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# THE SOURCE OF CALCIUM FOR MUSCARINIC-MEDIATED CATECHOLAMINE RELEASE FROM CAT ADRENALS

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SUMMARY

1. In view of conflicting reports on the source of $Ca^{2+}$ needed to trigger the secretory response to muscarinic stimulation of chromaffin cells, we have reinvestigated this problem in the cat adrenal gland perfused with oxygenated Krebs solution at 37 °C. Above a basal rate of secretion of 60 ng/30 s of total catecholamines, 5 s pulses of 100 $\mu$M-methacholine evoked 10-fold increases of secretion. This response was entirely mediated by muscarinic receptors, since it was blocked by submicromolar concentrations of atropine but not by *d*-tubocurarine.

2. Delayed application of methacholine pulses after $Ca^{2+}$ removal from the Krebs solution led to a progressive decline of the secretory response with a $t_{\frac{1}{2}}$ of 15 s. Secretion was blocked by 85% after a 60 s period of $Ca^{2+}$ deprivation; extension of the external $Ca^{2+}$ ($Ca_o^{2+}$) wash-out period up to 5 min did not further reduce the secretory response.

3. When EGTA (1 mM) was present in the 0 $Ca^{2+}$ solution, the rate of decline of methacholine responses, as a function of the time of exposure to 1 mM-EGTA, was similar to that obtained with 0 $Ca^{2+}$. Again, about 15–20% of the secretory response was resistant even to prolonged periods of washing out with the 0 $Ca^{2+}$-EGTA solution.

4. The $Ca^{2+}$ ionophore ionomycin (1 $\mu$M) first decreased and then accelerated the rate of decline of methacholine responses upon $Ca_o^{2+}$ wash-out. Particularly relevant is the complete blockade of secretion when the $Ca_o^{2+}$ wash-out is performed in the presence of this ionophore. This suggests the existence of a small intracellular functional $Ca^{2+}$ store sensitive to ionomycin.

5. After abolition of the secretory response through 60 s periods of wash-out with a 0 $Ca^{2+}$-EGTA–ionomycin solution, followed by delayed 5 s methacholine pulses after $Ca_o^{2+}$ reintroduction, the glands instantly recovered their normal muscarinic-mediated secretory response. This suggests that upon muscarinic stimulation, $Ca^{2+}$ required by the secretory machinery to trigger such response immediately comes from extracellular sources. How $Ca_o^{2+}$ gains the cell interior so fast upon muscarinic stimulation is unknown; we have previously suggested that the muscarinic receptor in the cat chromaffin cell could be coupled to an ionophore channel which might be chemically activated by muscarinic agonists.

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6. Secretory responses to 5 s pulses with 35 or 100 mM-$K^+$ declined faster ($t_{\frac{1}{2}}$ of 3 and 6 s, respectively) upon $Ca_o^{2+}$ wash-out than those of methacholine. It seems, therefore, that $K^+$-evoked secretion depends on $Ca_o^{2+}$ entry in a manner more astringent than the muscarinic-mediated secretory response.

7. Secretion evoked by the selective nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP, 5 s pulses of 100 $\mu$M) declined with the time of $Ca_o^{2+}$ removal with a $t_{\frac{1}{2}}$ of 8–9 s. In the case of acetylcholine (100 $\mu$M for 5 s), the decline of secretion upon $Ca_o^{2+}$ removal exhibited a $t_{\frac{1}{2}}$ of 12 s.

8. In conclusion, the $Ca^{2+}$ required by the secretory machinery in cat chromaffin cells stimulated with acetylcholine, DMPP, methacholine or high $K^+$ seems to come mostly from the extracellular milieu. However, an intracellular $Ca^{2+}$ pool probably located in the smooth endoplasmic reticulum seems to contribute a small proportion to the secretory response triggered by muscarinic–cholinergic receptor stimulation. The mobilization of the intracellular $Ca^{2+}$ pool seems to be unable by itself to trigger a substantial secretory response in the absence of extracellular $Ca^{2+}$.

## INTRODUCTION

The $Ca^{2+}$ dependence and the source of $Ca^{2+}$ required to trigger adrenomedullary catecholamine release by muscarinic receptor stimulation is still controversial. For instance, in 1966 Poisner and Douglas demonstrated in the perfused cat adrenal gland that the absence of external $Ca^{2+}$ ($Ca_o^{2+}$) practically abolished the secretory responses to muscarine, pilocarpine and methacholine, and we have recently corroborated this finding (Borges, Ballesta & García, 1987; Ballesta, Borges, García & Hidalgo, 1989). On the other hand, Knight & Baker (1986) observed that in chicken adrenal cells both $Na^+$ and $Ca^{2+}$ ions were required to trigger the muscarine-evoked secretory response. Furthermore, Kim & Westhead (1989) observed that muscarine-evoked secretion from superfused bovine adrenal chromaffin cells was abolished in the absence of $Ca_o^{2+}$. In contrast, Harish, Kao, Raffaniello, Wakade & Schneider (1987) observed that muscarine-evoked secretion from perfused rat adrenals is well maintained in $Ca^{2+}$-free solutions, and Nakazato, Ohga, Oleshansky, Tomita & Yamada (1988) have presented some results in this direction: acetylcholine and pilocarpine evoked an initial secretory response in perfused guinea-pig adrenals when $Ca^{2+}$ was removed; however, in the case of pilocarpine the response was only 10% of that obtained in the presence of $Ca^{2+}$ and disappeared on challenging the gland for a second time.

Judging by the literature, the issue on whether extracellular $Ca^{2+}$ ions or rather $Ca^{2+}$ from intracellular storage sites ($Ca_i^{2+}$) are predominantly required to trigger and maintain the muscarinic-mediated secretory response, remains unsettled. We undertook again this problem based on the following premises:

(i) A model with a sound muscarinic receptor-mediated secretory response, the cat adrenal gland, was selected. This model has been widely used in various laboratories since the pioneering experiments of Dale (1914) and Feldberg, Minz & Tsudimura (1934), followed later by reports from Douglas & Poisner (1965) (who first demonstrated the preferential release of adrenaline by muscarinic stimulation), Lee & Trendelenburg (1967), Lee (1972), Kirpekar, Prat & Schiavone (1982), Borges, Sala & García (1986), Borges *et al.* (1987) and Ballesta *et al.* (1989).

