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Effects of Various Neurotransmitters on the Intracellular Cacium Concentration in Trigeminal Ganglion Neurons

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Functional neurotransmitter receptors which elevate intracellular free calcium concentration ($[Ca^{2+}]_i$) have been identified in freshly isolated trigeminal ganglion (TG) neurons in neonatal rat pups. Measurement of $[Ca^{2+}]_i$ was performed using fura-2 acetoxymethyl ester (Fura-2) based ratio microspectrofluorimetry. In our experiments only small sized neurons (<30 μm) according to somatic diameter were used. In single intact neurons, the expression percentage and magnitudes of $[Ca^{2+}]_i$ increase responding to each agonist were determined. Application of bradykinin (BK), substance P (SP), calcitonin gene related peptide (CGRP), ATP and capsaicin elevated $[Ca^{2+}]_i$ in the expression percentages of 24%, 37%, 31%, 77% and 50%, respectively, and elevated $[Ca^{2+}]_i$ from 1.63±0.04 (Mean±S.E) to 1.92±0.05, from 1.36±0.08 to 1.88±0.12, from 1.33±0.03 to 1.61±0.09, from 1.35±0.04 to 2.2±0.15 and from 1.26±0.06 to 2.9±0.26, respectively. The purinergic receptor which respond to ATP was most frequently expressed (77%). A $[Ca^{2+}]_i$ increase in response to all neurotransmitters was transient in nature, except in response to capsaicin. Increase of $[Ca^{2+}]_i$ evoked by capsaicin was not transient: $[Ca^{2+}]_i$ was maintained at a higher concentration than the prestimulus level. The magnitude of $[Ca^{2+}]_i$ increase evoked by capsaicin was bigger than for any other neuroransmitters. Our results show that a subpopulation of trigeminal ganglion neurons with small diameter express functional receptors responsible for elevating $[Ca^{2+}]_i$ coexisting with other neurotransmitter receptors in the early neonatal stage. Our data may have implications for studying orofacial pain transmission which is associated with intracellular calcium increase.

Key words : neurotransmitter, intracellular calcium, trigeminal ganglion, micro-spectrofluorimetry

Introduction

Orofacial sensations are transmitted to the CNS through the trigeminal ganglion (TG) neurons and many neurotransmitters are involved in this sensory pathway. Calcitonin gene-related peptide (CGRP) was found to be released from cultured rat trigeminal ganglion cells (Mason *et al.*, 1984) and glutamate- immunoreactivity was also found in the trigeminal root ganglia (Kai-Kai and Howe, 1991). Lee *et al.* (1985) reported substance P coexisting with CGRP in the rat peripheral nervous system. Also, CCK-like immunoreactivity coexisting with substance P was observed in ocular sensory neurons (Yasuaki and Richard, 1986). Recently, Park *et al.* (1995) supported previous studies by finding that cholecystokinin was colocalized with substance P or CGRP in the trigeminal ganglion. Among the various sensory neuron populations, C and Aδ type neurons are known to be involved in pain transmission from orofacial regions and they belong to small to intermediate sized subneuronal populations.

Intracellular calcium plays an important role in sensory neurons. Increase of intracellular calcium activates release of neurotransmitters at the synaptic terminal (Smith and Augustine, 1988) and regulates neuronal excitability by affecting the membrane conductances including Ca^{2+} activated ion channels (Martty, 1989). Elevation of postsynaptic calcium activated synaptic transmission, which is related to long-term potentiation (Malenka *et al.*, 1989). Calcium ions also have a role in the regulation of mammalian intra-mitochondrial metabolism (McCormack *et al.*, 1990). Transcriptional programs could also be coordinated by N-methyl-D-aspartate sensitive glutamate receptor stimulation in primary cultures of cerebellar neurones (Szekely *et al.*, 1990). In spite of the critical role of intracellular calcium in primary afferent neurons, effects of various neurotransmitters on intracellular concentration of free calcium have not been sufficently studied.

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Recently, some of the receptor agonists have been shown to elevate $[Ca^{2+}]_i$ in dorsal root ganglion neurons from neonatal rats (Bowie *et al.*, 1994). However, there has been no attempt to study the effects of various neurotransmitters on the $[Ca^{2+}]_i$ in trigeminal ganglion neurons.

