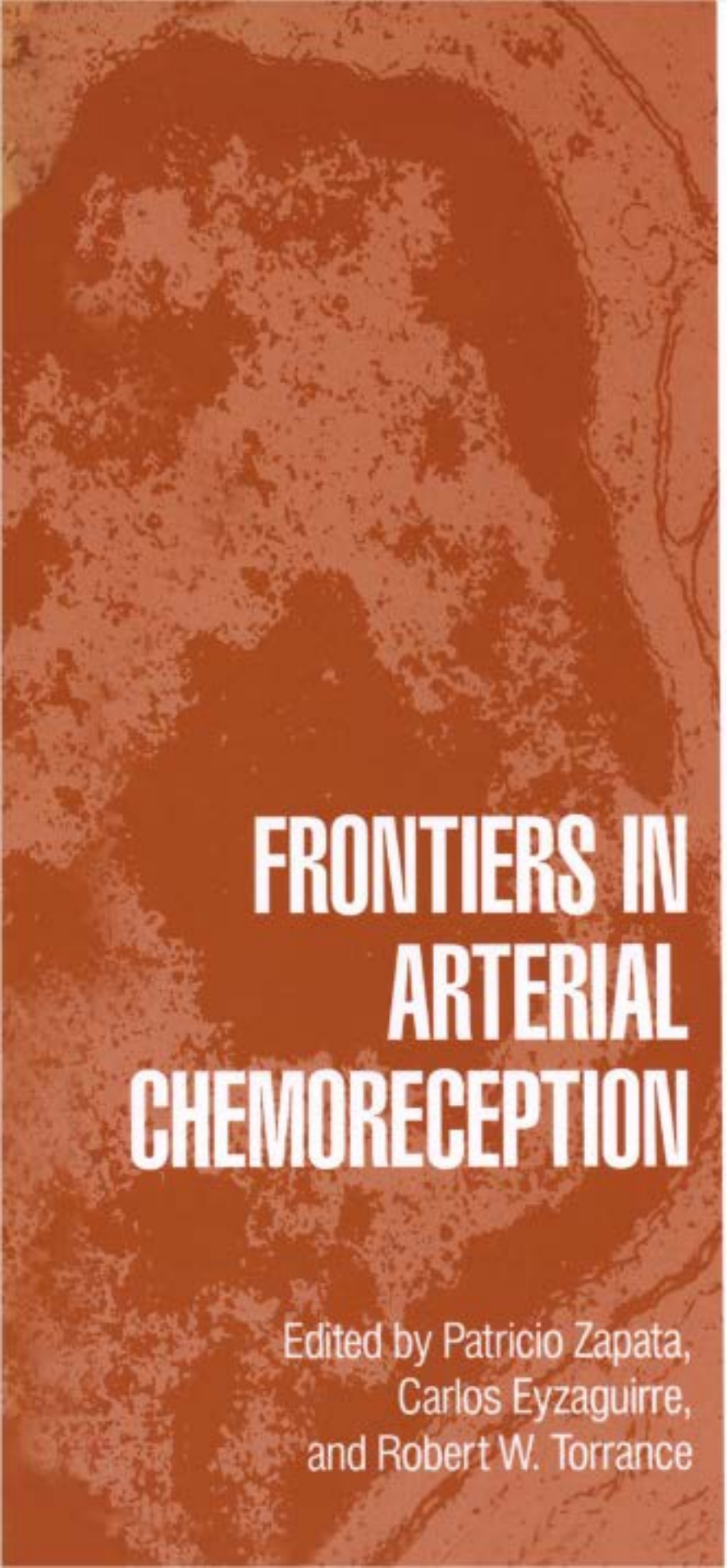

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INTRACELLULAR Ca^{2+} DEPOSITS AND CATECHOLAMINE SECRETION BY CHEMORECEPTOR CELLS OF THE RABBIT CAROTID BODY

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1. INTRODUCTION

The pivotal role of intracellular free [Ca^{2+}] fluctuations in the control of cellular functions such as contraction and secretion, including the release of neurotransmitters, was recognized many decades ago (see Rubin, 1982). More recently, the list of cellular functions triggered or modulated by the levels of Ca^{2+}_i has grown enormously. Additional functions regulated by $[Ca^{2+}]_i$ include neuronal excitability, synaptic plasticity, gene expression, cellular metabolism, cell division and differentiation, and programmed cell dead (Miller, 1991; Clapham, 1995). Paralleling the growth in this list of Ca^{2+}-controlled functions, a multiplicity of cellular mechanisms aimed at maintaining resting free $[Ca^{2+}]_i$ in the range of 100 nM for most cells has been described, allowing increases in Ca^{2+}_i levels that are specific in their magnitude, time course and spatial distribution, according to the cell function activated (Toescu, 1995).

