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Mechanism of damnacanthal-induced $[Ca^{2+}]_i$ elevation in human dermal fibroblasts

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Abstract

Damnacanthal is a potent and selective inhibitor of $p56^{lck}$ tyrosine kinase in a variety of tissues. We have found, however, using the Ca^{2+} microfluorimetry technique, that damnacanthal releases intracellular Ca^{2+} stores and promotes Ca^{2+} entry in human dermal fibroblasts. The effect of damnacanthal on the peak $[Ca^{2+}]_i$ values and the latent time to the peak was concentration-dependent. Damnacanthal releases Ca^{2+} from thapsigargin-sensitive Ca^{2+} stores, and the Ca^{2+} stores responding to damnacanthal were overlapped with those of bradykinin. Damnacanthal-induced Ca^{2+} entry was mediated by voltage-dependent and voltage-independent Ca^{2+} channels. This effect of damnacanthal on intracellular Ca^{2+} mobilization was also observed in cultured bovine coronary endothelial cells but not demonstrated in freshly isolated rat basilar smooth muscle cells. Our study suggests that damnacanthal increases intracellular Ca^{2+} by releasing Ca^{2+} from internal stores and promoting Ca^{2+} entry. The relationship between the actions of damnacanthal on tyrosine kinase and intracellular Ca^{2+} requires further investigation. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Tyrosine kinase; Damnacanthal; Dermal fibroblast; Ca^{2+}, intracellular

1. Introduction

Tyrosine kinases are involved in the regulation of rapid tissue responses such as contraction (Di Salvo et al., 1997; Hughes and Wijetunge, 1998) and delayed responses such as gene transcription (Gilmour and Reich, 1995), cell proliferation (Marais and Marshall, 1996) and apoptosis (Anderson, 1997). Tyrosine kinase inhibitors are extensively used in experimental studies and possess the potential in the treatment of human disorders (Al-Obeidi et al., 1998; Lawrence and Niu, 1998).One of the Src tyrosine kinase inhibitors, damnacanthal, was reported having a potent and selective inhibitory effect on $p56^{lck}$ tyrosine kinase activity (Faltynek et al., 1995). Damnacanthal inhibits $p56^{lck}$ autophosphorylation and the phosphorylation of an exogenous peptide by $p56^{lck}$. In several recent studies, damnacanthal was used as inhibitor for Src tyrosine kinase in a variety of tissues (Hiwasa et al., 1997,1999; Yuan et al., 1998; Zubkov et al., 1999).

We have found, using Ca^{2+} microfluorimetry technique, that damnacanthal produced a $[Ca^{2+}]_i$ mobilization in cultured human dermal fibroblasts. The Ca^{2+} stores and the Ca^{2+} pathways responding to damnacanthal were investigated with different Ca^{2+} channel inhibitors. The effect of damnacanthal on Ca^{2+} was also compared in cultured endothelial and freshly isolated smooth muscle cells.

2. Materials and methods

2.1. Cell culture

Neonatal normal human dermal fibroblast cells (NHDF-Neo) were purchased from Clonetics (San Diego, CA). Cells were cultured in fibroblast basal medium, supplemented with 2% fetal bovine serum, 1 ng/ml basic human fibroblast growth factor, antibiotics (gentamicin 50 μg/ml, amphotericin-B 50 ng/ml) and 5 μg/ml insulin in a 5% CO_2 incubator.

NHDF-Neo between 3rd and 5th passages was subcultured onto 25-mm round coverslips, 3–5 days prior to the start of experiment. Twenty-four hours before $[Ca^{2+}]_i$ measurements, culture medium was replaced by serum-free medium.

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2.2. $[Ca^{2+}]_i$ microfluorimetry

The buffer solution for $[Ca^{2+}]_i$ measurement was (in mM): NaCl 145, $CaCl_2$ 2, KCl 3, $MgCl_2$ 1, HEPES 10, glucose 10 (pH was adjusted to 7.4 with NaOH). Cells were loaded with the fluorescence indicator fura-2/acetoxymethylester (AM) (3 μM) for 2 h at room temperature and washed for another 2 h in fura-2 free buffer solution. After post-loading, the coverslips were placed in the bottom of a Plexiglas perfusion chamber (volume ~600 μl) with two openings at each end for perfusion and aspiration.

