THE ROLE OF DIHYDROPYRIDINE-SENSITIVE Ca²⁺ CHANNELS IN STIMULUS-EVOKED CATECHOLAMINE RELEASE FROM CHEMORECEPTOR CELLS OF THE CAROTID BODY

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Abstract—The present study utilized an *in vitro* preparation of the rabbit carotid body, with tissue catecholamine stores labeled by incubation with ³H-tyrosine. The goal was to characterize pharmacologically the voltage-dependent Ca²⁺ channels present in the type I (glomus) cells of this arterial chemoreceptor organ, and to elucidate their role as pathways for Ca²⁺ entry. We found that release of ³H-dopamine induced by high external potassium was over 95% dependent on external calcium concentration and that this release was 90–100% inhibited by the dihydropyridine antagonists, nisoldipine and nitrendipine, and was potentiated by the dihydropyridine agonist, BayK 8644. Therefore, any stimulus-induced, calcium-dependent release of ³H-dopamine that was inhibited by nisoldipine and potentiated by BayK 8644, was considered to be supported by Ca²⁺ entry into the cells via voltage-dependent Ca²⁺ channels. Significant differences were observed in the release of ³H-dopamine induced by 75 vs 25 mM K⁺. On prolonged stimulation, release induced by 75 mM K⁺ was large and transient, whilst that induced by 25 mM K⁺, although more moderate, was sustained. The release elicited by 75 mM K⁺ was inhibited approximately 90% by 1.5 mM Co²⁺ or 625 nM nisoldipine, while release by 25 mM K⁺ was completely blocked by 0.6 mM Co²⁺ or 125 nM nisoldipine. Low PO_2-induced release of ³H-dopamine was 95% dependent on Ca²⁺, and was inhibited by nisoldipine (625 nM) in a manner inversely proportional to the intensity of hypoxic stimulation, i.e. 79% inhibition at a PO_2 of 49 Torr, and 20% inhibition at PO_2 of 0 Torr. BayK 8644 potentiated the release induced by moderate hypoxic stimuli. Release elicited by high PCO_2/low pH, or by Na⁺-propionate or dinitrophenol-containing solutions, was approximately 80% Ca²⁺-dependent, and the dihydropyridines failed to modify this release.

It is concluded that type I cells possess voltage-dependent Ca²⁺ channels sensitive to the dihydropyridines, which in agreement with previous electrophysiological data should be defined as L-type Ca²⁺ channels. Calcium entry which supports the release of ³H-dopamine elicited by moderate hypoxia should occur mainly through these channels while the release induced by strong hypoxic stimuli will be served by Ca²⁺ entry which occurs in part via voltage-dependent Ca²⁺ channels, and in part through an additional pathway, probably a Na⁺/Ca²⁺ exchanger. The insensitivity to dihydropyridines of the release of ³H-dopamine induced by high PCO_2/low pH, Na⁺-propionate and dinitrophenol may indicate a complete loss of efficacy of the drugs to modulate Ca²⁺ channels under these conditions or more likely, that other mechanisms are activated, probably the Na⁺–Ca²⁺ exchanger.

Carotid body (CB) chemoreceptors are thought to be composite receptors in which the type I (glomus) cells detect changes in blood PO_2, PCO_2 and pH and respond with the release of neurotransmitters to activate the closely apposed chemosensory nerve terminals.[8,20] One such neurotransmitter that has received considerable attention in recent years and is known to be released by the type I cells is dopamine (DA). This biogenic amine has been shown to be released in proportion to both the intensity of stimulation and the resultant sensory discharge recorded from the carotid sinus nerve (CSN). This relationship between stimulus intensity, CSN sensory discharge, and DA release has been shown to be Ca²⁺-dependent,[40] and is known to occur with both physiological (low PO_2, low pH)[21,41,42] and pharmacological (cyanide, dinitrophenol, 2-deoxyglucose)[38,39] stimuli.

In a previous study,[3] it was postulated that type I cells might possess voltage-dependent Ca²⁺ channels. This suggestion was proposed to explain the Ca²⁺-dependent release of DA elicited by high external K⁺.[17] It was also shown in a subsequent preliminary study[39] that nitrendipine, a Ca²⁺ channel blocker of the dihydropyridine group, markedly inhibited the high K⁺ and low PO_2-induced release of DA. Quite recently, several different laboratories working with isolated type I cells have provided direct electrophysiological evidence for the presence of voltage-dependent Ca²⁺ channels in these cells.[16,17,32,33,53]

The present study was undertaken to characterize pharmacologically the type(s) of voltage-dependent Ca^{2+} channels present in the chemoreceptor type I cells of the CB, and to define the role of these Ca^{2+} channels in the response to different forms of chemoreceptor stimulation. Since the involvement of voltage-dependent Ca^{2+} channels in a cellular response couples a voltage dependency to that response,[27] the present study should reveal which chemoreceptor stimuli produce significant changes in the membrane potential of type I cells. Calcium entry into these cells was assessed by measuring the Ca^{2+}-dependent release of DA from the tissue. We chose this indirect method, instead of the measurement of $^{45}Ca^{2+}$ fluxes, because it has been found that, for a variety of tissues, there exists a very close relationship between Ca^{2+} influx and the intensity of the tissue's Ca^{2+}-dependent response.[6,13,29,30] Furthermore, the small size of the CB and the cellular heterogeneity of the tissue (type I and II cells, endothelial cells, neurons, etc.) make the measurement of $^{45}Ca^{2+}$ fluxes both technically difficult and of questionable significance. The release of DA, on the other hand, is a parameter specific to the type I cells.[20]

EXPERIMENTAL PROCEDURES

The present study utilized an *in vitro* CB preparation from adult (1.5–2.5 kg) New Zealand rabbits. The animals were anesthetized with Na^+-pentobarbitone (30–40 mg/kg, i.v.), tracheotomized, and both carotid bifurcations were exposed and removed for further dissection in a lucite chamber filled with ice-cold 100% O_2-equilibrated Tyrode's solution (in mmol/l: NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1.1; HEPES, 10 mM; glucose, 5.5 mM; pH adjusted with 1 N NaOH to 7.42). Carotid bodies were cleaned of surrounding tissue and incubated for 2 h with ^{3}H-tyrosine (20 μM, 30 Ci/mmol), as previously described.[19] Under these conditions, the organs synthesized approximately 20–22 pmol of ^{3}H-catecholamine/mg tissue (i.e. about 10 pmol, or more than 5×10^5 d.p.m./CB). Following incubation, the CBs were transferred to new vials containing 4 ml of tyrosine-free media and incubated for an additional 2 h to wash out the rapidly releasable pool of labeled catecholamine;[3] during this period the medium (4 ml) was replaced by prewarmed fresh solutions every 30 min and the samples were discarded. Thereafter, the collection of incubation media for analysis was grouped in stimulation cycles; one stimulation cycle consisted of a period of incubation with the stimulus (test) solution, preceded by a similar period and followed by several poststimulus periods of incubation with standard solution to determine basal responses and allow the preparation to recover, respectively. The duration of each of these periods in any given experiment is provided in the Results section. The number of stimulation cycles per experiment varied from one to four depending on the nature of the test incubation media. The stimulus-evoked release declined in successive presentation of the stimulus in parallel to the basal release and therefore the ratios of evoked to basal release remained constant throughout the experiments.[21] The incubation media were continuously bubbled with the appropriate gas mixture, and the PO_2 was monitored with an O_2 electrode in selected experiments. Under these experimental conditions, the threshold for DA release by low O_2 was approximately 75 Torr O_2 (i.e. normal saline equilibrated with 10% O_2/90% N_2).