Since Ca^{2+} cannot be metabolized, cells regulate their cytoplasmic levels of free Ca^{2+} through numerous binding proteins and influx and efflux mechanisms (Fig 1). Ca^{2+} influx to cell cytoplasm from the extracellular *milieu* occurs via voltage or receptor operated channels or via yet ill-defined capacitative pathways; the Na^+/Ca^{2+} exchanger can also produce in some circumstances net influx of Ca^{2+} (Miller, 1991; Clapham, 1995). Ca^{2+} efflux to the extracellular space occurs against electrochemical gradients, and thereby the pumping out of Ca^{2+} is directly (Ca^{2+} pump) or indirectly (Na^+/Ca^{2+}) coupled to the hydrolysis of ATP.

In addition to the extracellular space, there are intracellular stores, represented primarily by the smooth endoplasmic reticulum, capable of accumulating Ca^{2+} at very high concentrations (mM range) via specific Ca^{2+} ATPases (Fig 1). The smooth endoplasmic

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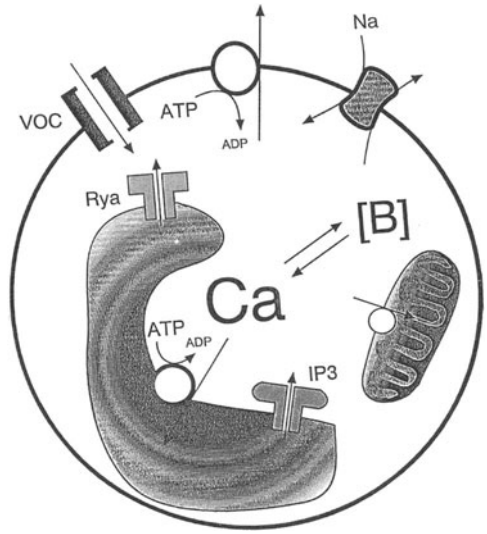

Figure 1. Model for the regulation of cellular calcium homeostasis. See text for an explanation.

reticulum of many cells may possess two specific mechanisms capable of mobilizing the accumulated Ca^{2+} in response to adequate signals, the inositol 1,4,5-triphosphate receptors (IP_3R) that on IP_3 binding are ensambled to produce a Ca^{2+} channel, and the ryanodine receptor-channel complex (RyR) equally permeant to Ca^{2+} (Pozzan et al, 1994; Simpson et al, 1995). Many extracellular signals, hormones and neurotransmitters, possess receptors in the plasma cell membrane that activate phospholipase C leading to the genesis of IP_3 in the cell interior and to the release of Ca^{2+} from the endoplasmic reticulum via the IP_3R (Clapham, 1995); the RyR are activated by local increases in free Ca^{2+}_i produced by Ca^{2+} entering via plasma cell membrane (López-López et al, 1995), and represent the substrate for the long time known process of Ca^{2+}-induced Ca^{2+} release (Simpson et al, 1995). Therefore, the endoplasmic reticulum may function as a sink or as a source for free cytoplasmic Ca^{2+} in different functional states of the cells, and additionally the sink or source function of the endoplasmic reticulum might vary from cell to cell, depending on the degree of expression of the repertoire of the molecules involved in the endoplasmic reticulum handling of Ca^{2+} (Simpson et al, 1995). At cytoplasmic levels of free Ca^{2+} higher than 500 nM (Clapham, 1995), *i.e.*, during cell activation, mitochondria may accumulate Ca^{2+}, thereby functioning as effective sinks to reduce cytoplasmic free Ca^{2+} concentration. Finally, many cytosolic constituents including proteins, and small organic molecules can bind Ca^{2+} ions contributing to modulate local levels of cytoplasmic free Ca^{2+} (Clapham, 1995; Toescu, 1995). The proteins with the capacity to bind Ca^{2+} may in turn acquire catalytic activities when their binding sites are occupied by the regulating ion.

In the carotid body (CB) chemoreceptor cells, our knowledge on the role of Ca^{2+} in cell signalling is rather restricted (see Gonzalez et al, 1994). It was only in 1968 that Eyzaguirre and Zapata showed the Ca^{2+} dependency of carotid sinus nerve (CSN) discharges elicited by hypoxia, acidity or flow interruption. In 1975, again Eyzaguirre's group (Eyzaguirre et al, 1975) reported that acetylcholine was incapable of generating ac-

