed in response to hypoxia alone. Using a stronger CO_2 stimulus of 20% (Fig. 2B), multiplicative interactions are detectable at some, but not all, levels of

hypoxia. These findings, whilst providing some evidence for multiplicative stimulus interactions, are not in full agreement with earlier studies of afferent chemoreceptor discharge (Fig. 1), which indicate that stimulus interaction occurs over a wide range of O_2 and CO_2 levels.

Effects of chemostimuli on catecholamine secretion

Direct measurements of stimulus-evoked transmitter release from individual type I cells are still in their infancy, and to date have been confined to the study of catecholamines released from type I cells (Hatton & Peers, 1997; Carpenter *et al.* 2000; Pardal *et al.* 2000). This is in large part due to the technical demands of monitoring release of various transmitters from isolated cells. Fortunately, catecholamines are amenable to such studies since they are easily oxidized and so can be monitored amperometrically (Chow & Von Ruden, 1995). The technique of amperometry allows placement of polarized carbon fibre microelectrodes close to individual cells, so that any released catecholamine diffusing to the electrode tip is instantly oxidized. This allows detection of exocytosis as a real-time electrical event. Amperometry has to date only been used to study catecholamine release from type I cells in a limited number of reports (see above), and the question of whether stimulus interaction occurs at the level of transmitter release from type I cells has yet to be addressed. However, progress has been made using the PC12 cell line, pioneered by Millhorn and colleagues as a model system for studying chemoreception at the single cell level (Zhu *et al.* 1996; Conforti & Millhorn, 1997).

PC12 cells are a well-established cell line having proved useful as a model system for studying a wide variety of cellular functions. They are derived from rat adrenal chromaffin tissue and synthesize, store and release catecholamines. Zhu *et al.* (1996) first demonstrated that these cells could be used to study chemoreception by demonstrating that they possess O_2 sensitive K^+ channels, and that hypoxia caused cell depolarization and a subsequent rise of $[Ca^{2+}]_i$. We used these cells to show that hypoxia could evoke quantal catecholamine release in a graded manner (Fig. 3, adapted from Taylor & Peers, 1998). The relationship between P_{O_2} and catecholamine release was qualitatively reminiscent of the relationship between P_{O_2} and rises of $[Ca^{2+}]_i$ (Fig. 2) and afferent chemosensory discharge. Subsequent studies established that hypoxia-evoked catecholamine secretion was entirely dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels (Taylor & Peers, 1998). In addition, we demonstrated that acidosis (caused either by simple reduction of extracellular pH, or by addition of weak acids to cause intracellular acidosis) was an effective secretagogue, and that acid-evoked

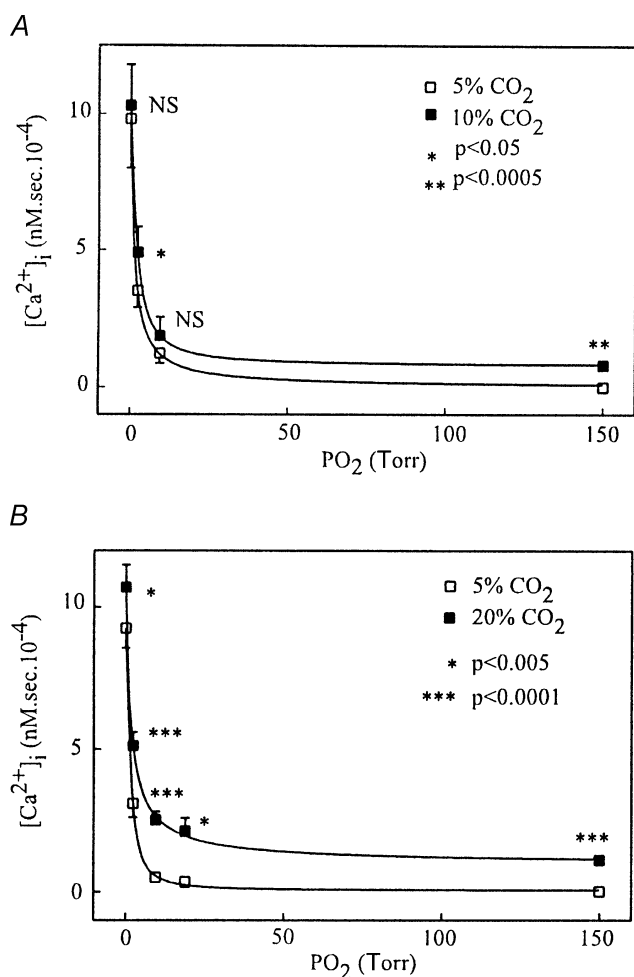


Figure 2. Plot of the mean relationship between $[Ca^{2+}]_i$ (measured fluorimetrically from type I cells using the $[Ca^{2+}]_i$ indicator Indo-1) and P_{O_2} under eucapnic conditions, and when CO_2 was raised to either 10% (A) or 20% (B). Taken from (Dasso *et al.* 2000) with permission.

transmitter release was also entirely dependent on voltage-gated Ca^{2+} entry (Taylor *et al.* 1999). Crucially, we also found evidence that stimulus interactions occurred at the level of catecholamine release (Fig. 4). Thus hypoxia was only a weak secretagogue when the background pH was alkaline, but when pH was reduced, hypoxia was far more effective. Analysis of exocytotic frequency as a function of P_{O_2} with varying pH levels indicated that pH affected the slope of the relationship between P_{O_2} and catecholamine secretion (Taylor *et al.* 1999). Thus, evidence of multiplicative stimulus interactions was found at the single cell level.