Digital $[Ca^{2+}]_i$ imaging was performed by video microfluorimetry using a cooled CCD camera (Princeton Instruments, Trenton, NJ) coupled to a Nikon Ecripse microscope (40× Fluor objective, Nikon, New York, NY) and software (Universal Imaging, West Chester, PA) on a personal computer. Sample illumination was supplied by a 75 W-Xenon arc lamp, and excitation wavelength was selected by a computer controlled filter wheel. Fluorescence imaging was obtained with alternating excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Data from regions of interest were displayed in real time and recorded to hard disk. Background fluorescence obtained from a cell-free portion of the same coverslip was subtracted from all recordings before calculation of the 340:380 ratio. EGTA (0.1 mM) was included in all experiments using Ca^{2+}-free extracellular buffer. Subsequent analysis converted the ratio to the $[Ca^{2+}]_i$ according to the following calibration method. Standardized chelated Ca^{2+}-free and high Ca^{2+} buffers (Molecular Probes, Eugene, OR) were made into ten varying proportions, with 2 μM fura-2 salt added, covering the range of Ca^{2+} from 17 to 39,800 nM. Ratio values for 340 to 380 nm fluorescence were determined from droplets of these standard mixtures of known Ca^{2+}, with the objective focused approximately 20 μm above the glass coverslip and with background from fura-2 free solution. The values of R_{min}, R_{max}, and the apparent K_d were determined and these values were then used to convert ratio data to approximate $[Ca^{2+}]_i$ values.

2.3. Chemicals

Fura-2/AM was obtained from Molecular Probes (Eugene, OR). Damnacanthal was obtained from Biomol (Plymouth meeting, PA). Media and reagents for tissue culture were purchased from Clonetics. Nicardipine, econazole, lanthanum, thapsigargin, bradykinin and the other chemicals were purchased from Sigma (St. Louis, MO).

2.4. Data analysis

Data are expressed as mean ± S.E.M. Statistical differences between the control and other groups were compared by using one-way analysis of variance (ANOVA) and then Scheffe's F procedure if significant variance was found; a value of $P < 0.05$ was considered statistically significant. Applications of agents or changing solutions are indicated in all figures by horizontal lines.

3. Results

The experiments described below were conducted with NHDF-Neo. Original traces of $[Ca^{2+}]_i$ mobilization were shown with several typical responses from cells. To avoid possible signal decay, which occurs after about 60 min, all experiments were done within 30 min. To avoid the possible intracellular Ca^{2+} store depletion, only one agent and one concentration were tested in each coverslip. Damnacanthal was diluted with the extracellular Ca^{2+} microfluorimetry buffer solution before use.

3.1. Damnacanthal produces Ca^{2+} release and entry

Application of damnacanthal (0.5 ~ 30 μM) produced a peak $[Ca^{2+}]_i$ elevation which gradually and slightly declined to a level markedly above the resting $[Ca^{2+}]_i$ level in the presence of the extracellular Ca^{2+} (Fig. 1). This effect of DC lasted for 30 min, the longest time studied. The onset of the $[Ca^{2+}]_i$ response (latency period) to damnacanthal was related to the concentration of damnacanthal: it required 5 min to produce a $[Ca^{2+}]_i$ elevation for a lower dose of damnacanthal (such as 0.5 μM), but a few seconds for a high dose (such as 30 μM). In the absence of extracellular Ca^{2+}, damnacanthal produced a transient peak $[Ca^{2+}]_i$ response which returned

