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The norepinephrine-sensitive Ca^{2+}-storage site differs from the caffeine-sensitive site in vascular smooth muscle of the rat aorta

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Using microfluorometry of quin 2, a Ca^{2+}-sensitive dye, we characterized the release and uptake of Ca^{2+} by the norepinephrine-sensitive Ca^{2+}-storage site and the caffeine-sensitive one. The norepinephrine-sensitive Ca^{2+}-storage site was readily depleted in Ca^{2+}-free medium and almost completely replenished by loading with 1.0 mM Ca^{2+} solution for 3 min, whereas the caffeine-sensitive site was scarcely affected. Furthermore, norepinephrine has little effect on the caffeine-sensitive Ca^{2+}-storage site in Ca^{2+}-free medium, and vice versa. We conclude that the location and mechanisms of release and uptake of Ca^{2+} of these two Ca^{2+}-storage sites differ in the case of rat aortic vascular smooth muscle cells in primary culture.

Ca^{2+} store; Norepinephrine; Caffeine; Quin 2; (Vascular smooth muscle cell, Rat aorta)

1. INTRODUCTION

The release of cellular Ca^{2+} plays a major role in contraction of both VSMCs and striated muscles [1-3]. Although NE [4,5] and caffeine [6] will release Ca^{2+} from the intracellular Ca^{2+}-storage sites, the sites sensitive to these two agents have not been clearly identified [7,8]. Using microfluorometry of quin 2, the release and uptake of calcium by the NE-sensitive storage and caffeine-sensitive sites in cultured rat VSMCs were investigated.

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Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; PSS, physiological saline solution; VSMCs, vascular smooth muscle cells; NE, norepinephrine

2. MATERIALS AND METHODS

Quin 2/AM was purchased from Dotite (Japan). Drugs used were l-norepinephrine hydrochloride (Sigma), caffeine (Sigma) and ionomycin (Calbiochem).

2.1. *Cell culture and quin 2 loading of cells*

VSMCs were cultured from the rat aortic media as described [9], primary cultures being used for all observations. On days 5-6, just before reaching confluency, the cultured cells on Lux chamber slides were loaded with quin 2 physiologically as the acetoxy methyl ester (quin 2/AM) as in [10,11]. The millimolar composition of the 'normal' PSS (pH 7.4 at 25°C) was: 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5.5 glucose, and 10 Hepes. The composition of Ca^{2+}-free PSS was similar to that of normal PSS, except that it contained 2 mM EGTA instead of 1 mM $CaCl_2$.

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2.2. *Microfluorometry of quin 2*

Changes in $[Ca^{2+}]_i$ were estimated by measuring the fluorescence change of the quin 2-Ca^{2+} complex in the cytosol of VSMCs. The fluorescence intensity in a spot ($< 1\,\mu m^2$) of the cytosol 3 μm from the nucleus was recorded using microfluorometry as in [10,11].

3. RESULTS AND DISCUSSION

As shown in fig.1A, marked fluorescence of the quin 2-Ca^{2+} complex was observed almost exclusively in the cytosol. When VSMCs were exposed to Ca^{2+}-free PSS containing 2 mM EGTA, $[Ca^{2+}]_i$ decreased exponentially, reaching a steady-state level within 6 min (fig.1B). This steady-state level was retained for over 60 min. Both NE (10^{-5} M) and caffeine (10^{-2} M) induced a transient $[Ca^{2+}]_i$ elevation in Ca^{2+}-free PSS; there was a progressive increase in $[Ca^{2+}]_i$, which peaked at 2 or 0.5 min after application of NE or caffeine, respectively. The peak values obtained by application of NE or caffeine after 10 min in Ca^{2+}-free media were dose-dependent. Since both NE and caffeine induced $[Ca^{2+}]_i$ transients in the absence of extracellular Ca^{2+}, the observed $[Ca^{2+}]_i$ elevations were due to the release of Ca^{2+} from the intracellular storage sites. Using the method of Tsien et al. [12], an estimate of $[Ca^{2+}]_i$ was made. The maximum and minimum fluorescence signals were obtained by permeabilizing cells with 10^{-7} M ionomycin in the presence of excess Ca^{2+} (10^{-3} M) or 10 mM EGTA (approx. 10^{-9} M Ca^{2+}), respectively. Using these values, it can be calculated that the $[Ca^{2+}]_i$ of VSMCs in normal PSS, Ca^{2+}-free PSS (2 mM EGTA) for 10 min and the peak value induced by NE (10^{-5} M) in Ca^{2+}-free PSS for 10 min were 105 ± 19, 48 ± 6 and 403 ± 129 nM,