tion potentials in the CSN in Ca^{2+}-free solutions. Some years later, a few ultrastructural studies showed the appearance of omega exocytotic profiles and/or coated pits and vesicles in chemoreceptor cells of rat CBs incubated in Ca^{2+}-containing (but not in Ca^{2+}-free) rich K^+_e solutions or in the presence of the Ca^{2+} ionophore A23187, concluding that exocytosis was Ca^{2+}_e-dependent (Gronblad et al, 1980). Consistent with that, it was later shown that the release of dopamine (DA) elicited by hypoxia and high K^+_e was Ca^{2+}_e-dependent in >95% (Fidone et al, 1982; Almaraz et al, 1986; Obeso et al, 1992). More recently, three different laboratories (González et al, 1993; López-Barneo et al, 1993; Buckler & Vaughan-Jones, 1994) have used fluorescent dyes to measure $[Ca^{2+}]_i$ in isolated chemoreceptor cells, and have consistently found that more than 95% of the $[Ca^{2+}]_i$ rise produced by hypoxia is due to Ca^{2+} entering from the extracellular space. In the case of acidic stimulation the Ca^{2+}_e dependence of the release of DA was approximately 80% (Obeso et al, 1992) and the Ca^{2+}_i signal was reduced by a similar percentage in Ca^{2+}-free media (Buckler & Vaughan-Jones, 1993). At variance with those findings, Biscoe and Duchen (1990) reported that up to 40% of the hypoxic Ca^{2+}_i rise was due to Ca^{2+} entering the cytoplasm from intracellular stores, and Biscoe et al (1989) could not detect a rise in free Ca^{2+}_i during acidic stimulation. In addition, Lahiri et al (1995) have recently communicated that the anoxic CSN discharge was better preserved in Ca^{2+}-free media containing thapsigargin than in its absence, concluding that Ca^{2+} from intracellular deposits seems to contribute to the anoxic chemoreception.

2. METHODS

Using an *in vitro* preparation of the rabbit CB whose catecholamine deposits have been labeled by prior incubation of the organs with their natural precursor ^{3}H-tyrosine (Fidone & Gonzalez, 1982), we have studied the significance of intracellular Ca^{2+} stores as possible sources of Ca^{2+} for the secretory response, and as possible buffers (sinks) for the Ca^{2+} entering the cell cytoplasm during stimulation. The tools used in the experiments include: ATP and bradykinin as possible generators of IP_3, and thereby as possible activators of the IP_3R; ryanodine at submicromolar concentration and caffeine as activators of the RyR; thapsigargin as a blocker of the reticulum ATPase; ruthenium red as a blocker of the mitochondrial Ca^{2+} uniporter; and, ionomycin as a Ca^{2+} ionophore that allows rapid equilibration of Ca^{2+} throughout cellular membranes. The experiments have been performed in Ca^{2+}-containing and in Ca^{2+}-free solutions. The effects of the drugs have been tested on their ability to trigger a secretory response in basal normoxic conditions, to test for a role of intracellular Ca^{2+} stores as sources of Ca^{2+} for the secretory response, and on their ability to modify the intensity and time course of the secretory response elicited by hypoxia and high K^+_e. In this way, we have tested for the capacity of the intracellular stores to act as sources of Ca^{2+}, or as sinks of the Ca^{2+} entering from the extracellular space.

It should be noted that most of the experiments have been performed sampling the incubating solutions for their analyses in ^{3}H-catecholamine (^{3}H-CA) content every 2 min, thereby providing a high sensitivity of the overall procedures capable of detecting <6 fmol of labelled CA (<200 cpm), which after correction for the specific activity of ^{3}H-CA in the cells, represents approximately 120 fmol of total CA release, or an amount equivalent to the basal release/2 min in normoxic conditions. During stimulation (hypoxia, high K^+_e) the release increases markedly, and consequently the sensitivity of the radioisotopic method to detect the effect of a drug on the stimulus induced release decreases.

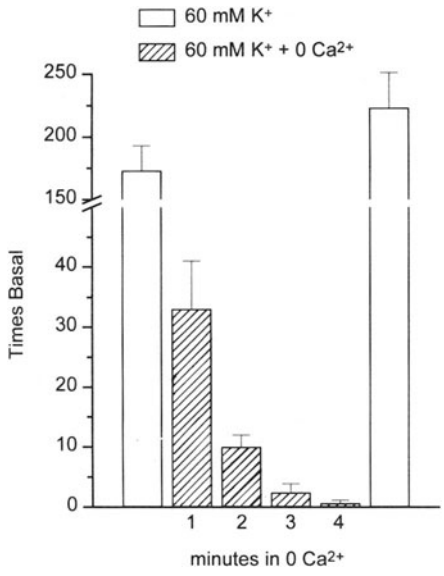

Figure 2. Time course of the disappearance of high external K^+ induced release of ^{3}H-catecholamines upon removal of calcium from the extracellular space. Empty bars: release response in Ca^{2+}-containing solutions at the beginning and the end of the experiments. Slashed bars: release response after incubation in Ca^{2+}-free solutions for the indicated periods. Release response (ordinate) is expressed as times basal release in normal K^+ solutions.