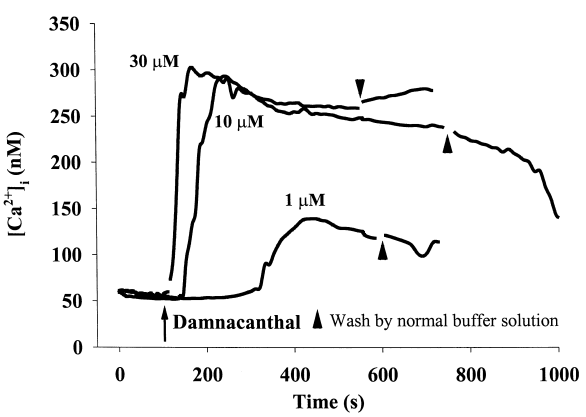

Fig. 1. Original tracings show the $[Ca^{2+}]_i$ elevation induced by damnacanthal (1, 10 and 30 μM) in NHDF-Neo in the presence of extracellular Ca^{2+}. The $[Ca^{2+}]_i$ response to damnacanthal is composed of a peak that is followed by a slightly reduced plateau $[Ca^{2+}]_i$ phase. The maximum $[Ca^{2+}]_i$ elevation to damnacanthal is concentration-dependent. The latency period (the time between the application of damnacanthal to the peak $[Ca^{2+}]_i$ response) of $[Ca^{2+}]_i$ response to damnacanthal is also concentration-dependent: a higher dose of damnacanthal produced a faster $[Ca^{2+}]_i$ elevation. The elevation induced by damnacanthal is largely resistant to washout with normal extracellular buffer solution.

more quickly to the basal level within 5 min (Fig. 2), indicating the plateau phase was Ca^{2+} entry from external space. The peak $[Ca^{2+}]_i$ value was significantly higher in the presence than in the absence of extracellular Ca^{2+} (Fig. 3), indicating the $[Ca^{2+}]_i$ peak was composed by both Ca^{2+} release from intracellular stores and Ca^{2+} influx from extracellular space. Re-administration of normal Ca^{2+} buffer after damnacanthal-stimulation triggered a large and quick Ca^{2+} influx (Fig. 2), which indicates that damnacanthal may deplete intracellular Ca^{2+} stores and depleted stores may in turn activate Ca^{2+} entry via voltage-independent or capacitative Ca^{2+} entry. An exception was observed that after high dose of damnacanthal (e.g., 30 μM) stimulation, the Ca^{2+} influx occurred slower than those occurred after other lower doses (Fig. 2).

The effect of damnacanthal on $[Ca^{2+}]_i$ was concentration-dependent, in the presence or absence of external Ca^{2+}, and 10 μM damnacanthal evoked an almost maximal response in the peak $[Ca^{2+}]_i$ response (Fig. 3). The onset of the peak $[Ca^{2+}]_i$ response (latency period), in the presence or absence of external Ca^{2+}, was also concentration-dependent that higher concentration of damnacanthal produced earlier $[Ca^{2+}]_i$ elevation (Fig. 3). The $[Ca^{2+}]_i$ elevation induced by damnacanthal in some cells was resistant to washout and this resistance was also concentration-dependent. Sustained $[Ca^{2+}]_i$ elevation remained after a 5 min washout with normal extracellular buffer, in 65.9, 76.2, 66, 27.3 and 9.3% of cells treated with 30, 10, 3, 1 and 0.5 μM of damnacanthal, respectively.

3.2. Damnacanthal releases Ca^{2+} from thapsigargin-sensitive stores

Ca^{2+} entry into cells in most of the cell types induces further Ca^{2+} release through ryanodine receptor from

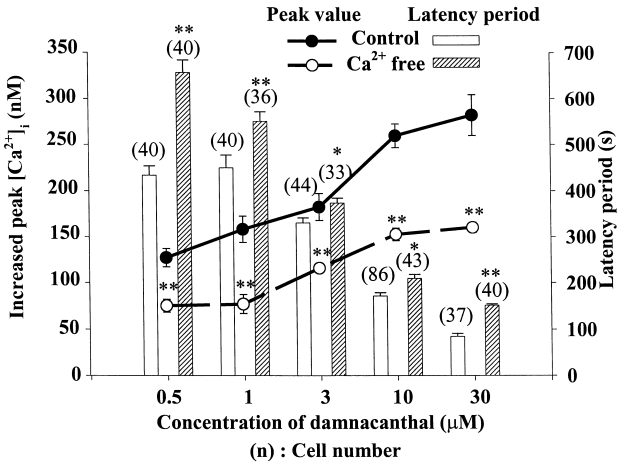

Fig. 3. Summary of the effect of damnacanthal on $[Ca^{2+}]_i$ in NHDF-Neo. The peak $[Ca^{2+}]_i$ value (left) and the latency period (right) to damnacanthal were concentration-dependent, in the presence and absence of extracellular Ca^{2+}. * or ** indicates $P < 0.05$ or $P < 0.001$ (vs. control, ANOVA). Bars represent S.E.M.

intracellular Ca^{2+} stores, and this event is called Ca^{2+}-induced Ca^{2+} release. Caffeine, an activator of Ca^{2+}-induced Ca^{2+} release, was applied to the cells but failed to produce any $[Ca^{2+}]_i$ elevation, at 3 mM ($n = 64$) and 10 mM ($n = 25$), in NHDF-Neo, indicating the lacking of Ca^{2+}-induced Ca^{2+} release in these cells (data not shown).