(ii) A high rate of perfusion of the gland (about 6-fold higher than in previous reports) was applied, so that a better temporal resolution could be achieved to correlate the $Ca_o^{2+}$ wash-out periods in the range of few seconds with the decline of the secretory response to various secretagogues. This high rate of perfusion of the gland also permitted the stimulation of the gland with the very short pulses of solutions containing various secretagogues.

(iii) New experimental protocols have been used. For instance, the depletion of $Ca_i^{2+}$ stores using the ionophore ionomycin, or the time course of recovery of the muscarinic secretory response, upon $Ca^{2+}$ reintroduction, in glands previously depleted of $Ca_i^{2+}$ and $Ca_o^{2+}$ through perfusion with 0 $Ca^{2+}$-EGTA–ionomycin-containing solutions.

Though still indirect, this strategy provides information on the rate of loss and recovery of the muscarinic-mediated secretory responses under experimental conditions which are likely causing a gradual exhaustion of $Ca_i^{2+}$ and $Ca_o^{2+}$ pools in adrenal medullary chromaffin cells.

## METHODS

### *Chemicals*

Methacholine chloride, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), acetylcholine chloride, atropine, *d*-tubocurarine chloride, ionomycin and EGTA were obtained from Sigma Chemical Co., Spain. All other chemicals used were reagent grade.

### *Catecholamine release from perfused cat adrenal glands*

Cats (2–4 g in weight) were anaesthetized with sodium pentobarbitone (40 mg/kg I.P.). Both adrenal glands from each animal were isolated and prepared for their retrograde perfusion as previously described (García, Hernández, Horga & Sánchez-García, 1980). They were perfused at a rate of 6 ml/min with normal Krebs–Tris solution at 37 °C and pH 7·4, continuously bubbled with pure $O_2$, and having the following composition (mM): NaCl, 144; KCl, 5·9; $MgCl_2$, 1·2; $CaCl_2$, 2·5; Tris, 10; and glucose, 11.

Glands were initially perfused for 1 h with Krebs solution, allowing them to adapt to the novel '*in vitro*' conditions. Later, glands were stimulated by switching from the normal Krebs–Tris solution to another similar solution containing a secretagogue for a short period of time, usually 5 s. A pulse was given every 15 min. The control pulses were applied in normal Krebs solution at the beginning ($P_1$ and $P_2$) and the end of each experiment ($P_{13}$), or along the entire experiment in the control glands. The other pulses ($P_3$–$P_{12}$) were applied after switching to a $Ca^{2+}$-free Krebs solution for a period of time between 0 and 300 s; the stimulus consisted of a 0 $Ca^{2+}$ solution containing the secretagogue and the collection of samples was done in the 0 $Ca^{2+}$ solution. Then, glands were perfused with the normal Krebs solution until the next pulse (see protocol on top of Fig. 4). The composition of the $Ca^{2+}$-free solution differed from the normal one in the following: $MgCl_2$, 3·7 mM and $CaCl_2$, 0 mM; thus, when $Ca_o^{2+}$ was removed, $MgCl_2$ was increased to have the total concentration of divalent cations constant.

The secretagogues used fell into four category groups: (a) methacholine, a pure muscarinic agonist in the cat adrenal gland (100 $\mu$M for 5 s (longer times were also used in one protocol)); (b) acetylcholine, the physiological transmitter at the splanchnic–chromaffin cell synapse, serving as stimulant of both nicotinic and muscarinic receptors (100 $\mu$M for 5 s); (c) DMPP, a pure nicotinic agonist (10 or 100 $\mu$M for 5 s); high $K^+$ solutions (35 or 100 mM-$K^+$, with equimolar reduction of the concentration of $Na^+$), to recruit through direct depolarization the voltage-dependent $Ca^{2+}$ channels of chromaffin cells.

### *Assay of total catecholamines present in perfusate fluid samples*

The basal rate of catecholamine release was monitored immediately before the pulse, or before switching to the 0 $Ca^{2+}$ solution by collecting a 30 s sample. Catecholamines released during each pulse were collected in two to four additional 30 s samples after the beginning of the pulse.

These fluid samples were collected in acidified chilled tubes (0·05 N-perchloric acid, final), and

the catecholamines fluorometrically assayed, directly, in 1 ml aliquots using the iodine oxidation procedure described by Shellenberger & Gordon (1971) to develop the total catecholamine fluorescence. Fluorescence units were converted into nanograms of total catecholamines through the use of appropriate standards of the pure amines. Data are expressed as nanograms per pulse with their S.E.M. They reflect the sum of the catecholamines present in the two stimulation samples after subtracting from them the basal catecholamine release (before the pulse) to obtain the net catecholamine release. In some figures, data were normalized to 100% of the initial secretion obtained with each secretagogue in the presence of 2·5 mM-$Ca^{2+}$ or in 0 $Ca^{2+}$ solution. Statistical differences between means were calculated by using Student's $t$ test for non-paired data. Level of significance was established at the value of $P < 0{\cdot}05$.

## RESULTS

### *Secretory responses to methacholine as a function of the length of its pulses*

The aim of these preliminary experiments was to define reproducible secretory responses to methacholine with the shortest possible stimuli. This could be achieved thanks to the high rate of perfusion of the gland (6 ml/min). Glands were challenged with 100 $\mu$M-methacholine pulses of increasing duration (5–60 s) at 15 min intervals, and in the presence of normal Krebs–Tris solution. As seen in Fig. 1, the secretory responses were proportional to the duration of the pulse; the longer the stimulation time, the greater the release of catecholamines, suggesting that, contrary to nicotinic responses, muscarinic-meditated secretory responses undergo little desensitization (Kirpekar *et al.* 1982; Ladona, Aunis, Gandía & García, 1987). Since a substantial response was obtained with 5 s pulses, this time was selected for subsequent experiments. The shorter the duration of the pulses, the better discrimination between the times selected to deplete $Ca_i^{2+}$ and $Ca_o^{2+}$ before applying each stimulus.