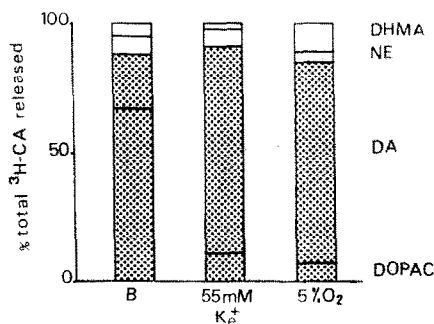All collected samples were added to a carrier mixture containing 0.3 M acetic acid, 1 mM ascorbic acid, and

Fig. 1. Profile of ^{3}H-catechols (^{3}H-CA) released from the carotid body following incubation with ^{3}H-tyrosine. ^{3}H-Catechols present in the alumina eluates were taken as 100%. Note that the relative proportions of ^{3}H-DA and ^{3}H-DOPAC are reversed under basal (B) vs stimulus conditions (55 mM K^+ and 5% O_2/95% N_2-equilibrated media; 10 min), owing to saturation of degradation mechanisms during stimulation. Nonetheless, DA + DOPAC still represent over 85% of ^{3}H-catechols under all conditions.

100 μM unlabeled DA. Radiolabeled catechols in the incubation media were analysed by adsorption to alumina at pH 8.6, and elution with 1 N HCl.[54] Since the CB receives a rich sympathetic innervation,[19] the ^{3}H-catechols released may have two origins, the type I cells and the sympathetic nerve endings. ^{3}H-DA is synthetized and stored exclusively in type I cells while ^{3}H-norepinephrine (NE) is synthetized and stored, both in type I cells and in the sympathetic endings;[19] therefore to assess the origin of the ^{3}H-catechols released it was necessary to identify its chemical nature. To do so, the alumina eluates were vacuum-concentrated to dryness, resuspended in 40 μl of mobile phase[22] containing 5 μg of unlabeled DA, NE, dihydroxyphenyl acetic acid (DOPAC) and dihydroxymandelic acid (DHMA), and separated using thin-layer chromatography according to the method of Fleming and Clark.[22] The labeled compounds were eluted and counted as previously described.[25] The profiles of released ^{3}H-catechols under basal conditions and in response to high K^+ and low PO_2 stimulation are shown in Fig. 1. Observe that regardless of the expected variations[18,21,48] in the ^{3}H-DA to ^{3}H-DOPAC ratios between basal and stimulus samples, ^{3}H-DA + ^{3}H-DOPAC (the main catabolite of ^{3}H-DA in the CB)[24] represent over 85% of the total ^{3}H-catechols released, indicating that type I cells are their main source. Because the same analytical profile was found in pilot experiments for all the stimuli tested in this study, in most of the replicate experiments the alumina eluates were directly counted and ^{3}H-catechol release was taken as a measure of ^{3}H-DA release from type I cells. Results are expressed as c.p.m. of ^{3}H-DA present in each collected sample, as the ratio of stimulus-evoked release to basal release (evoked/basal); or as per cent of tissue content.

Materials. 3,5-^{3}H-Tyrosine, 40–60 Ci/mmol was purchased from New England Nuclear and aluminum oxide (alumina) from Serva (Heidelberg). Nisoldipine and nitrendipine were a generous gift from Miles Laboratories, and BayK 8644 was provided by Prof. A. Garcia (U.A.M. Spain).

RESULTS

Calcium-dependent release of ^{3}H-dopamine

The Ca^{2+} dependency of ^{3}H-DA release observed in this study is summarized in Table 1. The results are similar to those previously reported for the cat CB.[3,38-40] Although not shown in the table, most of

Table 1. Ca^{2+} dependency of ^3H-dopamine release induced by different stimuli

Stimulus	Normal Ca^{2+}	0 M Ca^{2+}	Percentage reduction in 0 M Ca^{2+}
High K^+ (40 mM)	105 ± 8.2	4.80 ± 0.38*	95.60
Hypoxia (2% O_2)	28.6 ± 3.3	0.69 ± 0.05*	97.50
High PCO_2/pH 6.6	1.60 ± 0.12	0.31 ± 0.09*	81.00
Na^+-Propionate (15 mM)	1.02 ± 0.11	0.21 ± 0.06*	80.00
Dinitropyridine (2.5×10^{-4} M)	47.65 ± 3.82	9.00 ± 1.3*	79.00
Cyanide (10^{-4} M)	4.00 ± 0.66	0.93 ± 0.13*	76.75
Veratridine (5×10^{-5} M)	16.9 ± 1.4	1.6 ± 0.09*	90.50
Ouabain (10^{-5} M)	11.2 ± 1.5	0.80 ± 0.21*	93.00

Data are expressed as the ratios of evoked to basal release. Except for veratridine and ouabain, the protocol in these experiments was as follows: a stimulation cycle with Ca^{2+}-containing media was applied first, then two stimulation cycles with nominally free Ca^{2+} media, followed by a stimulation cycle with Ca^{2+}-containing media; the incubation with the test solutions was 10 min in all stimulation cycles. In the experiments with veratridine, pairs of CBs were incubated in Ca^{2+}-containing and in nominally free Ca^{2+} media 30 min prior to and during the application of veratridine (20 min); media were changed and collected for analysis every 10 min. The experiments with ouabain were identical except for the duration of the exposure to the test solution (30 min). In all the cases the data are means $\pm$ S.E. of the mean of six individual values. Differences of the mean evoked release in Ca^{2+}-containing vs Ca^{2+}-free media are statistically significant (*$P < 0.001$; paired Student's t-test).

the stimuli were tested at varying intensities, and the Ca^{2+} dependency of release was present at all stimulus intensities.

Time course of high K^+-induced release of ^3H-dopamine

The time course of ^3H-DA release induced by 75 and by 25 mM K^+ are compared in Fig. 2A; in both cases the media were changed and collected for analysis every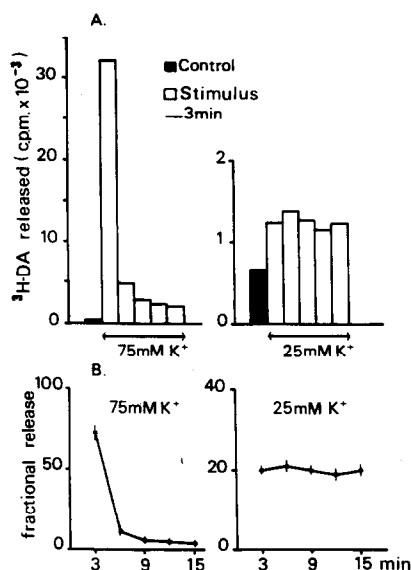 3 min for a total of 15 min. As shown in Fig. 2B, most of the ^3H-DA release evoked by 75 mM K^+ was collected in the first 3-min fraction, while with 25 mM K^+, release remained nearly constant for the duration of the stimulus. These results would suggest that Ca^{2+} entry inactivates rapidly during exposure to 75 mM K^+ but that entry is sustained during exposure to 25 mM K^+.