Thapsigargin produced a $[Ca^{2+}]_i$ elevation by inhibition of sarco-endoplasmic reticulum Ca^{2+}-ATPase (Treiman et al., 1998). In the absence of external Ca^{2+}, pretreatment of cells with thapsigargin (1 μM, 5 min) produced a transient Ca^{2+} elevation (not shown). Pre-treatment with thapsigargin abolished the peak $[Ca^{2+}]_i$ response and prolonged the latency period to damnacanthal (10 μM) applied subsequently, indicating that damnacanthal releases Ca^{2+} from the thapsigargin-sensitive Ca^{2+} stores. Fig. 4 summarized

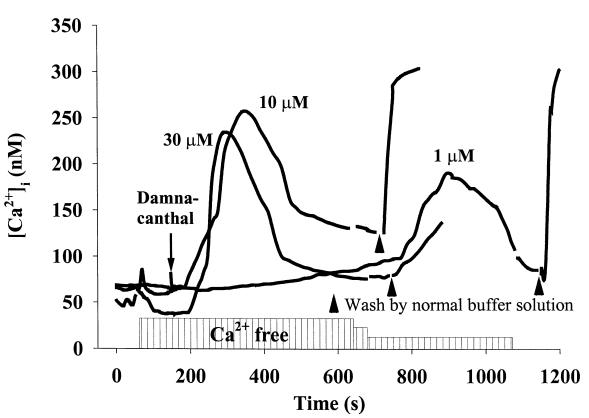

Fig. 2. Original tracings show the $[Ca^{2+}]_i$ elevation induced by damnacanthal (1, 10 and 30 μM) in the absence of extracellular Ca^{2+}. Damnacanthal produced smaller and transient peak $[Ca^{2+}]_i$ responses that decayed quickly to a level close or slightly above the base-line. Re-administration of the normal Ca^{2+} buffer triggered large and quick $[Ca^{2+}]_i$ elevations.

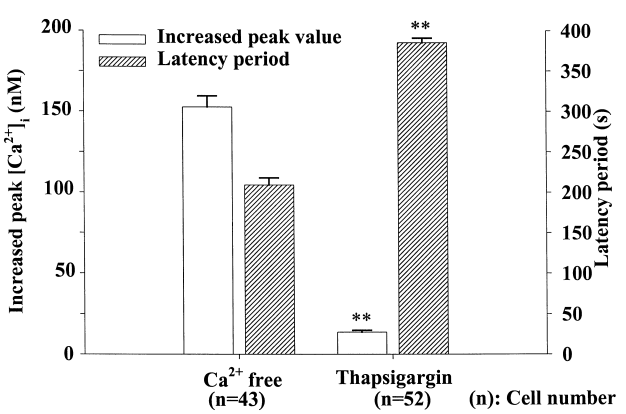

Fig. 4. The inhibitory effect of thapsigargin. The peak $[Ca^{2+}]_i$ elevation and the latency period to damnacanthal in the absence of extracellular Ca^{2+} was shown on the left side. Pre-incubation with thapsigargin doubled the latency period and almost abolished the $[Ca^{2+}]_i$ response to damnacanthal. ''*n*'' equals the number of cells studied. ** indicates $P < 0.001$ (thapsigargin vs. Ca^{2+} free) (ANOVA). Bars represent S.E.M.

the inhibitory effect of thapsigargin on damnacanthal-induced Ca^{2+} release.