The inset of Fig. 1 shows the profile of the secretory response to 5 s pulses of methacholine (100 $\mu$M). The basal secretion amounted to $60 \pm 5$ ng/30 s. The first 30 s sample including the 5 s pulse with methacholine contained about a 10-fold higher concentration of catecholamines; then, the secretion obtained in subsequent wash-out samples quickly declined to reach almost basal levels at the fourth sample collected.

### *The methacholine secretory effects are specifically mediated by muscarinic receptors*

That the methacholine-secretory effects are mediated only by muscarinic receptors is shown in Fig. 2*A*. Individual glands were sequentially challenged with methacholine pulses (100 $\mu$M for 5 s) in the absence (initial secretion) and the presence of increasing, cumulative concentrations of atropine or *d*-tubocurarine. At $10^{-8}$ M, atropine already blocked the methacholine secretory effects completely. So, it can be inferred that the concentration giving 50% of maximal inhibition, $IC_{50}$, for atropine to block the methacholine secretory effects was in the nM range. In contrast, the nicotinic receptor blocker *d*-tubocurarine, if anything, facilitated the secretory effects of methacholine. The opposite occurred with DMPP, whose secretory actions were dose-dependently inhibited by *d*-tubocurarine and decreased only at concentrations of atropine of 1 $\mu$M or over. Thus, we are pretty sure that in the present experimental conditions (i.e. 5 s pulses of 100 $\mu$M-methacholine) we are dealing with a fairly pure muscarinic receptor-associated secretory response.

*Decline of secretory response to delayed additions of methacholine after calcium removal*

These experiments were performed to obtain information on how fast the secretory response disappeared upon removal of external $Ca^{2+}$ ions from the medium perfusing

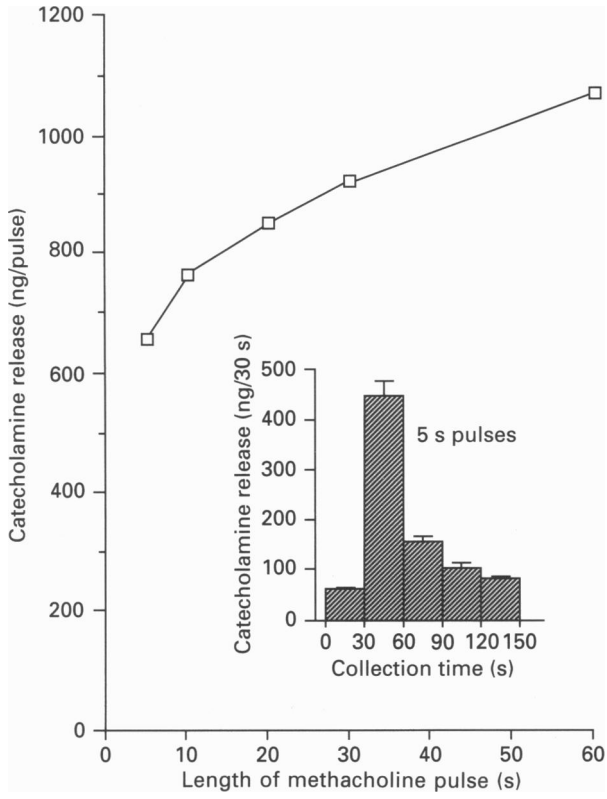


Fig. 1. Secretion of catecholamines evoked by methacholine pulses (100 $\mu$M) of the duration shown on the abscissa. After equilibration, each individual gland was sequentially stimulated at 15 min intervals with methacholine. Total catecholamine released in each pulse was estimated by collecting four 30 s samples during and after the drug pulse; a sample pre-pulse of 30 s duration was also collected to measure the basal output of catecholamines, which was subtracted from the evoked-release samples to obtain the net catecholamine release. Data are means of two glands. Inset: profile of secretion obtained in thirty-six glands with 5 s pulses of 100 $\mu$M-methacholine. Data are means $\pm$ S.E.M.

the gland. They were designed so that a series of thirteen subsequent 5 s pulses of 100 $\mu$M-methacholine at 15 min intervals ($P_1$–$P_{13}$) was applied to the same gland. Before carrying out any ionic manipulation it was necessary to test whether, in the presence of 2·5 mM-$Ca^{2+}$, there were any major changes in the extent of methacholine secretion from $P_1$ to $P_{13}$. This is shown in Fig. 3, where it can be appreciated that along the thirteen pulses, the secretion of catecholamines was maintained between 500 and 600 ng/pulse.

The decline of secretory responses to delayed additions of methacholine after $Ca^{2+}$ removal was studied according to the protocol shown on top of Fig. 4. Again, thirteen methacholine pulses were applied to the gland. $P_1$ and $P_2$ were given in normal Krebs–Tris solution, to test the extent of the secretory response in normal conditions