The unsustained release of ^3H-DA during exposure to 75 mM K^+ is, in fact, due to inactivation of Ca^{2+}, as confirmed by Fig. 3. The first panel of Fig. 3 illustrates a typical profile of declining release in 75 mM K^+. In a second CB (middle panel) removal of Ca^{2+} from the incubation medium not only abolished the immediate release of ^3H-DA, but also prevented its subsequent release following re-introduction of Ca^{2+} into the medium. This suggests that prior depolarization of the cells in a Ca^{2+}-free
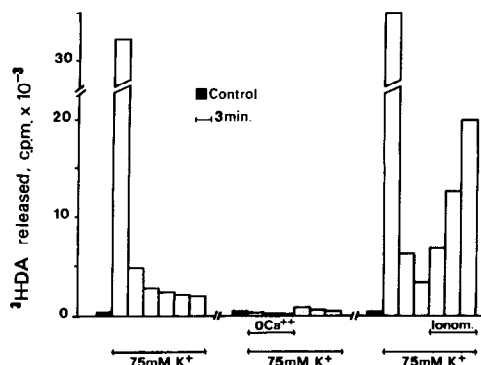

Fig. 2. Time course of K^+-induced release of ^3H-DA. (A) Two single stimulus cycles for 75 (left) and 25 (right) mM K^+. (B) Mean fractional release ($\pm$ S.E.) of ^3H-DA in response to 75 and 25 mM K^+. Total ^3H-DA released during the 15-min incubation with high K^+ was taken as 100%, and the fraction collected in each 3-min period is expressed as a per cent of this total. Data are from six experiments.

Fig. 3. Effects of 0 M Ca^{2+} and ionomycin (30 μM) on ^3H-DA release induced by 75 mM K^+. The experiment shows that neither depletion of releasable ^3H-DA stores, nor adaptation of exocytotic activity are responsible for accommodation of release induced by 75 mM K^+.

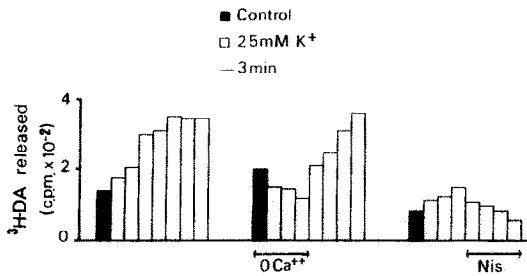Fig. 4. Effects of 0 M Ca^{2+} and nisoldipine (125 nM) on ^{3}H-DA release induced by 25 mM K^+. The experiment demonstrates the involvement of dihydropyridine-sensitive Ca^{2+} channels in the sustained release of ^{3}H-DA induced by 25 mM K^+.

medium prevented subsequent entry of Ca^{2+} apparently by inactivating the pathways for Ca^{2+} entry. Data obtained using a third CB (right panel), appeared to support this notion: introduction of ionomycin, a Ca^{2+} ionophore, during the declining phase of release reactivated the release process. This precluded the possibility that exhaustion of the releasable ^{3}H-DA pool, or loss of sensitivity to Ca^{2+} by the exocytotic machinery, was responsible for the unsustained release normally observed during exposure to 75 mM K^+.

Very different results were seen with 25 mM K^+. The left panel of Fig. 4 shows the normal time course of ^{3}H-DA release during exposure to 25 mM K^+; in the middle panel, removal of Ca^{2+} during the initial exposure to 25 mM K^+ abolished the increased release, but did not prevent reactivation of increased release upon reintroduction of Ca^{2+}. Nisoldipine reduced release when introduced late in the incubation period (Fig. 4, right panel), indicating that secretion depends on Ca^{2+} entry into type I cells via a dihydropyridine-sensitive pathway (see also below). These findings also indicate that, unlike the case for 75 M K^+, predepolarization with 25 mM K^+ does not inactivate the pathway for Ca^{2+} entry.

Pharmacology of high K^+-induced release of ^{3}H-dopamine

The effects of different concentrations of Ca^{2+} in the incubation media on the basal (5 mM K^+) and high K^+ (25 and 75 mM)-evoked release of ^{3}H-DA are shown in Fig. 5. In these experiments, 25 mM and 75 mM K^+ were applied for 10 min, and the media were sampled for analysis every 10 min. In the left of the figure, the plot of ^{3}H-DA release vs Ca^{2+} concentration shows that basal release did not change appreciably at low Ca^{2+}, but tended to decrease as Ca^{2+} was increased. Evoked release in 25 mM K^+ was abolished (over 95%) in 0 mM Ca^{2+}, was maximum at 0.5 mM but then declined at higher Ca^{2+}. The release evoked by 75 mM K^+ was likewise abolished in 0 mM Ca^{2+}, and increased dramatically with increasing Ca^{2+}. To the right in Fig. 5 a plot of the ratio of evoked release to basal release as a function of Ca^{2+} illustrates the reduction of the evoked response to 25 mM K^+ at 10 mM Ca^{2+}, and the progressive increase in the evoked release with 75 mM K^+ with increasing Ca^{2+}. These changes in CB ^{3}H-DA secretion with increasing Ca^{2+} are similar to those observed with other tissues.[43] They presumably reflect the increase in the threshold for Ca^{2+} channel activation with increasing Ca^{2+} that was evident with small depolarizations (25 mM K^+), but which disappeared upon stronger depolarizations (75 mM K^+).[27,43] The inset in the figure shows the protocol of sample collection for analysis.

Cd^{2+} and Co^{2+}, well-known Ca^{2+} channel blockers, produced a dose-dependent inhibition of the ^{3}H-DA release induced by 25 and 75 mM K^+, as shown in Fig. 6. Both responses to elevated K^+ were completely blocked by 100 μm Cd^{2+}. Variation in the effect of different Cd^{2+} concentrations on basal release precluded an accurate calculation of an IC_{50} for release evoked by 25 mK K^+, but did not interfere significantly in calculating a value for the much larger release evoked by 75 mM K^+. For this we found an
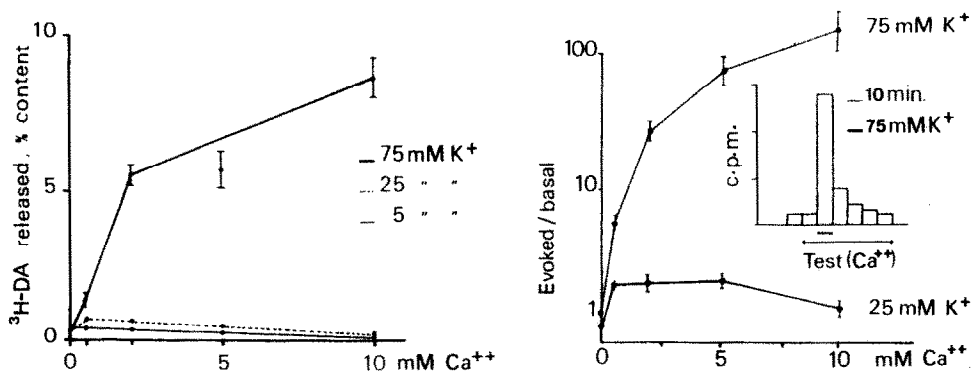