3. RESULTS AND DISCUSSION

Figure 2 shows the time course of extracellular Ca^{2+} washing out. In designing these experiments, the advantage that the release induced by high external K^+ is almost totally dependent on Ca^{2+} entering from the extracellular space was taken. Therefore, the minimum time of incubation in Ca^{2+} free medium required to abolish high K^+_e induced release would be equivalent to the time required to wash out Ca^{2+} ions from the incubating vial and the CB extracellular space. As shown in Figure 2, one min of incubation in 0 Ca^{2+} reduced markedly the release response, after 4 min in 0 Ca^{2+} the release was practically abolished, and on reintroduction of Ca^{2+} the release response recovered completely. In a similar experiment using hypoxic stimulation (2% O_2-equilibrated solutions, 2 min), the time course of inhibition was comparable; in fact, after 4 min in 0 Ca^{2+} the release induced by hypoxia was completely abolished. These experiments confirm previously reported findings with longer times of incubation in Ca^{2+} free solutions (*e.g.* Obeso et al, 1992), and suggest that intracellular Ca^{2+} stores do not play a significant role in mediating the secretory response to hypoxia, unless chemoreceptor cells have an unusually fast-emptying intracellular Ca^{2+} deposits. On the other hand, the present findings are consistent with observations of Fidone (see Fidone et al, 1977) and Torrance (1977) showing that the half-time for bicarbonate and sucrose wash out, respectively, was close to half a minute.

Next we performed a series of experiments aimed at mobilizing intracellular Ca^{2+} stores. In a first group of experiments we tested for the ability of ATP to mobilize Ca^{2+} stores. In a recent study, Spergel & Lahiri (1993) showed that ATP and analogs, but not adenosine, were able to increase CSN discharges in a dose-dependent manner, concluding that the CB expresses surface P_2 type ATP receptors. Since P_2 receptors are coupled to β type phospholipase C, it should be expected that their activation will lead to the formation of IP_3 and activation of IP_3R. ATP at 100 and 1500 μM did not affect the basal normoxic release of DA either in Ca^{2+} containing or in Ca^{2+} free solutions suggesting that the ATP receptors responsible for the activation of CSN discharges are located in a structure different from chemoreceptor cells. Bradykinin, a potent mobilizer of Ca^{2+} from intracellular

deposits and potent secretagogue in chromaffin cells (Augustine & Neher, 1992), was also without effect on the basal release of ^{3}H-CA from the CB, suggesting that chemoreceptor cells do not possess bradykinin receptors or that the endoplasmic reticulum Ca^{2+} stores are really small. The same was true for the rest of the agents tested including ionomycin in Ca^{2+}-free solutions.

In conclusion, the participation of endoplasmic reticulum and mitochondria as sources of Ca^{2+} capable of altering the secretory response of chemoreceptor cells appear to be of secondary importance, implying that chemoreceptor cells must rely almost exclusively on plasma membrane mechanisms to maintain their Ca^{2+} homeostasis. Although the precise identity of the Ca^{2+} stores sites is not fully clear, it appears that the smooth endoplasmic reticulum (SER) may be the principal place where the intracellular Ca^{2+} ATPase is located and thereby the SER could be the main place where Ca^{2+} is stored (Pozzan et al, 1994). If this is the case in chemoreceptor cells our findings would correlate with the apparently rear SER profiles present in the cells that appear to be located mainly in the processes of chemoreceptor cells (Verna, 1975; McDonald, 1981). It should be mentioned that papers consistent with our conclusion have been presented in this Symposium. Thus, He et al (1996) communicated that endothelin I increases up to five times the normoxic basal levels of IP_3, and therefore it should be expected that IP_3R of the endoplasmic reticulum represents the target of the IP_3 formed. Although the cellular element in which the increase of IP_3 had occurred was not identified, it was shown that endothelin I did not affect the basal release of CA or the Ca^{2+}_i levels in chemoreceptor cells, but still endothelin I was effective in potentiating the release of DA and the Ca^{2+}_i rise produced by hypoxia. Those findings imply that chemoreceptor cells possess endothelin receptors, and therefore it could be expected that IP_3 has increased in chemoreceptor cells. Therefore, the lack of effect on the basal release of CA and Ca^{2+}_i would imply that the cellular deposits of Ca^{2+} sensitive to IP_3 are really small. The effects observed during stimulation would be produced via voltage-dependent Ca^{2+} channels, as it is the case in other systems. These pieces of information urge to explore the dynamics of intracellular Ca^{2+} stores in a more direct manner and to correlate the data with other functions of the chemoreceptor cells, besides the release of neurotransmitters.

4. ACKNOWLEDGMENTS

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