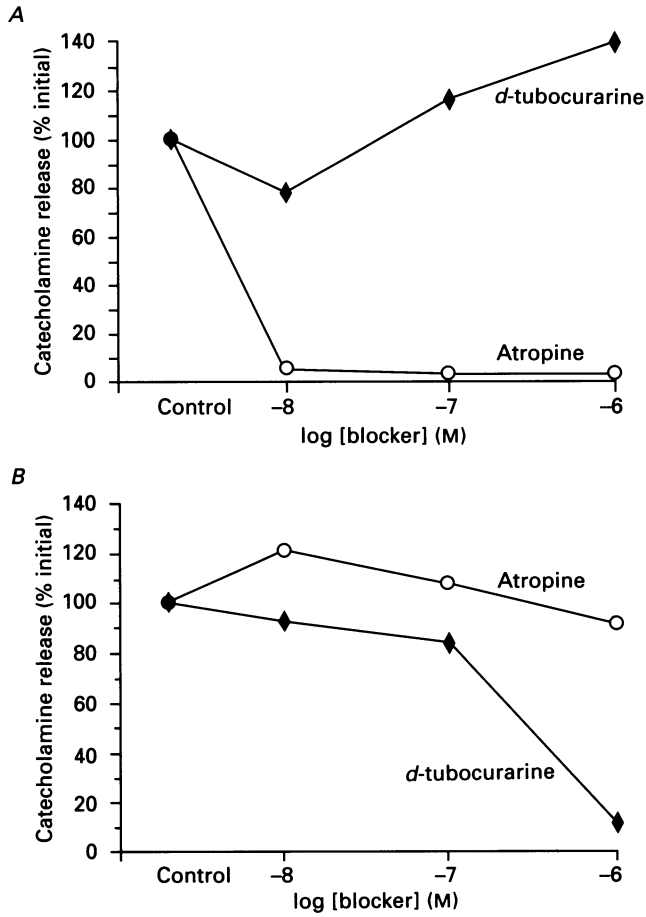


Fig. 2. Effects of atropine and *d*-tubocurarine on the secretory responses to methacholine (*A*) or DMPP (*B*). In *A*, sequential pulses of methacholine (100 $\mu$M for 5 s) were given at 15 min intervals in the absence (initial secretion) and the presence of increasing cumulative concentrations of atropine or *d*-tubocurarine (abscissa). In *B*, a similar experiment was performed but DMPP pulses (10 $\mu$M for 5 s) were used to trigger secretion. Data are normalized to a percentage of the initial secretion obtained in the absence of blocking agents; they are representative of single glands.

(2·5 mM-$Ca^{2+}$ present before, during and after methacholine stimulation). In $P_3$, $Ca_o^{2+}$ was suddenly decreased to 0 mM (nominal, compensated for by equimolar concentrations of $Mg^{2+}$), at the same time as the methacholine pulse was applied. Samples to collect the released catecholamines were also in 0 $Ca^{2+}$. Once $P_3$ ended, glands were perfused with normal Krebs solution for 15 min. A 2 s period of perfusion with 0 $Ca^{2+}$ solution preceded $P_4$. From $P_5$ to $P_{11}$ this period in 0 $Ca^{2+}$ solution was

increased to 5, 10, 20, 30, 60, 120 and 300 s respectively. Finally, $P_{12}$ was given following the same protocol as for $P_3$, and $P_{13}$ imitated $P_1$ and $P_2$.

When methacholine was applied simultaneously with $Ca^{2+}$ deletion ($P_3$ in Fig. 4) catecholamine release decreased by 30% with respect to the control release ($P_1$ and

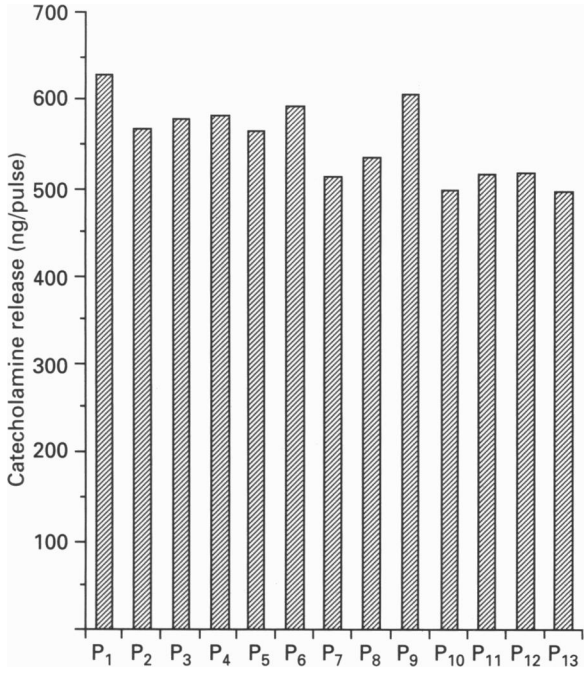


Fig. 3. Reproducibility of the secretory response to 5 s pulses of methacholine (100 $\mu$M) applied at 15 min intervals to adrenals perfused continuously during the entire experiment with normal Krebs–Tris solution containing 2·5 mM-$Ca^{2+}$. Data are expressed as net catecholamine release (evoked minus basal secretion) in ng/pulse (ordinate); they are the means of results obtained in two glands.

$P_2$). Then, as the time of $Ca^{2+}$ deletion increased before the methacholine challenge, secretion declined gradually to reach 15% of the initial secretion after a $Ca_o^{2+}$ wash-out period of 60 s. It is interesting to note that though small, a secretory response was preserved even after 2–5 min wash-out of $Ca_o^{2+}$. In $P_{12}$ (simultaneous deletion of $Ca_o^{2+}$ and methacholine stimulation) a partial recovery of secretion was observed. Recovery was complete in $P_{13}$ (methacholine challenge in the presence of 2·5 mM-$Ca^{2+}$).

*Effects of EGTA and ionomycin on the decline of secretory responses to delayed additions of methacholine after calcium removal*

We reasoned that a $Ca^{2+}$ chelator should clean up the extracellular $Ca^{2+}$ quicker during the pre-methacholine period of $Ca_o^{2+}$ wash-out, and that this manipulation could accelerate the rate of decline of methacholine responses. When EGTA (1 mM) was present in the 0 $Ca^{2+}$ solution, the rate of decline of methacholine responses as a function of the time of exposure to 1 mM-EGTA was slightly accelerated with

respect to that in 0 $Ca^{2+}$ (Fig. 5). Thus, the $t_{\frac{1}{2}}$ responses declined from 15 s in 0 $Ca^{2+}$ to 8 s in 0 $Ca^{2+}$-EGTA (Table 1). However, the secretory response was not abolished even after 120 s wash-out with 0 $Ca^{2+}$-EGTA, suggesting that in addition to the simple wash-out of extracellular $Ca^{2+}$, some other factor must contribute a small