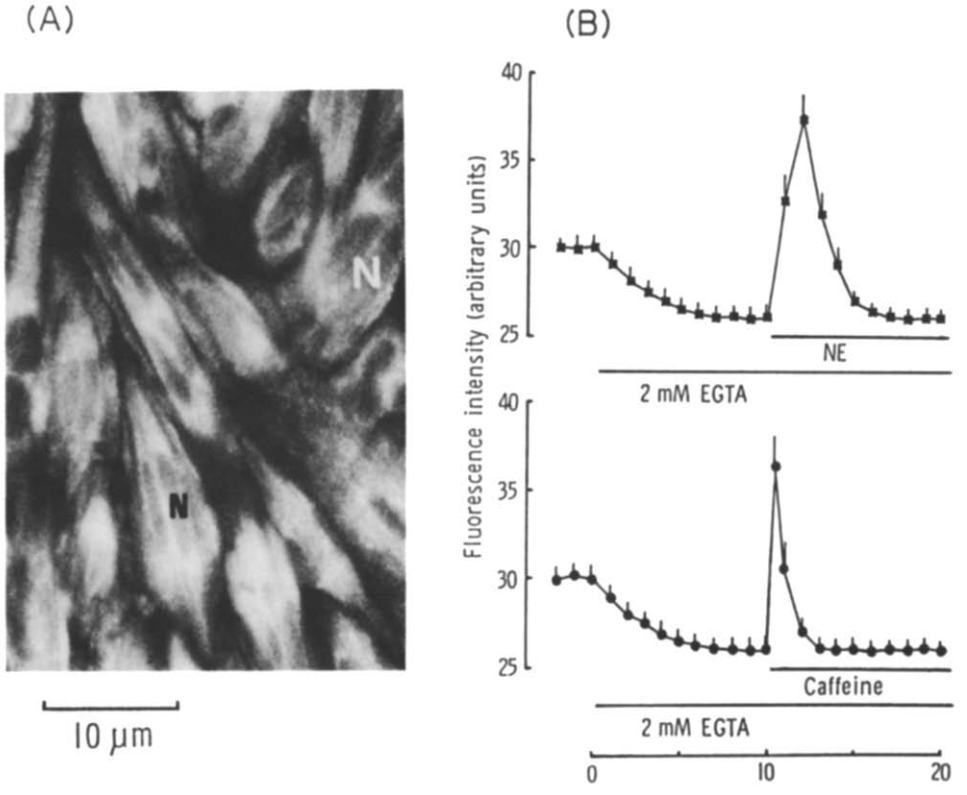

Fig.1. (A) Fluorescence photomicrograph of quin 2-loaded cells 2 min after addition of 10^{-5} M NE in Ca^{2+}-free PSS containing 2 mM EGTA (N, nucleus). (B) Typical time courses of the effects of (■) 10^{-5} M NE (upper), and (●) 10^{-2} M caffeine (lower) on the fluorescence signal in VSMCs in Ca^{2+}-free PSS containing 2 mM EGTA. Data are means ± SD of 8 cells.

respectively ($n=5$). The resting level of $[Ca^{2+}]_i$ in normal PSS found here is similar to those reported in [13–16].

Fig.2 depicts the effects of the duration of exposure to Ca^{2+}-free medium on the peak levels of the Ca^{2+} transients induced by the first application of NE (10^{-5} M) and caffeine (10^{-2} M) (doses sufficient to exert maximum elevations of $[Ca^{2+}]_i$ [10,17]). After 10 min exposure to Ca^{2+}-free medium, the longer the duration of exposure, the lesser the extent of the NE-induced elevation of $[Ca^{2+}]_i$, while that of caffeine was little affected. The half-times for decay of the peak levels of NE- and caffeine-induced elevations of $[Ca^{2+}]_i$ in Ca^{2+}-free medium were 13 and 190 min, respectively. When VSMCs were incubated with 1.0 mM Ca^{2+} PSS for 3 min, and then incubated again with Ca^{2+}-free media for 1 min prior to the application of NE after 15 min or longer in Ca^{2+}-free media, the extent of peak elevation of $[Ca^{2+}]_i$ by NE was large and independent of the duration of the first exposure to Ca^{2+}-free media. This observation indicates that, although the NE-induced $[Ca^{2+}]_i$ elevation decreases time-dependently, there may be no discernible damage in the Ca^{2+}-release mechanisms mediated by NE in Ca^{2+}-free solution for at least 60 min. The NE-sensitive stored Ca^{2+} is rapidly depleted in Ca^{2+}-free solution and rapidly replenished in 1.0 mM Ca^{2+} PSS.