The aim of this experiment is to determine how functional neurotransmitter receptors which elevate intracellular Ca^{2+} are expressed especially in neonatal rats. For this purpose, a variety of receptor agonists - substance P, bradykinin, CGRP, ATP and capsaicin - were tested for their ability to increase $[Ca^{2+}]_i$ measured using microscope photometry. We recorded all $[Ca^{2+}]_i$ from only small neurons (< 30 μ), since we were interested in the effects of various agonists on $[Ca^{2+}]_i$, particularly emphasized on orofacial pain transmission. Part of this work was published in abtract form in the 27 th Annual Meeting Society for Neuroscience, 1997 (Lee *et al.*, 1997).

Materials and Methods

Preparation of trigeminal ganglion (TG) neurons

After decapitation of neonatal (three to seven day old) Sprague-Dawley rats, TGs of both sides were removed. The TGs were then immersed in a cold Ca^{2+}/Mg^{2+}-free phosphate buffered saline (CMF-PBS) solution which was oxygenated with a 95/5% gaseous mixture of O_2/CO_2. The connective tissues surrounding the neurons were carefully removed with fine forceps. The TGs were then chopped into small pieces and incubated for 20 min at 37°C in a collagenase (0.2 mg/ml)/ dispase (0.4 u/ml) enzyme mixture. After enzymatic treatment, TGs were washed 2 times with Ca^{2+}/Mg^{2+}-free PBS solution and suspended in 2 ml of Dulbecco's Modified Eagle's Medium (Sigma, U.S.A) for trituration. The trituration was done by 5 times passages with a series of fire-polished Pasteur pipettes.

Fluorescent dye loading and $[Ca^{2+}]_i$ measurement

A microscope photometry technique with Ca^{2+} sensitive dye, fura-2 acetoxymethyl ester (fura-2, Sigma) was used to determine $[Ca^{2+}]_i$. Isolated TG neurons were loaded by 5 μM fura-2 in Tyrode balanced salt solution of 2 ml for 30 min at room temperature (25°C). After washing the neurons with Tyrode balanced salt solution 3 times, the TG neurons were kept on ice until use. For experiments, only small diameter TG neurons (< 30 μ) were chosen using the scale indented eyepiece of microscope. After TG neurons adhered to the bottom of the tissue bath, precoated with Cell-Tak, they were visualized using an epifluorescence microscope (Axiovert 10, Zeiss, Germany). For excitation of the fluorescent probe fura-2, a collimated beam of light from a 75 W Xenon arc lamp was passed through a dual spectrophotometer (Photon Technology International; PTI, U.S.A.). This altered wavelengths from 340 to 380 nm by means of a spinning chopper (60Hz). The emitted light, selected at 510 nm, was collected by a photomultiplier.

Data analysis

Data were analysed using Felix software (PTI, U. S.A). To estimate $[Ca^{2+}]_i$ from dual-wavelength fluorescence data, some data were converted to concentrations using a calibration protocol, which was performed with TG neurons using calcium-ionophore, 10 μM ionomycin and 5 mM EGTA.

Solutions

The $[Ca^{2+}]_i$ measurement was done in the bath when the solution was perfused at 37°C. The flow rate was 1 ml/min and the cell chamber capacity was 300 μl. All the agonists was added to normal bath solution and the concentration mentioned in this experiment is the final concentration. The composition of control Tyrode solution was (mM/L); NaCl 120, KCl 5, $CaCl_2$ 5, $MgCl_2$ 1, glucose 10, HEPES (free acid) 5. The pH was adjusted to 7.4 pH with NaOH. Calcium free Tyrode solution was made by omitting $CaCl_2$ and adding I mM EGTA. All the reagents of collagenase, fura-2, substance P, bradykinin, calcitonin gene related peptide (CGRP), ATP, capsaicin and thapsigargin were purchased from Sigma except dispase (grade II), which was purchased from Boehringer Manheim (Germany).