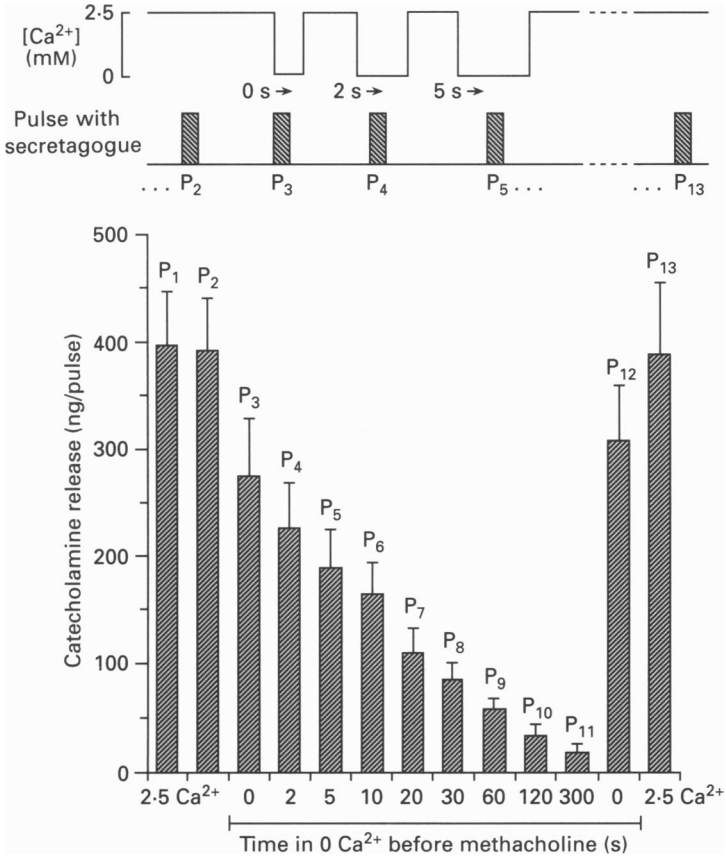


Fig. 4. Secretory responses to delayed additions of methacholine (100 $\mu$M for 5 s) after $Ca^{2+}$ removal for the times shown in the abscissa. The experimental protocol is described on top of the figure. See text for further details. Data represent the net catecholamine released (evoked minus basal) by each methacholine challenge ($P_1$–$P_{13}$); they are means ± S.E.M. of seven glands.

percentage to the secretory response, i.e. intracellular methacholine-sensitive $Ca^{2+}$ pools.

Since the $Ca^{2+}$ ionophore ionomycin quickly depletes $Ca_i^{2+}$ from intracellular stores (Fonteríz, López, García-Sancho & García, 1991), we performed experiments in which the 0 $Ca^{2+}$ solution used to wash out $Ca_o^{2+}$ before challenging the gland with methacholine contained 1 $\mu$M-ionomycin. At earlier times of washing out with ionomycin solution (2–10 s) there was a delay in the decay of methacholine-evoked secretion (Fig. 5). This was likely to be due to $Ca_o^{2+}$ entry inside chromaffin cells provoked by ionomycin from the extracellular milieu still insufficiently free of the cation, and/or to $Ca^{2+}$ mobilization by ionomycin from intracellular stores (Fonteríz

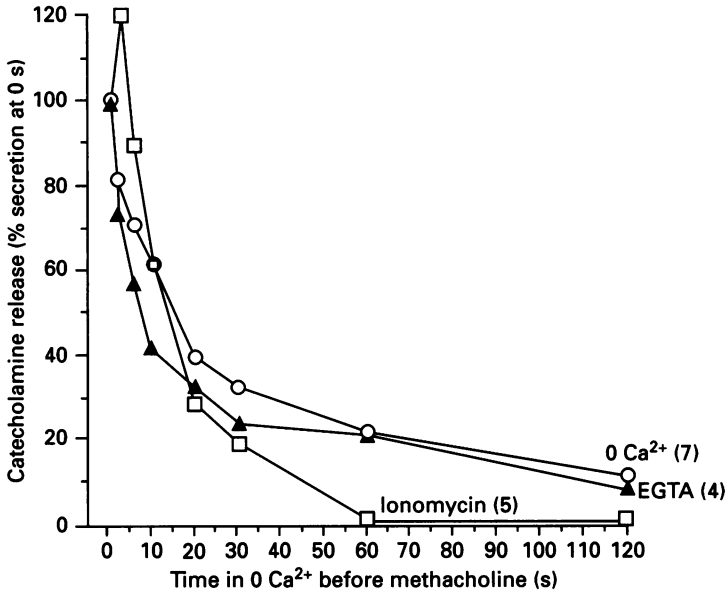


Fig. 5. Secretory responses to delayed additions of methacholine (100 $\mu$M for 5 s) after $Ca^{2+}$ removal for the time length shown in the abscissa. Experimental protocol was as in Fig. 4, but the 0 $Ca^{2+}$ solution contained in one case EGTA (1 mM) and in the other ionomycin (1 $\mu$M). Data were normalized as a percentage of secretion obtained when methacholine is added simultaneously with $Ca^{2+}$ removal ($P_3$ in the protocol shown on top of Fig. 4). They are means ± S.E.M. of the number of glands shown in parentheses.

TABLE 1. Estimated $t_{\frac{1}{2}}$ for the decay of catecholamine release, provoked by increasing periods of $Ca^{2+}$ deletion before applying each secretagogue for 5 s

| Secretagogue | $n$ | Initial catecholamine release (ng/pulse) | $t_{\frac{1}{2}}$ for decay upon $Ca^{2+}$ removal (s) |
|---|---|---|---|
| Methacholine (100 $\mu$M) | 7 | 396 | 15 |
| Methacholine (0 $Ca^{2+}$-EGTA) | 4 | 428 | 8 |
| Methacholine (0 $Ca^{2+}$-ionomycin) | 5 | 343 | 14 |
| Acetylcholine (100 $\mu$M) | 3 | 768 | 13 |
| DMPP (10 $\mu$M) | 4 | 1307 | 8 |
| DMPP (100 $\mu$M) | 5 | 1649 | 9 |
| $K^+$ (35 mM) | 4 | 578 | 3 |
| $K^+$ (100 mM) | 4 | 3478 | 6 |

Approximate $t_{\frac{1}{2}}$ values were estimated from the curves drawn in Figs 5 and 7; $n$, number of glands used with each secretagogue. Protocol as in Fig. 4.