Fig. 5. Effect of Ca^{2+} concentration on ^{3}H-DA release under resting conditions and in response to high K^+. Left: release expressed as per cent of tissue content. Right: ratio of evoked to basal release. Inset: sequence of medium renewal and collection for analysis. Each data point represents the mean ± S.E. of at least four samples.
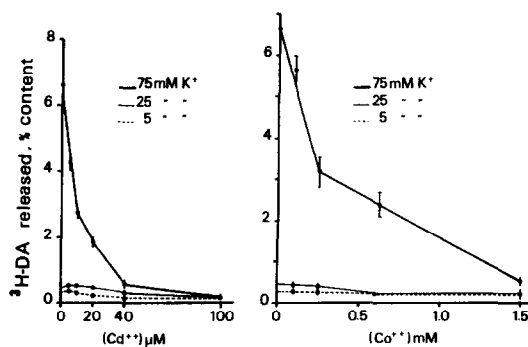
Fig. 6. Effects of Cd^{2+} (left) and Co^{2+} (right) on ^{3}H-DA release under resting conditions (5 mM K$^+$) and during high K$^+$ (25 and 75 mM). Experimental protocol as in Fig. 5. Cd^{2+} or Co^{2+} was introduced 10 min before and during the incubation with high K$^+$. Release is expressed as per cent of tissue content. Data points are mean ± S.E. of four to six experiments.

IC$_{50}$ of $10\,\mu$M, which is lower than the $56\,\mu$M observed for PC12 cells by Freedman *et al.*[23] We found that Co^{2+} at any concentration did not appreciably modify the basal release, but at 0.6 mM completely abolished the release evoked by 25 mM K$^+$, and at 1.5 mM inhibited 93% of the release induced by 75 mM K$^+$. The IC$_{50}$ for the Co^{2+} effect on the 75 mM K$^+$-induced release was 0.3 mM, with maximum inhibition at 1.5 mM. Both values are comparable to those reported for PC12 cells.[23]

Neither nisoldipine (Fig. 7A) nor nitrendipine (Fig. 7B) modified the basal release of ^{3}H-DA. However, both exhibited a dose-dependent inhibitory effect on the release induced by 25 and 75 mM K$^+$. At 10^{-9} M, nisoldipine inhibited 50% and 10% of the release induced by 25 and 75 mM K$^+$, respectively. As shown in the inset, this difference was maintained throughout the range of nisoldipine concentrations (Fig. 7A).
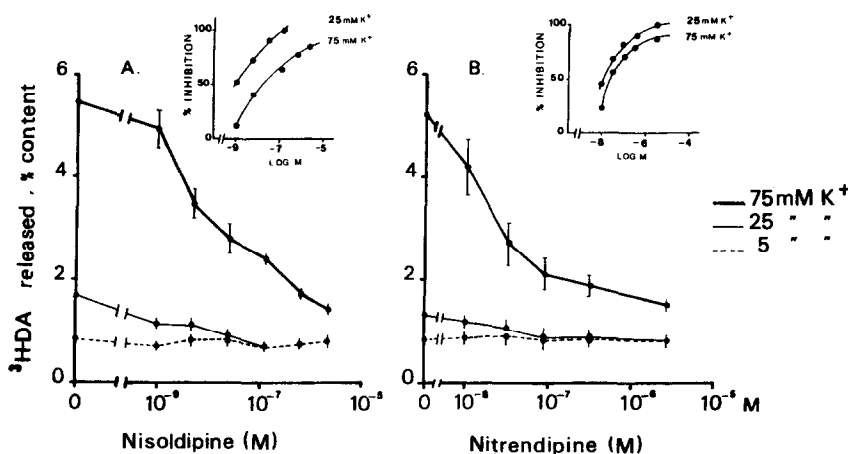
Nitrendipine, on the other hand, required higher concentrations to inhibit release, and exhibited less marked differences in its effects on 25 and 75 mM K$^+$-induced release (inset Fig. 7B). These data demonstrate that the voltage-dependent Ca^{2+} channels in the CB type I cells are dihydropyridine sensitive. Figure 8 illustrates further proof of the dihydropyridine sensitivity of the Ca^{2+} channels in type I cells; BayK 8644, a Ca^{2+} channel agonist belonging to the dihydropyridine family,[49] markedly potentiated the release of ^{3}H-DA by 30 mM K$^+$.

Effects of dihydropyridines on low PO$_2$-induced release of ^{3}H-dopamine

The participation of Ca^{2+} channels in the response to low PO$_2$ was assessed using stimuli of moderate intensity (10 min incubation with 7% O$_2$ equilibrated media; PO$_2$ = 49 Torr). The results of a single experiment with two stimulation cycles are shown in Fig. 9A (left). Introduction of nisoldipine (625 nM) into the media 10 min prior to presentation of the second stimulus inhibited the response. The inhibition of the evoked release from four such experiments had a mean of 79 ± 7% (Fig. 9A, right). In Fig. 9B, BayK 8644 was used in a similar protocol and produced a five-fold increase in the release of ^{3}H-DA in response to 7% O$_2$. These data implicate dihydropyridine-sensitive Ca^{2+} channels in the mediation of Ca^{2+} entry during low PO$_2$-induced release of ^{3}H-DA.

The effects of 625 nM nisoldipine on the low PO$_2$-induced release of ^{3}H-DA are summarized in Fig. 10. The inhibitory effect of this dihydropyridine decreased as the intensity of the low PO$_2$ stimulus was increased. Even at high stimulus intensities (e.g. 2% O$_2$/98% N$_2$ media) over 95% of ^{3}H-DA release was Ca^{2+}-dependent (see Table 1), suggesting that as the intensity of hypoxic stimulation increases, the role

Fig. 7. Effects of nisoldipine (left) and nitrendipine (right) on the release of ^{3}H-DA under resting conditions (5 mM K$^+$) and during high K$^+$ (25 and 75 mM) stimulation. Experimental protocol as in previous figure, nisoldipine and nitrendipine were present in the incubation media 10 min prior and during high K$^+$ stimulation. Release is expressed as per cent of tissue content. Insets: dose–response inhibition curves. All data points are means ± S.E. of four to six values.
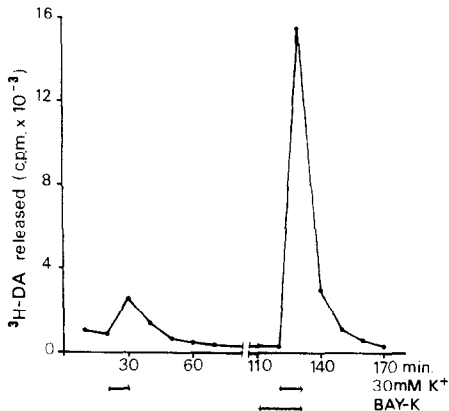
Fig. 8. Effect of the dihydropyridine Ca^{2+} channel agonist BayK 8644 ($1 \mu M$) on 30 mM K^+ induced release of ^3H-DA. Note that BayK 8644 did not affect basal release.