As shown in fig.3A, when VSMCs were repeatedly exposed to 10^{-5} M NE in Ca^{2+}-free PSS containing 2 mM EGTA for 5 min with an interval of 2 min, the peak level of the fluorescence increase on a second exposure to NE was about 30% of that observed with the first. The fluorescence increase on a third exposure was little, if any. Thus, the NE-sensitive Ca^{2+}-storage site of VSMCs was practically depleted with three applications of 10^{-5} M NE, in Ca^{2+}-free medium. When 10^{-2} M caffeine was subsequently applied to VSMCs in Ca^{2+}-free medium after the third exposure to NE, a transient elevation of $[Ca^{2+}]_i$ occurred, the peak level being neither greater nor smaller than that induced by the same dose of caffeine, for the same

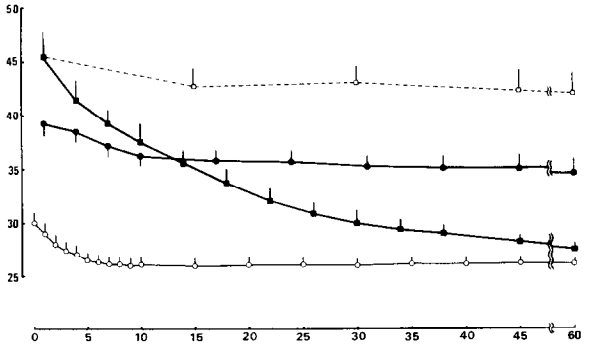

Fig.2. Effects of Ca^{2+}-free medium on peak levels of the fluorescence signal induced by the first application of 10^{-5} M NE (■) and 10^{-2} M caffeine (●). (○) Fluorescence levels of control cells, incubated only in Ca^{2+}-free PSS containing 2 mM EGTA. The NE-induced peak signals recovered to the steady level when Ca^{2+}-depleted cells were loaded with 1.0 mM Ca^{2+} for 3 min, and then incubated again with Ca^{2+}-free media for 1 min prior to application of NE (□---□). Data are means ± SD of 5 experiments. Ordinate, fluorescence intensity (arbitrary units); abscissa, time (min) in Ca^{2+}-free solution.

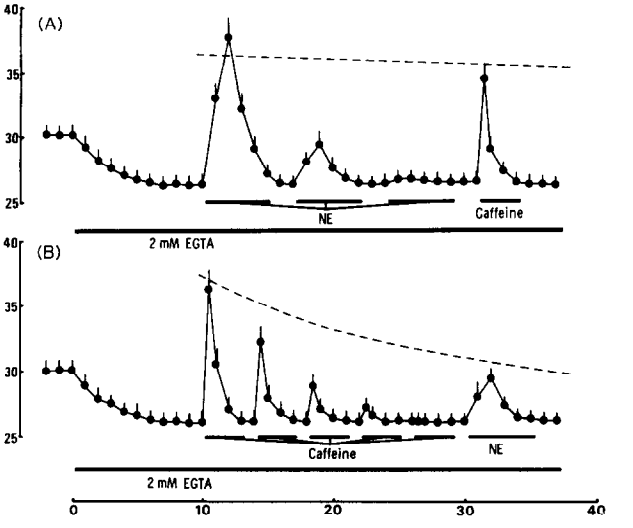

Fig.3. (A) Effect of repetitive application of 10^{-5} M NE on subsequent elevation of cytosolic Ca^{2+} induced by 10^{-2} M caffeine in Ca^{2+}-free PSS containing 2 mM EGTA. Data are means ± SD of 5 experiments. (B) Effect of repetitive application of 10^{-2} M caffeine on subsequent elevation of cytosolic Ca^{2+} induced by 10^{-5} M NE in Ca^{2+}-free PSS containing 2 mM EGTA. Data are means ± SD of 5 experiments. (– – –) Time courses of peak levels of fluorescence induced by the first application of 10^{-2} M caffeine (A) and 10^{-5} M NE (B) in Ca^{2+}-free media; obtained from fig.2. Ordinate, fluorescence intensity (arbitrary units); abscissa, time (min) in Ca^{2+}-free solution.