Results

Increase of $[Ca^{2+}]_i$ evoked by depolarization

Fig. 1 shows a transient increase of $[Ca^{2+}]_i$ in a single TG neuron evoked by high K bath solution (50 mM KCl) for 10 sec (starting points are indicated by arrow). The KCl is replaced with an equimolar amount of NaCl in the normal bath solution. The number of Y axis means values of 340/380 nm ratio fluorescence of Ca^{2+}. As shown in this figure, $[Ca^{2+}]_i$ rapidly increased from 1.5

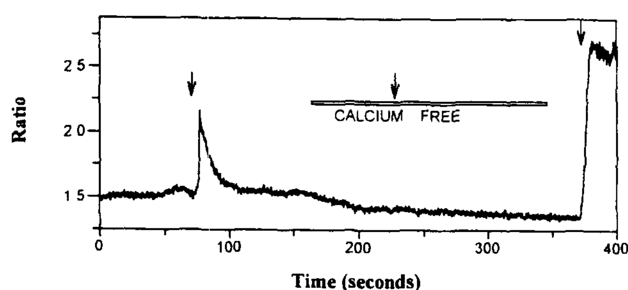

Fig. 1. Transient increase of $[Ca^{2+}]_i$ in a single TG neuron evoked by 50 mM KCl. Arrow indicates the time when bath solution was changed to high K. Under the Ca^{2+} free solution (white bar), an increase of $[Ca^{2+}]_i$ is not observed. The number on the Y axis means values of 340/380 nm ratio of Ca^{2+}.

(110 nM) in the resting state to 2.2 (380 nM) by 50 mM KCl and decreased to prestimulus level of $[Ca^{2+}]_i$ over 50 sec. However, in the Ca^{2+} free bath solution, increase of $[Ca^{2+}]_i$ was not observed by 50 mM KCl (second arrow on the white bar). When bath solution was changed again to normal bath solution which contain 5 mM Ca^{2+}, $[Ca^{2+}]_i$ increased to 2.7 (580 nM).

Depolarization induced $[Ca^{2+}]_i$ increase was always observed from the neurons we tested (n=6). The average increase of $[Ca^{2+}]_i$ was from 1.4±0.05 to 2.58±0.16. Our results indicate that depolarization induced increase of $[Ca^{2+}]_i$ is due to Ca^{2+} influx from the extracellular bath solution and suggests existence of voltage-dependent Ca^{2+} channels in these neurons.

Increase of $[Ca^{2+}]_i$ evoked by various neurotransmitters

We tested effects of BK, SP, CGRP and ATP on the $[Ca^{2+}]_i$. Fig. 2A shows a transient increase of $[Ca^{2+}]_i$ by stimulation with 1 μM BK followed by 1 μM SP in a single TG neuron. The 1 μM BK clearly increased $[Ca^{2+}]_i$ from 1.62 (140 nM) to 1.89 (275 nM). When the $[Ca^{2+}]_i$ was decreased to the prestimulus level, the TG neuron was stimulated again with 1 μM SP. The 1 μM SP also increased $[Ca^{2+}]_i$ to 1.88 (260 nM) which indicates that BK and SP functional receptors coexist in the same neuron.

Fig. 2B shows a representative transient increase of $[Ca^{2+}]_i$ by 1 μM CGRP and 0.1 mM ATP. The 1 μM CGRP increased $[Ca^{2+}]_i$ from 1.28 (60 nM) in resting state to 1.55 (175 nM). When the $[Ca^{2+}]_i$ was decreased to prestimulus level after washout, the neuron was stimulated again with 0.1 mM ATP,

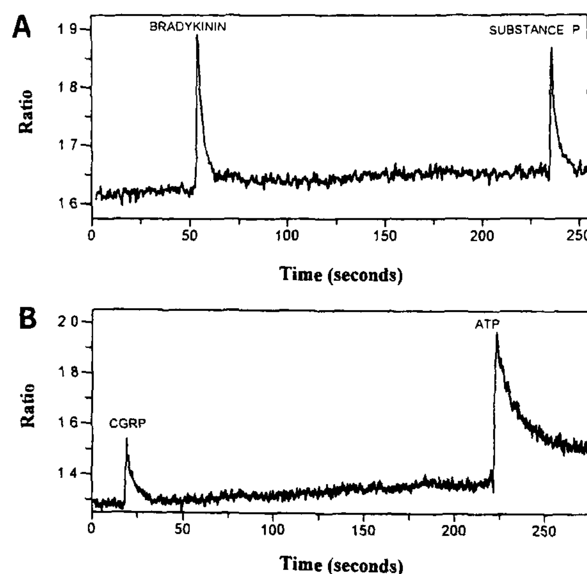

Fig. 2. (A) A representative figure of transient increase of $[Ca^{2+}]_i$ evoked by 1 μM BK and 1 μM SP in a single TG neuron. (B) Increase of $[Ca^{2+}]_i$ evoked by 1 μM CGRP and 0.1 mM ATP.