Fig. 10. Decreasing potency of nisoldipine (625 nM) in inhibiting the release of ^3H-DA induced by increasingly strong hypoxic stimuli. Experimental protocol as in Fig. 9. Duration of low PO_2 incubations was 10 min. Means $\pm$ S.E. of the mean of six or more samples.

of Ca^{2+} channels in mediating Ca^{2+} entry becomes less important.

To test the possibility that the loss of efficacy of nisoldipine with increasing strength of the hypoxic stimuli is related to the proportionally increasing acidification that is produced during low PO_2 stimulation,[15,46] we utilized a strong hypoxic stimulus of shorter duration (2% O_2; 3 min). The rationale for these experiments was that acidic stimuli evoke Ca^{2+}-dependent release that was insensitive to dihydropyridines (see Table 1 and below) and that intracellular acidification produced under low O_2 increased dramatically between 3 and 10 min (see Ref. 15). In these experiments (Fig. 11), pairs of CBs were subjected to two cycles of low PO_2 stimulation; one CB served as
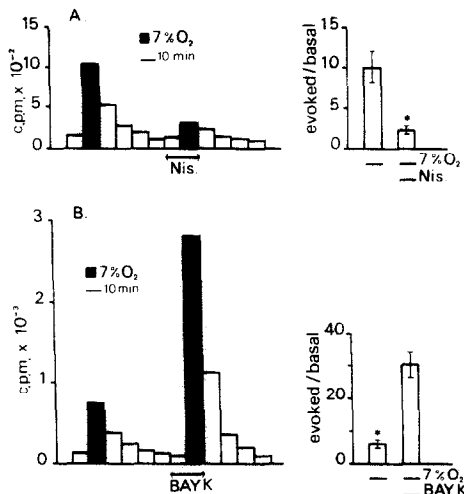
a control while the second was used to assess the effect of nisoldipine during the second stimulus cycle. The ratios of evoked release in the first and second cycles from four such experiments are shown in the right hand panel of Fig. 11. It is evident from a comparison with the results of Fig. 10, that shortening the duration of the stimulus increased the efficacy of nisoldipine inhibition of the low PO_2-induced release (29% vs 73%).

Effects of dihydropyridines on the release of ^3H-dopamine induced by different stimuli

In addition to high K^+ (a depolarizing agent useful in revealing the participation of voltage-dependent Ca^{2+} channels in depolarization-mediated transmitter release) and low PO_2 (the principal natural stimulus for the carotid chemoreceptors), we also examined the effects of the dihydropyridines on the secretory response evoked by other natural and pharmacological stimuli of the carotid chemoreceptors (Table 2). Stimuli that evoke only a moderate release of ^3H-DA (20% CO_2/pH 6.6 and Na^+-propionate) were employed to test the effects of the Ca^{2+} channel agonist,

Fig. 9. Dihydropyridine and low PO_2-induced release of ^3H-DA. (A) Left: effect of nisoldipine (625 nM) on release induced by 7% O_2-equilibrated media (10 min; black bars). (A) Right: evoked to basal release ratios from four experiments ($x \pm$ S.E.). (B) Left: effect of $1 \mu M$ BayK 8644 was tested. (B) Right: mean evoked to basal release ratios. Differences of the mean evoked release in control vs test conditions were statistically significant, *$P < 0.01$; paired Student's t-test.
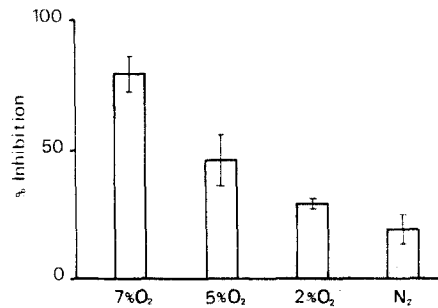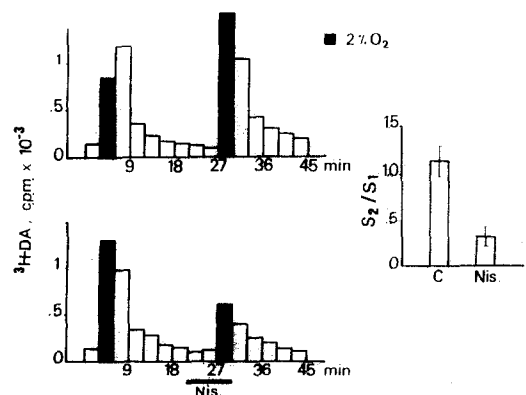

Fig. 11. Effect of nisoldipine on ^3H-DA release induced by severe hypoxia. Duration of stimulus (2% O_2 in N_2), 3 min (black bars). Left: comparison of control (top) and nisoldipine (625 nM)-treated (bottom) carotid bodies. Right: ratios of evoked release (second/first stimulus presentation). Means $\pm$ S.E. of such four paired experiments.

Table 2. Dihydropyridine sensitivity of ^{3}H-dopamine release evoked by different stimuli

Stimulus	Control	Dihydropyridine	Percentage effect
20% CO_2/pH 6.6	1.6 ± 0.17	1.5 ± 0.21†	—
NaPr (15 mM)	1.0 ± 0.16	1.1 ± 0.17	—
Dinitropyridine (2.5×10^{-4} M)	48.0 ± 7.0	47.4 ± 8.5‡	—
Cyanide (2.5×10^{-4} M)	17.8 ± 2.1	11.4 ± 1.0**‡	−36%
Veratridine (5×10^{-5} M) 20 min	22.8 ± 4.0	16.4 ± 1.9*‡	−28%
Veratridine (5×10^{-5} M) 3 min	9.5 ± 1.6	5.0 ± 0.9**‡	−47%

†BayK 8644, 1 μM; ‡nisoldipine, 625 nM. Experimental protocols as in Fig. 9; duration of exposure to test solutions 10 min in all the cases except when indicated otherwise. Data are means ± S.E. of the means of four or more individual values. Differences of the mean evoked release in control vs test conditions were statistically significant for cyanide and veratridine (*$P < 0.05$; **$P < 0.02$, paired t-test).