time of exposure to Ca^{2+}-free medium, without the repetitive application of NE. This observation indicates that the caffeine-sensitive Ca^{2+}-storage site was not affected by prior treatment with NE. Conversely, as shown in fig.3B, when VSMCs were repeatedly exposed to 10^{-2} M caffeine in Ca^{2+}-free PSS containing 2 mM EGTA for 3 min with an interval of 1 min, the peak level of $[Ca^{2+}]_i$ elevation was reduced gradually with each application, the fifth application producing little or no cellular response. Thus, the caffeine-sensitive Ca^{2+}-storage site was practically depleted after the fifth application of caffeine. When 10^{-5} M NE was subsequently applied after depletion of the caffeine-sensitive Ca^{2+}-storage site, there was an elevation of $[Ca^{2+}]_i$, the peak level being in good accord with that induced by the same dose of NE for the same duration of exposure to Ca^{2+}-free medium and without the repetitive application of caffeine. These findings indicate that the NE-sensitive Ca^{2+}-storage site differs from the caffeine-sensitive site in VSMCs in primary culture.

The effects of Ca^{2+} loading on the NE- and caffeine-sensitive Ca^{2+}-storage sites in Ca^{2+}-depleted VSMCs are shown in fig.4. When VSMCs were incubated with 1.0 mM Ca^{2+} PSS for 3 min after the depletion of caffeine-sensitive stored Ca^{2+} by a five-step application of caffeine in Ca^{2+}-free PSS, the $[Ca^{2+}]_i$ transient induced by subsequent application of caffeine was practically negligible (fig.4A). In contrast, the NE-sensitive Ca^{2+}-storage site was almost completely replenished in such cases (fig.4B). Thus, the NE-sensitive Ca^{2+}-storage site once depleted is readily replenished by loading with 1.0 mM Ca^{2+} PSS for 3 min, whereas the caffeine-sensitive Ca^{2+}-storage site is barely affected. The findings that Ca^{2+} in the NE-sensitive storage site is readily depleted and replenished indicate that the site is located in close proximity to the cell membrane. Daniel and coworkers [7,18] demonstrated that Ca^{2+} binds at the cell membrane of the rabbit aortic smooth muscle, and Brockerhoff [19] proposed that cells store calcium in the hydrogen belt of their membranes, on the cytoplasmic side, with the Ca^{2+} captive in cages formed by the phosphate and carbonyl oxygens of two acidic phospholipid molecules, e.g. phosphatidylinositol and phosphatidylserine. As there are differences in the rates of Ca^{2+} depletion and replenishment between the two Ca^{2+}-storage

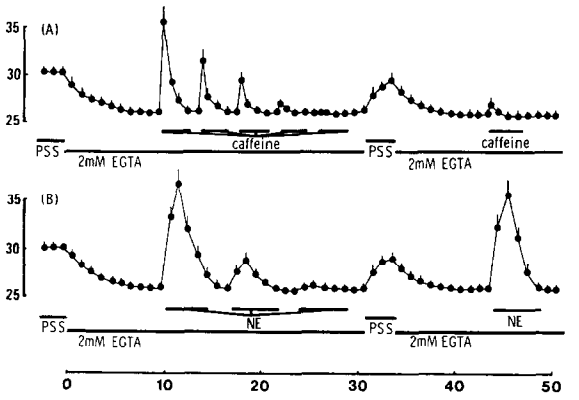

Fig.4. Effects of Ca^{2+} loading on the NE- and caffeine-sensitive Ca^{2+}-storage sites of Ca^{2+}-depleted cells in Ca^{2+}-free medium. (A) Effect of Ca^{2+} loading with 1.0 mM Ca^{2+} PSS for 3 min on the caffeine-induced Ca^{2+} transient after depletion of the caffeine-sensitive Ca^{2+}-storage site by five applications of caffeine in Ca^{2+}-free PSS. (B) Effect of Ca^{2+} loading with 1.0 mM Ca^{2+} PSS for 3 min on the NE-induced Ca^{2+} transient after depletion of the NE-sensitive Ca^{2+}-storage site by three applications of NE in Ca^{2+}-free PSS. Data are means ± SD of 5 experiments. Ordinate, fluorescence intensity (arbitrary units); abscissa, time sequence (min) in Ca^{2+}-free solution and 1 mM Ca^{2+}-PSS solution.

sites and NE has little effect on the caffeine-sensitive Ca^{2+}-storage site in Ca^{2+}-free medium, and vice versa, we tentatively conclude that the location and mechanisms of release and uptake of Ca^{2+} of these two Ca^{2+}-storage sites in cultured VSMCs differ; at least, the NE-sensitive Ca^{2+}-storage site does not overlap with the caffeine-sensitive site.

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