3.3. Damnacanthal produces Ca^{2+} entry

The following studies were performed to identify the Ca^{2+} pathways responding to damnacanthal. Nicardipine, a voltage-dependent Ca^{2+} channel (VDCC) blocker, and econazole, a voltage-independent Ca^{2+} hhannel (VICC) inhibitor (a cytochrome *P*450 inhibitor), were incubated with cells for 2 min before application of damnacanthal in the presence of these inhibitors (Fig. 5). Nicardipine (1 μM) prolonged the latency period and reduced markedly the peak $[Ca^{2+}]_i$ responses to damnacanthal, however, without effect on the plateau phase of $[Ca^{2+}]_i$ elevation (Figs. 5 and 6). Econazole (10 μM) reduced both the peak and plateau $[Ca^{2+}]_i$ responses to damnacanthal without increase the latency period of damnacanthal (Figs. 5 and 6). These results suggested that VDCC and VICC pathways may be involved in damnacanthal-induced Ca^{2+} influx, and Ca^{2+} influx via VDCC may potentiate Ca^{2+} release from the intracellular Ca^{2+} store in the absence of Ca^{2+}-induced Ca^{2+} release. The Ca^{2+} entry pathway is mainly depend upon the econazole-sensitive Ca^{2+} pathway.

Divalent or trivalent cations such as nickel, cobalt, and lanthanum have been recognized to block the non-selective cation channels as well as VDCC, VICC and other Ca^{2+} pathways (Fasolato et al., 1994). Lanthanum (1 mM) was used in this study and failed to attenuate either the peak or the plateau $[Ca^{2+}]_i$ responses induced by damnacanthal (10 μM), but prolonged markedly the latency period of $[Ca^{2+}]_i$ response to damnacanthal (Figs. 5 and 6). Damnacanthal (10 μM) produced the peak $[Ca^{2+}]_i$ response within 4 min in control studies, in the presence of econazole, or in the absence of extracellular Ca^{2+}, but damnacanthal failed to produce any $[Ca^{2+}]_i$ response in the presence of lanthanum in the first 4 min (Fig. 6). The average latency of damnacanthal-induced $[Ca^{2+}]_i$ response in the presence of lanthanum or nicardipine was 8 and 6 min, respectively.

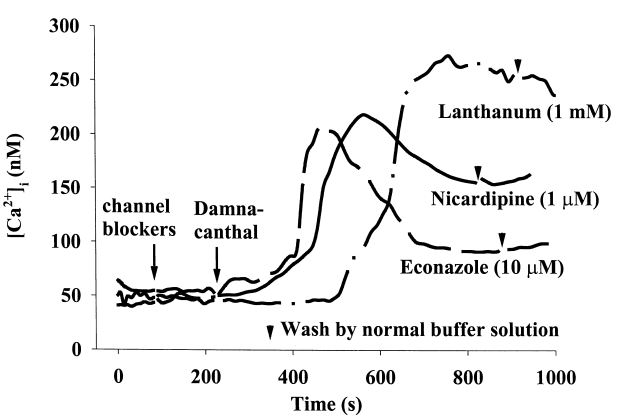

Fig. 5. Original tracings show the $[Ca^{2+}]_i$ elevation induced by damnacanthal in the presence of Ca^{2+} channel blockers. Damnacanthal produced smaller peak $[Ca^{2+}]_i$ responses in the presence of nicardipine and econazole. The $[Ca^{2+}]_i$ response to damnacanthal decayed quickly to a lower level in the presence of econazole but not nicardipine. Lanthanum prolonged the latency period to damnacanthal but failed to reduce either the peak or the plateau phase of $[Ca^{2+}]_i$ elevation.