BayK 8644 (1 μM); stronger pharmacological stimuli were used to test the effects of the antagonist nisoldipine (625 nM). The protocol for these experiments was identical to that described above for the experiments of Fig. 9. Our findings from these experiments, summarized in Table 2, are as follows: (i) it appears that high PCO_2/low pH, Na$^+$-propionate, and the protonophore dinitrophenol, do not trigger the activation of voltage-dependent Ca^{2+} channels because release is unaffected by the dihydropyridines. It is noteworthy that in other preparations these three stimuli produce intracellular acidification and fail to appreciably modify the membrane potential.[25,50] This suggests that the coupling between the increase in intracellular H$^+$ and the Ca^{2+} dependent release of DA from type I cells follow different pathways than those used by low PO_2; (ii) cyanide is a powerful stimulant classically used with carotid chemoreceptors,[5] but its mechanism of action, although probably related to histotoxic hypoxia, is not understood.[9,10,39] Whatever its precise mechanism of action, it is likely that it involves depolarization of the type I cells, because cyanide-induced release of ^{3}H-DA is partially sensitive to blockers of voltage-dependent Ca^{2+} channels; (iii) it has recently been shown that rabbit type I cells are excitable cells that possess tetrodotoxin-sensitive Na$^+$ channels.[17,32,33,53] We further showed that the ^{3}H-DA release induced by veratridine, a Na$^+$ channel activator, is Na$^+$ and Ca^{2+} dependent, and tetrodotoxin sensitive.[45] It was therefore of interest to explore the effects of nisoldipine on the veratridine-induced release. Table 2 shows that this release is partially sensitive to the dihydropyridine, and that the sensitivity increases as veratridine exposure decreases.

DISCUSSION

The results show that high K$^+$-induced release of ^{3}H-DA from type I cells is over 95% dependent on Ca^{2+}, is inhibited in a dose-dependent manner by Cd^{2+} and Co^{2+}, and is modulated by dihydropyridines. We also found that low PO_2-induced release is likewise Ca^{2+} dependent, modulated by dihydropyridines, and that this modulation is dependent on the intensity of hypoxic stimulation. The release of ^{3}H-DA induced by acidifying stimuli also exhibited a marked Ca^{2+} dependence, but was insensitive to modulation by dihydropyridines.

In a previous publication from our laboratory, before it was known that type I cells were electrically excitable, Almaraz et al.[3] postulated the existence of voltage-dependent Ca^{2+} channels in these cells to explain the release of ^{3}H-DA induced by high K$^+$. Quite recently, the presence of Ca^{2+} channels in type I cells has been directly confirmed by several groups in electrophysiological studies of freshly dissociated cells.[16,17,32,33,53] Analysis of the Ca^{2+} tail currents, recorded at instantaneous repolarizations, revealed a large component with a rapid decay and a small component which deactivated slowly, equivalent to about 95% and 5% of the total Ca^{2+} current amplitude, respectively.[53] However, because of a slow time course of inactivation, insensitivity of the closing kinetics to long conditioning pulses and the lability of Ca^{2+} current following intracellular dialysis during whole-cell patch-clamp recordings, two Ca^{2+} currents could not be resolved. It was therefore concluded that most, if not all, of the Ca^{2+} current was mediated by rapidly deactivating (L-type) Ca^{2+} channels.[53] Our present data are consistent with these electrophysiological findings in showing that Ca^{2+} entry into type I cells activated by high K$^+$ are dihydropyridine sensitive, a characteristic of L-channels;[35,52] this conclusion also agrees with the reported potentiation of Ca^{2+} currents recorded in dissociated chemoreceptor cells by BayK 8644.[26] However, Akaike et al.[1,2] have described Ca^{2+} channels that behave kinetically as T-type channels but nonetheless are sensitive to dihydropyridines.

In order to explain differences in the time course of the secretory response induced by 25 mM and 75 mM K$^+$, Almaraz et al.[3] postulated the existence of two types of Ca^{2+} channels in type I cells. However, the available data more likely favor the existence of a single type of voltage-dependent Ca^{2+} channel in type I cells,[53] despite the finding in the present study of different sensitivities to nisoldipine and Co^{2+} in the release response induced by 25 mM and 75 mM K$^+$, and the two components found in the analysis of the

Ca^{2+} tail currents,[53] both of which could be viewed as compatible with the existence of two types of Ca^{2+} channels. With the knowledge that type I cells generate action potentials,[17,33,53] it is possible now to explain differences in the time course of release in response to 25 and 75 mM K^+ with a single set of Ca^{2+} channels of the L-type. The large, transient release in response to 75 mM K^+ would result from the maximal, or near maximal, activation of Ca^{2+} channels that within a few seconds completely inactivate.[53] The magnitude of the sustained depolarization with 75 mM K^+ ensures continued inactivation, with the consequence that the secretory response subsides. At the same time, the high level of depolarization should favor the entry of Ca^{2+} via the Na^+/Ca^{2+} exchanger,[11] and thereby account for both the small response to 75 mM K^+ late in the stimulus period, as well as the small difference between the Ca^{2+} dependency and the sensitivity to channel blockers. The moderate, sustained release in response to 25 mM K^+ would result from the depolarization of type I cells to the activation threshold for Na^+ and Ca^{2+} channels, and the consequent generation of action potentials.[28] Repolarization of the action potential resulting from the activation of voltage-gated K^+ channels, both Ca^{2+} dependent and independent[17,53] will limit the entry of Ca^{2+} to the duration of the action potentials. At the same time repolarization will allow the removal of inactivation from Ca^{2+} channels, thereby permitting their recruitment by successive action potentials. The slow kinetics of Ca^{2+} channel activation and the interspike intervals will limit the entry of Ca^{2+} and therefore the release response.

Dihydropyridine inhibition and potentiation of low PO_2-induced release of ^3H-DA is evidence for the participation of voltage-gated Ca^{2+} channels in the physiological response of the carotid chemoreceptors. At the same time the data indicate the existence of additional pathways for Ca^{2+} entry during hypoxic stimulation: low PO_2-induced release of ^3H-DA is over 95% Ca^{2+} dependent at all intensities of the stimulus and the blockade of the release by dihydropyridines ranges from 79% with mild hypoxic stimulus to about 20% under anoxia. The recent finding that chemoreceptor cells exhibit a K^+ current that is reversibly inhibited by low oxygen pressure[16,32,33] suggests a possible mechanism for the depolarization required to activate the Ca^{2+} channels. The additional pathways for Ca^{2+} entry activated during hypoxic stimulation could be related to the concomitant acidification produced under low O_2 that is parallel to the intensity and duration of the hypoxia.[14,15,51] The data presented in Figs 10 and 11 support this contention; as the intensity and duration of the hypoxic stimuli increase the sensitivity to dihydropyridines decreases and tends towards the insensitivity of the release response observed with pure acidic stimuli (see Table 2). It would appear then that under in vitro conditions the participation of dihydropyridine-sensitive voltage-gated Ca^{2+} chan-

nels during strong hypoxia is limited to the initial moments of the stimulus and thereafter the pathways for Ca^{2+} entry activated by acidic stimuli are those that support the Ca^{2+}-dependent release of ^3H-DA. In apparent contradiction with this interpretation is the reported inhibition by low pH of the O_2-sensitive K^+ current in dissociated chemoreceptor cells;[33] more recently, however, it has been also shown that Na^+ and Ca^{2+} currents in these cells are similarly inhibited by low pH[32] (see also Ref. 36). It must be pointed out also that in vivo, physiological hypoxia is accompanied by systemic alkalosis. Situations comparable to the in vitro conditions are seen in vivo at extremely low PO_2, and in certain lung pathologies and circulatory shock.