*et al.* 1991). At later times, however (30–120 s of ionomycin wash-out), the opposite occurred; the decline of secretion was accelerated. Overall, the $t_{\frac{1}{2}}$ calculated from the decline curve was 14 s, very similar to that seen without ionomycin. Though this parameter seemed to be unmodified, an interesting feature emerged from experiments with ionomycin. Catecholamine release was completely abolished at 60 and 120 s of wash-out periods. Thus, the 15% component of secretion refractory to the washing

out with 0 $Ca^{2+}$ or 0 $Ca^{2+}$-EGTA solutions, seems to be dependent on a functional intracellular $Ca^{2+}$ pool which is depleted only by ionomycin. Again, these findings are in accord with recent data from our laboratory measuring $Ca_i^{2+}$ transients in Fura-2-loaded bovine adrenal chromaffin cells. Cells suspended in 0 $Ca^{2+}$-EGTA solutions for 2–5 min retained intact an ionomycin-sensitive intracellular $Ca^{2+}$ pool.

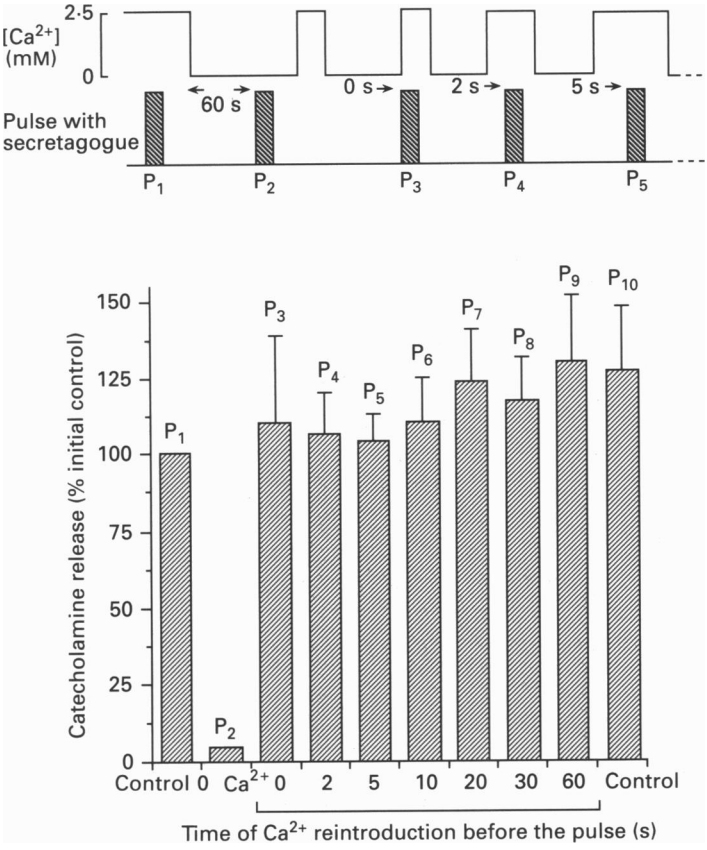


Fig. 6. Recovery of the methacholine secretory response upon $Ca^{2+}$ reintroduction to glands previously depleted of $Ca_o^{2+}$ and $Ca_i^{2+}$ by perfusing them for 60 s with a 0 $Ca^{2+}$ solution containing 1 mM-EGTA and 1 $\mu$M-ionomycin. Methacholine pulses (100 $\mu$M for 5 s) were applied simultaneously with the $Ca^{2+}$ reintroduction (0 s time in abscissa of $P_3$) or 2, 5, 10, 20, 30 or 60 s after reintroducing $Ca^{2+}$. Data are normalized to a percentage of the initial control secretion ($P_1$) obtained with a pulse of methacholine given in the presence of normal Krebs solution containing 2·5 mM-$Ca^{2+}$. They are means ± S.E.M. of four glands.

*Recovery of the secretory response from calcium-depleted glands upon the delayed addition of methacholine after calcium reintroduction*

The combination of EGTA and ionomycin, in the absence of $Ca_o^{2+}$ ($Mg^{2+}$ used as substitute), should cause the depletion of external as well as internal stores of $Ca^{2+}$; this is true in bovine chromaffin cell suspensions loaded with Fura-2 (Fonteríz *et al.* 1991). Under these conditions (perfusion for 60 s with a 0 $Ca^{2+}$ solution containing

1 mM-EGTA and 1 $\mu$M-ionomycin), methacholine is not provoking a significant secretory signal ($P_2$ in Fig. 6). After cell $Ca^{2+}$ depletion, reintroducing methacholine simultaneously with $Ca^{2+}$ ($P_3$ in Fig. 6) restores the secretory response to control levels. In subsequent secretory pulses, when $Ca^{2+}$ was reintroduced 2, 5, 10, 20, 30

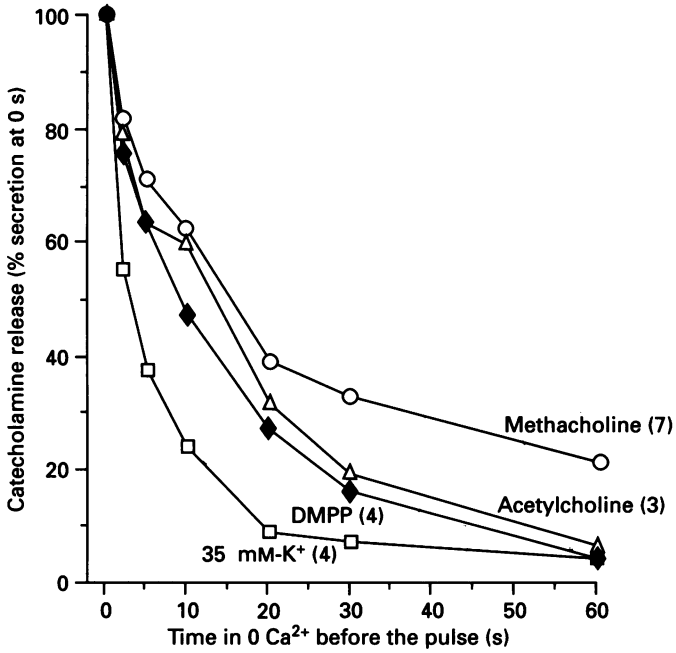


Fig. 7. Rates of decline of the secretory responses to delayed additions of methacholine (100 $\mu$M for 5 s), acetylcholine (100 $\mu$M for 5 s), DMPP (10 $\mu$M for 5 s) and $K^+$ (35 mM for 5 s), upon progressive wash-out of $Ca_o^{2+}$. Experimental protocol as in Fig. 4. In abscissa, the time of perfusion of the glands with 0 $Ca^{2+}$ solution before each secretagogue challenge is shown. Data were normalized to 100% of the secretion obtained with each secretagogue when added simultaneously with $Ca^{2+}$ deletion (pulse $P_3$ in protocol shown in Fig. 4). They are means $\pm$ S.E.M. of the number of glands shown in parentheses.

and 60 s prior to the methacholine pulse, the extent of the secretory response was similar to control levels. Thus, it seems that in chromaffin cells depleted of $Ca_i^{2+}$, the reintroduction of $Ca^{2+}$ almost instantaneously provokes the full recovery of the methacholine release response.