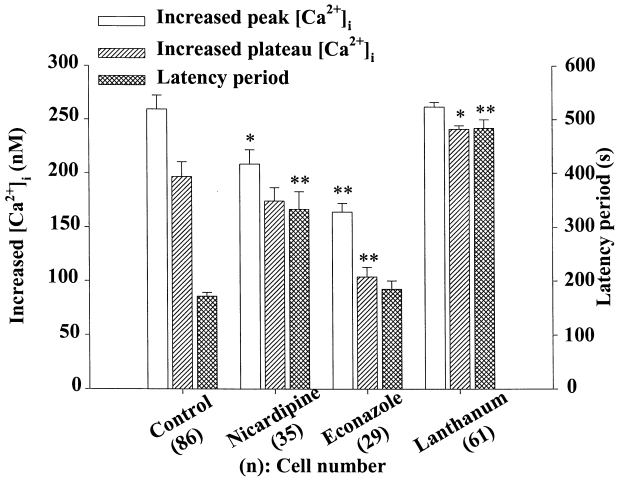

Fig. 6. The inhibitory effects of Ca^{2+} channel blockers on the peak, plateau $[Ca^{2+}]_i$ response and latency period were shown. Nicardipine prolonged the latency period from about 4 to 6 min and reduced the peak but not plateau phase of $[Ca^{2+}]_i$ elevation. Econazole reduced both the peak and plateau $[Ca^{2+}]_i$ elevations, however, without any effect on the latency period. Lanthanum abolished the $[Ca^{2+}]_i$ elevation in the first 4 min. However, $[Ca^{2+}]_i$ elevation reached the control level in the presence of lanthanum after 8 min (prolonged the latency period). * or ** indicate $P < 0.05$ or $P < 0.001$ (vs. control, ANOVA). Bars represent S.E.M.

3.4. Relationship between damnacanthal- and bradykinin-sensitive intracellular stores

Since damnacanthal releases Ca^{2+} from intracellular stores, it is important to know if damnacanthal shares similar Ca^{2+} stores with other receptor agonists. Bradykinin (0.001–10 μM) produced a consistent $[Ca^{2+}]_i$ elevation in all cells studied ($n = 66$ (14, 10, 16, 12 and 14 cells for 0.001, 0.01, 0.1, 1 and 10 μM, respectively, data not shown), and we tested the relationship between damnacanthal- and bradykinin-sensitive intracellular Ca^{2+} stores.

In the absence of extracellular Ca^{2+}, bradykinin (1 μM) produced a transient $[Ca^{2+}]_i$ elevation in cells (Fig. 7). Another application of bradykinin, 5 min later, failed to produce a $[Ca^{2+}]_i$ increase, indicating the first application of bradykinin emptied bradykinin-sensitive Ca^{2+} stores. In the presence of bradykinin and in the absence of extracellular Ca^{2+}, subsequent addition of damnacanthal (10 μM) produced smaller $[Ca^{2+}]_i$ changes ($n = 39$) than changes produced by damnacanthal alone (without pre-treatment with bradykinin). This result indicates that bradykinin shares part of the damnacanthal-sensitive intracellular Ca^{2+}

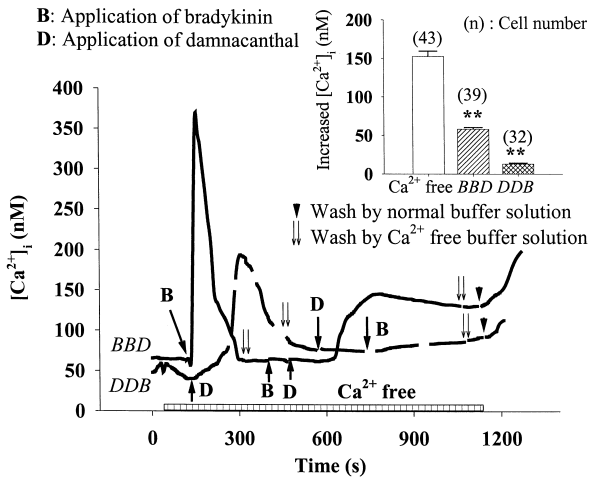

Fig. 7. Original tracings show damnacanthal- and Bradykinin-induced $[Ca^{2+}]_i$ elevation. *BBD* study (solid line): in the absence of extracellular Ca^{2+}, bradykinin (1 μM) produced a transient $[Ca^{2+}]_i$ elevation. Second application of bradykinin failed to produced a $[Ca^{2+}]_i$ elevation. Application of damnacanthal (10 μM), in the presence of bradykinin, produced smaller $[Ca^{2+}]_i$ changes. *DDB* study (dashed line): in the absence of extracellular Ca^{2+}, damnacanthal (10 μM) produced a transient $[Ca^{2+}]_i$ rise, and the second application of damnacanthal failed to produce any $[Ca^{2+}]_i$ change. In the presence of damnacanthal, bradykinin failed to produce a marked $[Ca^{2+}]_i$ change. Inset shows the summary of these studies that damnacanthal-sensitive intracellular Ca^{2+} stores overlap and may be larger than those of bradykinin-sensitive stores. ** indicates $P < 0.001$ (vs. Ca^{2+} free levels, ANOVA). Bars represent S.E.M. ''B'' indicates application of bradykinin. ''D'' indicates application of damnacanthal.

stores and that depletion of bradykinin-sensitive intracellular Ca^{2+} stores also partially emptied damnacanthal-sensitive intracellular Ca^{2+} stores.