Since no compelling evidence for stimulus interactions was found at the level of K^+ channel inhibition or at the level of rises of $[\text{Ca}^{2+}]_i$, the question arises of how such interactions were apparent at the level of transmitter release. The answer may lie in two key observations. First, while interactions were not apparent at the level of $[\text{Ca}^{2+}]_i$

rises (Fig. 2), there clearly were larger rises of $[\text{Ca}^{2+}]_i$ in response to hypoxia when CO_2 levels were raised (Dasso *et al.* 2000). These larger rises were generally modest, but the fact that they were seen at all is crucial. The second observation is not taken from studies of the carotid body, but instead comes from much earlier work in which the release of acetylcholine as a function of extracellular $[\text{Ca}^{2+}]$ was examined in motor nerve terminals, using the classic frog neuromuscular junction preparation (Dodge & Rahamimoff, 1967). In these studies, it was established that the relationship between $[\text{Ca}^{2+}]$ and acetylcholine release (measured as an end-plate potential) was not linear, but was in fact a power relationship (in this case, acetylcholine release was proportional to $[\text{Ca}^{2+}]^{3.9}$). This means that only a small additional rise of $[\text{Ca}^{2+}]_i$ is required to observe a substantial increase in transmitter release. Such small additional rises were reported in type I cells when CO_2 was raised during hypoxia (Dasso *et al.* 2000), and are likely

Figure 3.

Hypoxia-evoked catecholamine secretion from a single PC12 cell. Left, upper trace shows P_{O_2} levels measured in the same position of a perfusion chamber as where a PC12 cell was placed for study. Lower trace shows amperometric recording from a single cell. Note the appearance of spike-like events as the P_{O_2} declines. Each spike corresponds to the oxidation of the released contents of a single vesicle of catecholamine. Right, plot of the relationship between mean spike frequency and P_{O_2} , determined from experiments such as those shown on the left. Taken from (Taylor & Peers, 1998) with permission.

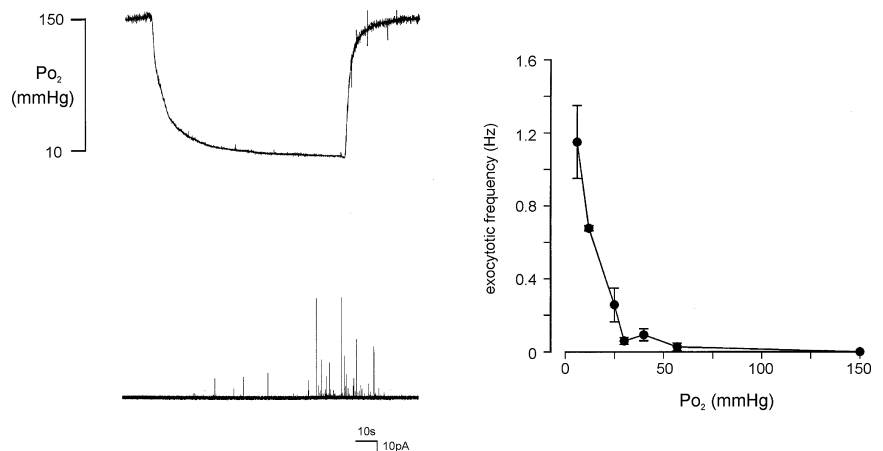
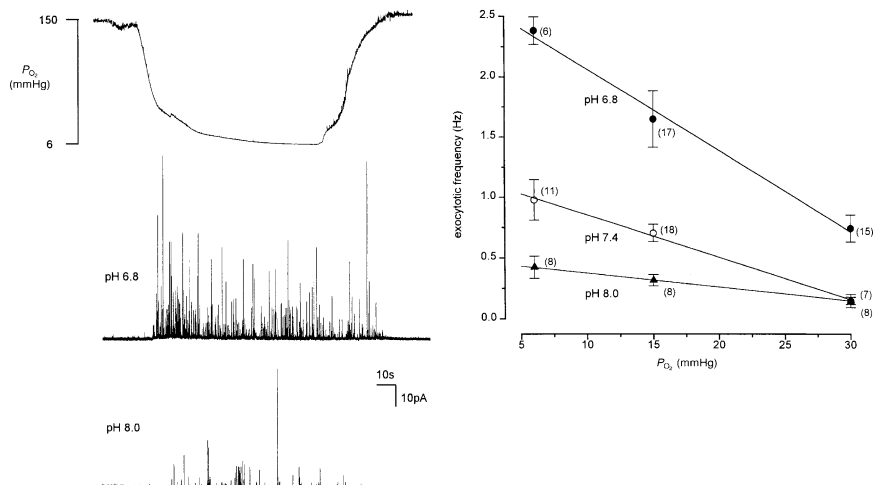


Figure 4.

Left, upper trace indicates P_{O_2} levels as in Figure 3. Below are shown two amperometric traces from individual PC12 cells in response to hypoxia either under acidic (middle trace) or alkaline (lower trace) conditions, as indicated. Right, plot of the relationship between mean spike frequency and P_{O_2} at three different pH levels. Note the increased slope of the relationship as background pH levels become more acidic. Taken from (Taylor *et al.* 1999) with permission.



to account for the large rises in catecholamine exocytosis observed in PC12 cells.

Clearly, much further work is required before these ideas can be established as accounting for multiplicative interactions of chemostimuli in the carotid body. Primarily, the experiments reported in the model chemoreceptor PC12 cell need to be repeated in type I cells, and additional mechanisms within the carotid body (such as, for example, dysinhibition of retrograde tonic nitric oxide or carbon monoxide release (Prabhakar, 1999)). However, the long-standing possibility that the relationship between $[Ca^{2+}]_i$ and transmitter release is supralinear in the carotid body type I cell, as it is in other synaptic preparations, is worthy of further consideration.

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