*Comparison of the rates of decline of the secretory responses to delayed additions of methacholine, acetylcholine, DMPP or potassium, upon progressive wash-out of extracellular calcium*

Experiments using a protocol similar to that in Fig. 4 (removal of $Ca_o^{2+}$ for increasing time periods, before applying a given stimulus also in the absence of this cation) were performed with high $K^+$ solutions. A concentration of $K^+$ (35 mM) and a duration of the pulse (5 s) was selected so that the size of its secretory responses was similar to that obtained with methacholine. Figure 7 shows that the secretory response to $K^+$ declined much faster than those to methacholine. The $t_{\frac{1}{2}}$ for the decay

was about 15 s for methacholine and only 3 s for 35 mM-$K^+$ (Table 1). Figure 7 also shows the decline curves for acetylcholine and DMPP which occupied an intermediate position between those of methacholine and 35 mM-$K^+$. Similar experiments were also performed using 100 $\mu$M-DMPP or 100 mM-$K^+$ for 5 s (not shown in Fig. 7, but see $t_{\frac{1}{2}}$ in Table 1).

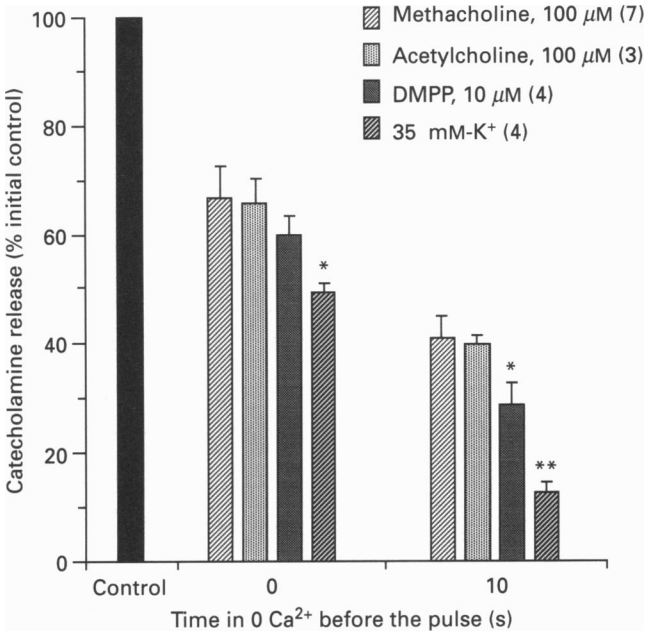


Fig. 8. Decrease of the secretory responses to various secretagogues after $Ca^{2+}$ depletion for 0 and 10 s. At 0 s, removal of $Ca^{2+}$ with simultaneous addition of the secretagogues was performed. At 10 s, glands were perfused for 10 s with 0 $Ca^{2+}$ before adding the secretagogue. Data were normalized to 100% of the initial secretion obtained with each secretagogue in the presence of 2·5 mM-$Ca^{2+}$ (mean of $P_1$ and $P_2$ in Fig. 4). Data are means ± S.E.M. of the number of glands shown in parentheses. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, both with respect to methacholine.

Table 1 shows the $t_{\frac{1}{2}}$ for the rates of decay of secretory responses to various secretagogues, upon washing out $Ca_o^{2+}$. They were estimated graphically by taking the figures closer to the 50% reduction of secretion from curves similar to those presented in Fig. 7. All secretory pulses lasted 5 s. The fastest rate of decay was observed with 35 mM-$K^+$ followed by 100 mM-$K^+$. An intermediate position between 35 mM-$K^+$ and methacholine was occupied by DMPP and acetylcholine.

In Fig. 8 a comparison is shown of the decline of secretion evoked by methacholine, acetylcholine, DMPP, and $K^+$ upon $Ca^{2+}$ removal. It seems that when the secretagogue is added simultaneously with $Ca^{2+}$ deletion (0 s in the histogram), the decrease in the secretory response observed is quite similar with methacholine, acetylcholine and DMPP, suggesting that a continued supply of $Ca_o^{2+}$ is required to maintain the secretory response once started. $K^+$ secretory responses declined faster even at 0 s ($P < 0{\cdot}05$ compared with methacholine). At later stages of $Ca^{2+}$ deletion

(i.e. 10 s), the decline of secretion is more rapid with $K^+$ and DMPP than with methacholine and acetylcholine, suggesting that the supply of $Ca^{2+}$ from intracellular sources to the secretory machinery, to maintain the secretory response longer, might be meaningful only for the muscarinic component of the acetylcholine-evoked secretion.

## DISCUSSION

By using manipulations aimed at restricting the availability of $Ca^{2+}$ to the secretory machinery, either from intracellular or extracellular sources, we expected to clarify the contribution of each $Ca^{2+}$ pool (intracellular or extracellular) to the triggering of the catecholamine release response mediated by muscarinic receptors in the cat adrenal medulla chromaffin cell. In the case of nicotinic or high $K^+$ stimulation, it seems that L-type, dihydropyridine-sensitive voltage-dependent $Ca^{2+}$ channels are fully responsible for the control of the cat adrenal medulla secretory response triggered by high $K^+$ (García, Sala, Reig, Viniegra, Frias, Fonteríz & Gandía, 1984) or nicotinic stimulation (Ladona *et al.* 1987). Therefore, it is plausible that only $Ca_o^{2+}$ passing through those channels is responsible for the triggering of catecholamine release evoked by DMPP or high $K^+$ stimulation. So, it seems logical that upon removal of $Ca_o^{2+}$, those responses are suppressed quickly. In fact, only 10 s of $Ca^{2+}$ removal led to a reduction of 70% of the secretory response to DMPP, and of 85% in the case of $K^+$.