On the contrary, damnacanthal shares most of the bradykinin-sensitive intracellular Ca^{2+} stores. In the absence of external Ca^{2+}, damnacanthal (10 μM) induced a transient $[Ca^{2+}]_i$ rise, and the second application of damnacanthal failed to produce any $[Ca^{2+}]_i$ changes (Fig. 7). In the presence of damnacanthal and in the absence of external Ca^{2+}, bradykinin failed to produce marked $[Ca^{2+}]_i$ elevation ($n = 32$). These studies suggest that damnacanthal-sensitive intracellular Ca^{2+} stores almost fully overlap the bradykinin-sensitive intracellular stores.

3.5. Effect of damnacanthal in endothelial and smooth muscle cells

The effect of damnacanthal on intracellular Ca^{2+} in fibroblasts was compared with cultured bovine coronary artery endothelial cells (purchased from Clonetics, passage 3–5) and freshly isolated rat basilar artery smooth muscle cells (Zhang et al., 1995). Application of damnacanthal (30 μM) to the endothelial cells induced a similar $[Ca^{2+}]_i$ elevation but to a smaller degree (the peak $[Ca^{2+}]_i$ value was 122.7 ± 27.4 nM and the latency period was 155.6 ± 32.0 s, $n = 31$) than the $[Ca^{2+}]_i$ elevation in the NHDF-Neo. In rat basilar smooth muscle cells, damnacanthal produced a small $[Ca^{2+}]_i$ response (not markedly different from the resting level) in only 16% of the cells studied ($n = 50$, data not shown).

4. Discussion

We have obtained the following observations in this study. (1) Damnacanthal, a selective inhibitor of $p56^{lck}$ tyrosine kinase activity, increased $[Ca^{2+}]_i$ by both releasing intracellular Ca^{2+} stores and promoting Ca^{2+} entry in NHDF-Neo. The peak $[Ca^{2+}]_i$ value and the latency period preceding the onset of the $[Ca^{2+}]_i$ peak in the damnacanthal response were concentration-dependent. (2) The intracellular Ca^{2+} stores, which responded to damnacanthal application, were thapsigargin-sensitive and overlapped the bradykinin-sensitive stores. (3) VDCC and VICC pathways may be involved in damnacanthal-induced Ca^{2+} entry. (4) Damnacanthal produced a similar response in cultured bovine coronary artery endothelial cells.

The peak $[Ca^{2+}]_i$ value produced by damnacanthal is composed of Ca^{2+} release from intracellular Ca^{2+} stores and Ca^{2+} entry, and the plateau $[Ca^{2+}]_i$ value is mostly Ca^{2+} entry from external space. Our studies demonstrated that the damnacanthal-sensitive Ca^{2+} stores overlap the thapsigargin-sensitive and bradykinin-sensitive Ca^{2+} stores and that damnacanthal-sensitive Ca^{2+} stores are larger than the bradykinin-sensitive Ca^{2+} stores. We speculate that the action of damnacanthal may be similar to the action of thapsigargin: by inhibition of Ca^{2+}-ATPase in the sarco-endoplasmic reticulum, since we do not have evidence that damnacanthal generates inositol 1,4,5-triphosphate (IP_3) (as does bradykinin) which could release Ca^{2+} from IP_3 receptors and thapsigargin-sensitive Ca^{2+} stores. This speculation that damnacanthal raises $[Ca^{2+}]_i$ not by generating IP_3 is supported by several observations in this study. First, $[Ca^{2+}]_i$ elevations induced by damnacanthal or thapsigargin require similar latency periods and that this latency period is dependent upon the concentration of damnacanthal and thapsigargin. G-protein coupled receptor agonists that generate IP_3 usually produce a fast peak $[Ca^{2+}]_i$ response without an apparent latency period. Second, damnacanthal and thapsigargin produced similar $[Ca^{2+}]_i$ elevations in which the peak and plateau $[Ca^{2+}]_i$ phases are not clearly distinguished. On the contrary, G-protein coupled receptor agonists (such as bradykinin) produce a sharp spike-like $[Ca^{2+}]_i$ peak response that is followed by a prolonged and much smaller plateau phase. Third, pre-treatment of cells with thapsigargin abolished the effect of damnacanthal. Fourth, the possibility of a Ca^{2+}-induced Ca^{2+} release mechanism was excluded in our study since caffeine failed to produce any $[Ca^{2+}]_i$ changes.