Regarding the nature of the pathways used for influx of Ca^{2+} into chemoreceptor cells during acidic stimulation, our data are compatible with two possibilities: (i) Ca^{2+} enters the cells via the voltage-gated Ca^{2+} channels, which became insensitive to dihydropyridines under the specific conditions set by the stimuli, and (ii) Ca^{2+} influx into chemoreceptor cells occurs through a dihydropyridine-insensitive pathway. Although it is not possible to distinguish between these alternatives without direct recordings of Ca^{2+} currents under the same conditions, there are other data that favor the second alternative. For example, on theoretical grounds it should not be expected that dinitrophenol produces any significant depolarization at the plasma membrane level. In fact it has been shown that dinitrophenol produces an intense acidification in lymphocytes without any appreciable change in their membrane potential.[25] It has also been shown that the other two acidifying stimuli used in the present study do not produce any significant change in the membrane potential of snail neurons.[50] Therefore, it appears inconceivable that these stimuli activate voltage-gated Ca^{2+} channels. In addition, a recent publication from our group suggested that the Ca^{2+}-dependent release of ^3H-DA elicited by acidifying stimuli would be mediated by influx of Ca^{2+} via the Na^+/Ca^{2+} exchanger;[39] it was suggested also that the accumulation of Na^+ accompanying proton extrusion during an acidic load, would be responsible for the reversal of the Na^+/Ca^{2+} exchanger.[39] Confirmation of these suggestions has been obtained.[44] The release of DA induced by acidic stimuli was found to be Na^+-dependent and inhibited by blockers of the Na^+-dependent proton-extruding mechanisms indicating that entry of Na^+ is the driving force for Ca^{2+} entry during acidic stimulation. This information, coupled to the complete insensitivity of the acidic stimuli release response to BayK 8644 and to nisoldipine, supports the conclusion that transduction of the acidic stimuli does not involve depolarization of type I cells nor participation of voltage-gated Ca^{2+} channels.

The time-dependence of nisoldipine on veratridine-induced ^3H-DA release, i.e. the inhibition produced by the Ca^{2+}-channel block decreased with the pro-

longed exposure to veratridine (see Table 2), may be explained as follows. It is conceivable that, after an initial burst of Ca^{2+} influx via Ca^{2+} channels, the sustained depolarization produced by the alkaloid permanently inactivates the voltage-dependent Ca^{2+} channels in the chemoreceptor cells.[53] With time, the depolarization itself, and the intracellular accumulation of Na^+ produced by veratridine,[4,12] will trigger Na^+/Ca^{2+} exchange that will provide the influx of Ca^{2+} to support the veratridine-induced release.[11] A Na^+/Ca^{2+} antiporter may also be an important mechanism in the secretory response induced by cyanide.[10]

REFERENCES

1. Akaike N., Kanaide H., Kuga T., Nakamura M., Sadoshima J. and Tomoike H. (1989) Low-voltage-activated calcium current in rat aorta smooth muscle cells in primary culture. *J. Physiol.* **416**, 141–160.
2. Akaike N., Kostyuk P. G. and Osipchuk Y. V. (1989) Dihydropyridine sensitive low threshold calcium channels in isolated rat hypothalamic neurones. *J. Physiol.* **412**, 181–196.
3. Almaraz L., Obeso A. and Gonzalez C. (1986) Effects of high potassium on the release of ^{3}H-dopamine from the cat carotid body *in vitro*. *J. Physiol.* **379**, 293–307.
4. Amy C. and Kirshner N. (1982) ^{22}Na$^+$ uptake and catecholamine secretion by primary cultures of adrenal medulla cells. *J. Neurochem.* **39**, 132–142.
5. Anichkov S. V. and Belen'kii M. L. (1963) *Pharmacology of the Carotid Body Chemoreceptors*. Macmillan, New York.
6. Baker P. F. and Knight D. E. (1984) Calcium control of exocytosis in bovine adrenal medullary cells. *Trends Neurosci.* **7**, 120–126.
7. Beaty G. N., Cota G., Siri L., Sanchez J. A. and Stefani E. (1987) Skeletal muscle Ca^{++} channels. In *Structure and Physiology of the Slow Inward Calcium Channel* (eds Venter J. C. and Triggle D.), pp. 123–140. Alan R. Liss, New York.
8. Belmonte C. and Gonzalez C. (1983) Mechanisms of chemoreception in the carotid body: possible models. In *Physiology of the Peripheral Arterial Chemoreceptors* (eds Acker H. and O'Reagan R. G.), pp. 197–220. Elsevier, Amsterdam.
9. Biscoe T. J. and Duchen M. R. (1989) Electrophysiological responses of dissociated type I cells of the rabbit carotid body to cyanide. *J. Physiol.* **413**, 447–468.
10. Biscoe T. J., Duchen M. R., Eisner D. A., O'Neill S. C. and Valdeolmillos M. (1989) Measurements of intracellular Ca^{++} in dissociated type I cells of the rabbit carotid body. *J. Physiol.* **416**, 421–434.
11. Blaustein M. P. (1988) Calcium transport and buffering in neurons. *Trends Neurosci.* **11**, 438–443.
12. Catterall W. A. and Nirenberg M. (1973) Sodium uptake associated with activation of action potential ionophores of cultured neuroblastoma and muscle cells. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3759–3763.
13. Collazos J. M. and Sanchez A. (1987) cAMP reduces the affinity of Ca^{2+}-triggered secretion in platelets. *Fedn Eur. biochem. Socs Lett.* **215**, 183–186.
14. Delpiano M. A. (1987) Glycolysis as a link for chemoreception? In *Chemoreceptors in Respiratory Control* (eds Ribeiro J. A. and Pallot D. J.), pp. 59–68. Croom Helm, London.
15. Delpiano M. A. and Acker H. (1985) Extracellular pH changes in the superfused cat carotid body during hypoxia and hypercapnia. *Brain Res.* **342**, 273–280.
16. Delpiano M. A. and Hefscheler H. (1989) Evidence for a PO_2-sensitive K^+ channel in the type I cells of the rabbit carotid body. *Fedn Eur. biochem. Socs Lett.* **249**, 195–198.
17. Duchen M. R., Caddy K. W. T., Kirby G. C., Patterson D. L., Ponte J. and Biscoe T. J. (1988) Biophysical studies of the cellular elements of the rabbit carotid body. *Neuroscience* **26**, 291–311.
18. Farnebo L. O. and Malmfors T. (1971) ^{3}H-Noradrenaline release and mechanical response in the field stimulated mouse vas deferens. *Acta physiol. scand, Suppl.* **371**, 1–18.
19. Fidone S. J. and Gonzalez C. (1982) Catecholamine synthesis in rabbit carotid body *in vitro*. *J. Physiol.* **333**, 69–79.
20. Fidone S. J. and Gonzalez C. (1986) Initiation and control of chemoreceptor activity in the carotid body. In *Handbook of Physiology. The Respiratory System II* (ed. Fishman A. P.), pp. 243–312. Am. Physiol. Soc., Bethesda, MD.
21. Fidone S. J., Gonzalez C. and Yoshizaki K. (1982) Effects of low oxygen on the release of dopamine from the rabbit carotid body *in vitro*. *J. Physiol.* **333**, 93–110.
22. Fleming R. M. and Clark W. G. (1972) Quantitative thin-layer chromatographic estimation of labelled dopamine and norepinephrine, their precursors and metabolites. *J. Chromat.* **52**, 305–312.
23. Freedman S. B., Dawson G., Villereal M. L. and Miller R. J. (1984) Identification and characterization of voltage-sensitive calcium channels in neuronal clonal cell lines. *J. Neurosci.* **4**, 1453–1467.
24. Gonzalez E., Rigual R., Fidone S. J. and Gonzalez C. (1987) Mechanisms for termination of the action of dopamine in carotid body chemoreceptors. *J. auton. nerv. Syst.* **18**, 249–259.
25. Grinstein S. and Cohen S. (1987) Cytoplasmic $[Ca^{2+}]$ and intracellular pH in lymphocytes. Role of membrane potential and volume-activated Na^+/H^+ exchange. *J. gen. Physiol.* **89**, 185–213.
26. Hescheler J. and Delpiano M. A. (1990) Ionic currents in carotid body type I cells and the effects of hypoxia and NaCN. In *Arterial Chemoreception* (eds Eyzaguirre C., Fidone S. J., Fitzgerald R. S., Lahiri S. and MacDonald D. M.), pp. 58–62. Springer, New York.
27. Hille B. (1984) *Ionic Channels of Excitable Membranes*. Sinauer, Sunderland, MA.
28. Kidokoro Y. and Ritchie A. (1980) Chromaffin cell action potentials and their possible role in adrenaline secretion from rat adrenal medulla. *J. Physiol.* **307**, 199–216.
29. Kilpatrick D. L., Slepetis R. J., Corcoran J. J. and Kirshner N. (1982) Calcium uptake and catecholamine secretion by cultured bovine adrenal medulla cells. *J. Neurochem.* **38**, 427–431.
30. Llinás R., Sugimori M. and Simon S. M. (1982) Transmission by presynaptic spike-like depolarization in the squid giant synapse. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2415–2419.