The fact that the methacholine-evoked secretory response is somehow less susceptible to $Ca_o^{2+}$ removal than the nicotinic or high-$K^+$ responses, strongly suggests that some factors, other than the availability of $Ca_o^{2+}$, partially contribute to such response. However, at least from our experiments, a prominent role of $Ca_i^{2+}$ can be discarded. This conclusion differs from that stated in a previous paper using a similar strategy (Harish *et al.* 1987). These authors concluded that 'catecholamine secretion from perfused rat adrenal glands can occur in the absence of extracellular calcium, presumably by mobilization of intracellular calcium'. From data in this report, we estimated that the $t_{\frac{1}{2}}$ for the decline of rat adrenal secretion evoked by muscarine injections was in the range of several minutes. This contrasts with the $t_{\frac{1}{2}}$ of 16 s that we obtained in the cat adrenal. Species differences, or differences in the protocol, pattern of stimulation or in the rate of perfusion of the organs could explain this discrepancy.

### *An intracellular calcium pool mobilized by muscarinic stimulation of chromaffin cells*

Up to now, fluorescent probes to directly measure $Ca_i^{2+}$ transients in response to muscarinic or nicotinic stimulation have been used only in bovine chromaffin cells. It is unfortunate that the only animal species showing a poor (O'Sullivan & Burgoyne, 1989; Kim & Westhead, 1989) or a non-existent secretory response to muscarinic stimulation (Fisher, Holz & Agranoff, 1981; Almazan, Aunis, García, Montiel, Nicolás & Sánchez-García, 1984; Livett & Boksa, 1984; Cheek & Burgoyne, 1985; Ballesta *et al.* 1989) was selected to perform such studies. It is obvious that the reason for such selection was the convenient large size of the bovine adrenal gland in comparison with the small amount of medullary tissue available in adrenals from cat, rat or guinea-pigs; though these tissues contain chromaffin cells with healthy

secretory responses (see references in the Introduction), the small amount of tissue available precludes studies of $Ca_i^{2+}$ transients in cell suspensions. However, measurements of $Ca_i^{2+}$ transients in single chromaffin cells isolated from these animal species, should allow their comparison with $Ca_i^{2+}$ transient studies performed in single bovine chromaffin cells loaded with Fura-2 (Cheek, 1989; Cheek, O'Sullivan, Moreton, Berridge & Burgoyne, 1989; O'Sullivan, Cheek, Moreton, Berridge & Burgoyne, 1989). These authors concluded that nicotinic and high-$K^+$ stimulation produced a $Ca_i^{2+}$ transient localized exclusively to the entire subplasmalemmal area of the cell. This agrees with our conclusion that $Ca_o^{2+}$ entering chromaffin cells is the immediate source of $Ca_i^{2+}$ made available to the secretory machinery being activated by nicotinic or high-$K^+$ stimulation.

In contrast to nicotine or high $K^+$, Cheek *et al.* (1989) concluded that 'in response to the ineffective secretagogues methacholine and muscarine, the rise in $Ca_i^{2+}$ originated only in one pole of the cell and even at the peak of the response $Ca^{2+}$ was still generally restricted to this same area of the cell'. Therefore, to cause exocytosis, the $Ca_i^{2+}$ rise must meet some specific spatial requirements, i.e. it should occur underneath the plasma membrane.

Other studies performed in suspensions of bovine chromaffin cells demonstrated a $Ca_i^{2+}$ rise by muscarinic stimulation, even in the absence of $Ca_o^{2+}$ (Kao & Schneider, 1985, 1986; Kim & Westhead, 1989), without concomitant release of catecholamines. The conclusion was that '$Ca^{2+}$ entering across the plasma membrane was much more effective at triggering exocytosis than was $Ca^{2+}$ released from internal stores' (Kim & Westhead, 1989).

Our results in the cat adrenal gland are in line with the suggestion of the importance of $Ca_o^{2+}$ even for the muscarinic-mediated secretory response in the following points: (i) the secretory response to methacholine was very fast, reaching a peak in a few seconds. A secretory response purely secondary to intracellular messenger formation should be delayed and develop slowly; (ii) removal of $Ca_o^{2+}$ caused a fast decline in the secretory response to methacholine. The rate of decline was affected little in the presence of EGTA; (iii) $Ca_i^{2+}$ reintroduction into glands whose external and internal $Ca^{2+}$ stores were depleted, provoked an instantaneous recovery of the muscarinic-mediated secretion.

Though these results emphasize the importance of $Ca_o^{2+}$ at triggering not only the nicotinic-, but also the muscarinic-mediated secretory response, some experimental designs suggest some role for $Ca_i^{2+}$ mobilized from intracellular stores. For instance: firstly, $Ca_o^{2+}$ removal, with or without EGTA, leaves intact about 15–20% of the muscarinic secretory response; and secondly, the component resistant to $Ca_o^{2+}$ wash-out is cancelled by ionomycin, an ionophore known to deplete $Ca_i^{2+}$ from intracellular stores.

In conclusion, using new experimental designs we corroborate in this study our earlier suggestion that $Ca^{2+}$ coming from external stores is a primary event at triggering the release of catecholamines from chromaffin cells of the cat adrenal medulla stimulated with muscarinic agonists. The almost instant recovery of the methacholine secretory response upon $Ca_o^{2+}$ reintroduction, in glands depleted of $Ca_i^{2+}$ and $Ca_o^{2+}$, is in line with our earlier suggestion that muscarinic stimulation of cat adrenal chromaffin cells might be associated with the activation of a $Ca_o^{2+}$-permeable

ionophoric channel (Borges *et al.* 1987). The mobilization of $Ca^{2+}$ from intracellular stores contributes little to the extent of this secretory response; however, it might be a contributing factor in the modulation of such response. The present study permits us to guess that important differences might exist between bovine and feline chromaffin cells, as far as the spatial distribution of $Ca_i^{2+}$ upon muscarinic stimulation is concerned. Only the use of video-imaging techniques in Fura-2-loaded single cells in both secreting (cat) and non-secreting cells (bovine), might clarify such differences.

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