Our results demonstrated clearly that the plateau phase of $[Ca^{2+}]_i$ induced by damnacanthal was Ca^{2+} entry from external space, since removal of external Ca^{2+} abolished the $[Ca^{2+}]_i$ plateau (Fig. 2). However, the Ca^{2+} entry pathways and the relationship between the initial entry and the release are not clear. A more prolonged latency period for damnacanthal was observed in the absence of extracellular Ca^{2+} than the latency in the presence of extracellular Ca^{2+}, indicating that Ca^{2+} entry not only composed the plateau $[Ca^{2+}]_i$ phase but also contributed to the peak $[Ca^{2+}]_i$ response. The initial Ca^{2+} entry pathways might involve both VDCC and VICC since nicardipine and econazole attenuated markedly the peak phase of $[Ca^{2+}]_i$. An interesting observation was that nicardipine but not econazole prolonged the latency period of $[Ca^{2+}]_i$ response to damnacanthal indicating probably that the initial Ca^{2+} entry from VDCC may play a role in the release of Ca^{2+} from intracellular Ca^{2+} stores. Lanthanum almost doubled the latency period of Ca^{2+} response to damnacanthal probably by blocking VDCC. However, the plateau $[Ca^{2+}]_i$ phase is mediated by VICC since only econazole reduced the plateau phase.

Damnacanthal has been used in several studies in different tissues as a potent and selective inhibitor (Faltynek et al., 1995) for Src tyrosine kinases at similar concentration as reported in this study (micromolar concentration) (Hiwasa et al., 1997,1999; Yuan et al., 1998; Zubkov et al., 1999). However, we did not notice any report studied the relationship between the actions of damnacanthal on tyrosine kinase and intracellular Ca^{2+}. There was no publication suggesting that other tyrosine kinase inhibitors may elevate intracellular Ca^{2+}. On the contrary, tyrosine kinase inhibitor such as genistein and tyrphostin have been reported to decrease intracellular Ca^{2+} by inhibiting Ca^{2+} release from internal stores and inhibiting Ca^{2+} entry (Di Salvo et al., 1997). Recently, Hiwasa et al. (1999) showed that damnacanthal (10 μg/ml) enhanced the phosphorylation of extracellular signal regulated protein kinase1/2 in UV^r-1 cells. It was not clear if this effect of damnacanthal is related to any $[Ca^{2+}]_i$ changes, even though elevation of $[Ca^{2+}]_i$ may lead to mitogen-activated protein kinase activation. Damnacanthal produced a similar $[Ca^{2+}]_i$ elevation in cultured bovine coronary artery endothelial cells but not in freshly isolated rat basilar smooth muscle cells in this study. It is not clear if the different actions of damnacanthal in fibroblasts, endothelial and smooth muscle cells are due to species, cerebral vs. peripheral, or culture vs. fresh isolation. This observation that damnacanthal failed to produce a $[Ca^{2+}]_i$ elevation in rat basilar smooth muscle cells, however, is consistent with a recent study of ours that damnacanthal (30 μM) did not change the tension of rabbit basilar artery (Zubkov et al., 1999). On the contrary, our results clearly demonstrated that damnacanthal produced a $[Ca^{2+}]_i$ elevation in both dermal fibroblasts and vascular endothelial cells. We conclude that damnacanthal inhibits tyrosine kinase and increases intracellular Ca^{2+} in dermal fibroblasts and endothelial cells. The relationship between the actions of damnacanthal on tyrosine kinase and intracellular Ca^{2+} requires further investigation.

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