31. Llinás R. and Yarome Y. (1981) Electrophysiology of mammalian inferior olivary neurones *in vitro*: different types of voltage dependent ionic conductances. *J. Physiol.* **315**, 549–567.

32. López-Barneo J., López-López J. R., Ureña J. and Gonzalez C. (1988) Chemotransduction in the carotid body: K current modulated by PO_2 in type I chemoreceptor cells. *Science* **241**, 580–582.

33. López-López J., Gonzalez C., Ureña J. and López-Barneo J. (1989) Low PO_2 selectively inhibits K channel activity in chemoreceptor cells of the mammalian carotid body. *J. gen. Physiol.* **93**, 1001–1014.

34. López-López J., Ganfornina M. D. and López-Barneo J. (1989) Efecto de la PO_2 y el pH sobre la actividad electrica de las células quimiorreceptoras del cuerpo carotídeo. Sevilla: III Congreso de la SEN, Abstr. p. 2.

35. Miller R. J. (1987) Calcium channels in neurones. In *Structure and Physiology of the Slow Inward Calcium Channel* (eds Venter J. G. and Triggle D.), pp. 161–246. Alan R. Liss, New York.

36. Moody W. (1984) Effects of intracellular H^+ on the electrical properties of excitable cells. *A. Rev. Neurosci.* **7**, 257–278.

37. Mullins L. J. (1984) An electrogenic saga: consequences of sodium–calcium exchange in cardiac muscle. In *Electrogenic Transport: Fundamental Principles and Physiological Implications*, p. 161. Raven Press, New York.

38. Obeso A., Almaraz L. and Gonzalez C. (1986) Effects of 2-deoxy-D-glucose on *in vitro* cat carotid body. *Brain Res.* **371**, 25–36.

39. Obeso A., Almaraz L. and Gonzalez C. (1989) Effects of cyanide and uncouplers on chemoreceptor activity and ATP content of the cat carotid body. *Brain Res.* **481**, 250–257.

40. Obeso A., Fidone S. and Gonzalez C. (1987) Pathways for calcium entry into type I cells: significance for the secretory response. In *Chemoreceptors in Respiratory Control* (eds Ribeiro J. A. and Pallot D. J.), pp. 91–98. Croom Helm, London.

41. Rigual R., Gonzalez E., Fidone S. and Gonzalez C. (1984) Effects of low pH on synthesis and release of catecholamines in the cat carotid body *in vitro*. *Brain Res.* **309**, 178–181.

42. Rigual R., Gonzalez E., Gonzalez C. and Fidone S. (1986) Synthesis and release of catecholamines by the cat carotid body *in vitro*: effects of hypoxic stimulation. *Brain Res.* **374**, 101–109.

43. Ritchie A. K. (1979) Catecholamine secretion in a rat pheochromocytoma cell line: two pathways for calcium entry. *J. Physiol.* **286**, 541–546.

44. Rocher A., Obeso A., Gonzalez C. and Herreros B. (1991) Ionic mechanisms for the transduction of acidic stimuli in the carotid body glomus cells. *J. Physiol.* **433**, 533–548.

45. Rocher A., Obeso A., Herreros B. and Gonzalez C. (1988) Activation of the release of dopamine in the carotid body by veratridine. Evidence for the presence of voltage-dependent Na^+ channels in type I cells. *Neurosci. Lett.* **94**, 274–278.

46. Roos A. and Boron W. F. (1981) Intracellular pH. *Physiol. Rev.* **61**, 296–434.

47. Rubin R. P. (1982) *Calcium and Cellular Secretion*, pp. 63–66. Plenum Press, New York.

48. Sarantos-Laska C., Majewski M., McCulloch M. W. and Rand M. J. (1980) Mechanism of noradrenaline release from rabbit atria induced by nicotinic agonists. *Arch. int. Pharmacodyn. Thér.* **247**, 294–305.

49. Scriabine A. (1987) Ca^{++} channel ligands: comparative pharmacology. In *Structure and Physiology of the Slow Inward Calcium Channel* (eds Venter J. C. and Triggle D.), pp. 51–70. Alan R. Liss, New York.

50. Thomas R. C. (1976) The effect of carbon dioxide on the intracellular pH and buffering power of snail neurones. *J. Physiol.* **255**, 715–735.

51. Torrance R. W. (1974) Arterial chemoreceptors. In *Respiratory Physiology* (ed. Widdicombe J. G.), pp. 247–271. University Park Press, Baltimore.

52. Tsien R. W., Lipscombe D., Madison D. V., Bley K. R. and Fox A. P. (1988) Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci.* **11**, 431–438.

53. Ureña J., López-López J. R., Gonzalez C. and López-Barneo J. (1989) Ionic currents in dispersed chemoreceptor cells of the mammalian carotid body. *J. gen. Physiol.* **93**, 979–1001.

54. Weil-Malherbe H. (1971) Determination of catecholamines. In *Analysis of Biogenic Amines and Their Related Enzymes* (ed. Glick D.), pp. 119–152. Interscience Publishers, New York.