which greatly increased $[Ca^{2+}]_i$ to 1.98 (300 nM). The magnitude of $[Ca^{2+}]_i$ increase associated with the use of ATP was much bigger than with other neurotransmitters, i.e., BK, SP and CGRP.

Increase of $[Ca^{2+}]_i$ evoked by capsaicin

An increase of $[Ca^{2+}]_i$ from 1.2 to 2.3 was evoked by 0.1 μM capsaicin for 15sec (Fig. 3A). In contrast to other agonists, the increase in $[Ca^{2+}]_i$ was not transient but was maintained at a higher level (2.1) than the prestimulus level after a short peak transient. However, the high plateau of $[Ca^{2+}]_i$ decreased to prestimulus level over 5 min in the Ca^{2+} free solution. Fig. 3B shows transient increase of $[Ca^{2+}]_i$ associated with 1 mM ATP, 50 mM KCl and 1 μM capsaicin, to compare the magnitudes of $[Ca^{2+}]_i$ increase and time courses evoked by three different stimulations. Each agonist increased from the prestimulus level of 1.25 to 2.25, 2.5, and 2.5, respectively. In contrast to the transient increase of $[Ca^{2+}]_i$ associated with ATP and 50 mM KCl, increased $[Ca^{2+}]_i$ evoked by capsaicin was maintained at higher levels in all tested neurons (n=11).

Dose dependency and expression percentage of $[Ca^{2+}]_i$ increase evoked by various agonists

Fig. 4A and 4B show summarized data including dose dependency and expression percentage of

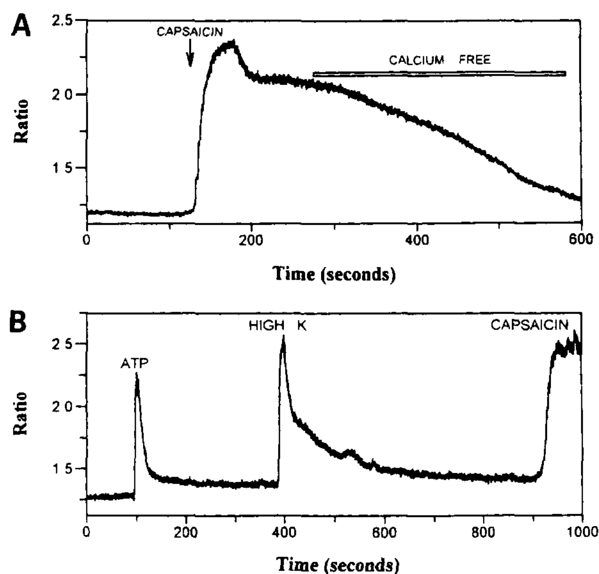

Fig. 3. (A) Increase of $[Ca^{2+}]_i$ by 0.1 μM capsacin. $[Ca^{2+}]_i$ was maintained at a higher level (2.1) than the prestimulus level after a short peak transient, but the plateau of $[Ca^{2+}]_i$ slowly decreased to a prestimulus level over 5 min under Ca^{2+} free. (B) shows coexistence of ATP and capsaicin receptors. Transient increase of $[Ca^{2+}]_i$ occurred with 1 mM ATP, 50 mM KCl and 1 μM capsaicin. Each stimulus increased $[Ca^{2+}]_i$ from prestimulus level of 1.25 to 2.25, 2.5 and 2.5, respectively.

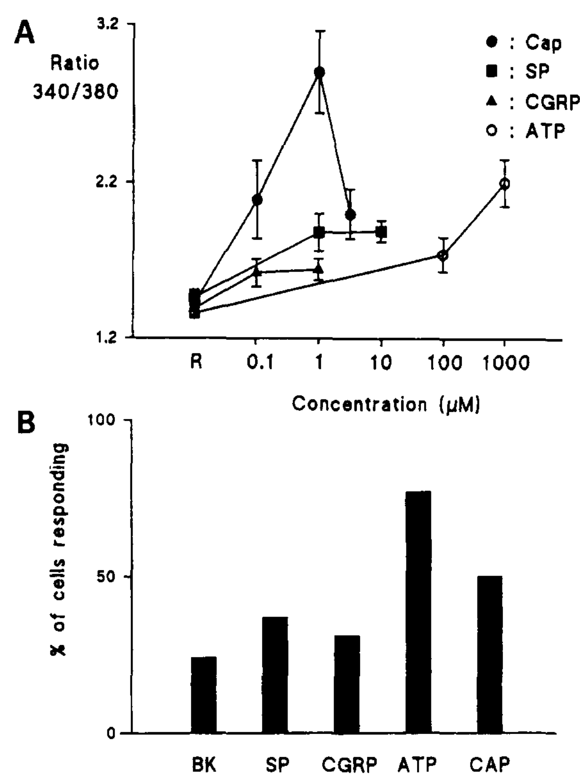

Fig. 4. (A) Dose dependency of agonists on elevating $[Ca^{2+}]_i$. Neither SP (■) nor CGRP (▲) show dose dependency at concentrations of 1 μM, 10 μM and 0.1 μM, 1 μM, respectively, but ATP (○) and capsaicin (●) show dose dependency at concentrations of 0.1 mM, 1 mM and 0.1 μM, 1 μM, respectively. (B) Expression percentage of trigeminal ganglion neurons responding to various agonists by elevating $[Ca^{2+}]_i$. The ATP receptor shows highest expression percentage (77%) and the BK receptor shows lowest expression percentage (24%).

each agonist on elevating $[Ca^{2+}]_i$. Transient increase of $[Ca^{2+}]_i$ by BK was observed in 6 of 25 neurons tested (24%, Fig. 4B). Neither SP (■) nor CGRP (▲) demonstrated dose dependency at concentrations of 1 μM, 10 μM, and 0.1 μM, 1 μM, respectively. Both 1 μM and 10 μM SP increased the average $[Ca^{2+}]_i$ from 1.36±0.08 (Mean±S.E) to 1.87±0.12 (n=7) and from 1.57±0.01 to 1.88±0.07 (n=8), respectively. Transient increase of $[Ca^{2+}]_i$ evoked by SP was observed in 15 of 40 total neurons (37%, Fig.4B). Meanwhile, 0.1 μM and 1 μM CGRP increased the average value of $[Ca^{2+}]_i$ from 1.33±0.03 to 1.62±0.09 (n=3) and from 1.41±0.06 to 1.64±0.07 (n=7). Transient increase of $[Ca^{2+}]_i$ evoked by CGRP was observed in 10 of 32 neurons (31%, Fig. 4B).

Contrary to the effect of SP and CGRP, both ATP (○) and capsaicin (●) showed clear dose dependency at concentrations of 0.1 mM, 1 mM and 0.1 μM, 1 μM, respectively. An increase in $[Ca^{2+}]_i$ from 1.4± 0.05 to 1.74±0.01 (n=4) and from 1.35±0.04 to 2.2±0.15 (n=11) was seen following application of 0.1 mM and 1 mM ATP, respectively. Maximal effect in elevating $[Ca^{2+}]_i$ was observed using 1 mM ATP. Of 18 neurons total, 14 neurons were responded to ATP (77%, Fig. 4B). Capsaicin increased $[Ca^{2+}]$i from 1.28±0.08 to 2.08±0.25 (n=4), from 1.26±0.06 to 2.9±0.26 (n=3) and from 1.7±0.13 to 2.0±0.16 (n= 3) at concentrations of 0.1 μM, 1 μM and 50 μM, respectively. We found that the maximal effect of capsain in elevating $[Ca^{2+}]_i$ occurred at 1 μM, and the effect of 5 μM capsaicin on elevating $[Ca^{2+}]_i$ was lower than 1 μM capsaicin. We could observe $[Ca^{2+}]_i$ increase evoked by capsaicin in 11 neurons of 22 neurons (50%, Fig. 4B).

Discussion

Intracellular free calcium concentration is strictly regulated in many exitable neurons, including cultured cerebellar neurons (Brorson *et al.*, 1991), sympathetic neurones in the bull-frog (Marrion and Adams, 1992), cultured spinal cord

neurones in the mouse (Mayer *et al.*, 1987), and dorsal root ganglion neurones in the rat (Thayer and Miller, 1990). The TG neurons convey sensory information which comes from the orofacial area, including pain and temperature. It might be that excitability of TG neurons, like DRG neurons also could be regulated by $[Ca^{2+}]_i$. We divided TG neurons into two groups, small and large, based on the diameter of their soma. We defined small neurons as being below 30 μ and large neurons as being more than 30 μ, criteria similar to those used for dividing TG neurons (Liu *et al.*, 1993). Bowie *et al.* (1994) reported that capsaicin increased $[Ca^{2+}]_i$ only in small (<17 μ) to intermediate sized neurons (<30 μ) and they were related to pain sensory transmission. It's known that C or Aδ fibers are involved in pain transmission and they belong to all small to intermediate sized neurons.

Depolarization induced increase of $[Ca^{2+}]_i$ was transient and was not observed in the Ca^{2+} free bath solution. However, when Ca^{2+} was added to the bath, $[Ca^{2+}]_i$ was increased again (Fig. 1). Our data indicate that the $[Ca^{2+}]_i$ increase induced by depolarization was due to Ca^{2+} influx. Two types of voltage dependent Ca^{2+} channels are reported in TG neurons (Oh, 1997). One is LVA I_{Ca} (low voltage activated calcium currents), which is blocked by amiloride, and the other is HVA I_{Ca} (high voltage activated calcium currents). The HVA I_{Ca} was further subclassified into L-type and N type according to sensitivity to blockade by nifedifine and ω-CgTx.

The profile of increase of $[Ca^{2+}]_i$ by various receptor agonists was similar, except for capsaicin. A transient increase in $[Ca^{2+}]_i$ was seen with use of BK, SP, CGRP and ATP; $[Ca^{2+}]_i$ rapidly increased following application of each neurotransmitter and gradually declined to the prestimulus level over 20~60 sec by spike broadening. We could find colocalization of BK and SP receptors (see Fig. 2A) and CGRP and ATP receptors in the same neurons (see Fig. 2B). Maximal response of ATP in elevating $[Ca^{2+}]_i$ was observed at 1 mM in the dose response curve in Fig. 4A. The percentage that respond to ATP by increasing $[Ca^{2+}]_i$ was also higher than any other receptor agonists (77%). The sequence of expression percentage of functional receptors was: ATP (77%)> capsaicin (50%)> SP (37%)> CGRP (31%)> BK (24%). The high expression percentages of capsaicin and SP, which are well known as neurotransmitters involved in pain transmission, correspond well with our results in the assumption that small neurons are related with pain. Our results showing an increase of $[Ca^{2+}]_i$ evoked by BK, SP and capsaicin was also similar to results seen using DRG neurons (Bowie *et al.*, 1994).

In contrast to the four receptor agonists, mentioned above, increase of $[Ca^{2+}]_i$ by capsaicin was not transient and showed biphasic states; rapid increase followed by transient small decrease, then sustained at a much higher $[Ca^{2+}]_i$ than prestimulus basal level. The effect of capsaicin on $[Ca^{2+}]_i$ was always irreversible, even at the lowest concentration of 0.1 μM. We found that sustained high level of $[Ca^{2+}]_i$ evoked by capsaicin was due to Ca^{2+} influx from the extracellular bath, since it decreased to prestimulus level in Ca^{2+} free bath solution (Fig. 3A). The maximal effect of capsaicin on elevating $[Ca^{2+}]_i$ was observed at 1 μM.

In summary, receptors of BK, SP, CGRP, ATP and capsaicin are expressed in neonatal TG neurons and all these receptors are involved in elevating $[Ca^{2+}]_i$, probably by Ca^{2+} influx via voltage dependent Ca^{2+} channels. Further study of the physiological roles of each neurotransmitter, particulary emphasizing orofacial pain transmission associated with intracellular calcium increase, is necessary.

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